Adipose-Derived Biogenic Nanoparticles for Suppression of Inflammation

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Extracellular vesicles secreted from adipose-derived mesenchymal stem cells (ADSCs) have therapeutic effects in inflammatory diseases. However, production of extracellular vesicles (EVs) from ADSCs is costly, inefficient, and time consuming. The anti-inflammatory properties of adipose tissue-derived EVs and other biogenic nanoparticles have not been explored. In this study, biogenic nanoparticles are obtained directly from lipoaspirate, an easily accessible and abundant source of biological material. Compared to ADSC-EVs, lipoaspirate nanoparticles (Lipo-NPs) take less time to process (hours compared to months) and cost less to produce (clinical-grade cell culture facilities are not required). The physicochemical characteristics and anti-inflammatory properties of Lipo-NPs are evaluated and compared to those of patient-matched ADSC-EVs. Moreover, guanabenz loading in Lipo-NPs is evaluated for enhanced anti-inflammatory effects. Apolipoprotein E and glycerolipids are enriched in Lipo-NPs compared to ADSC-EVs. Additionally, the uptake of Lipo-NPs in hepatocytes and macrophages is higher. Lipo-NPs and ADSC-EVs have comparable protective and anti-inflammatory effects. Specifically, Lipo-NPs reduce toll-like receptor 4-induced secretion of inflammatory cytokines in macrophages. Guanabenz-loaded Lipo-NPs further suppress inflammatory pathways, suggesting that this combination therapy can have promising applications for inflammatory diseases.

1. Introduction

Extracellular vesicles (EVs) are naturally occurring nanoparticles secreted by all cells, and serve important roles in many physiological processes, including immunomodulation.[1] Increasing evidence has shown that therapeutic effects of cell therapy are elicited through the release of secreted EV cargo, such as RNA, proteins, and lipids.[2–6] EVs have many advantages over cell therapy, including easier storage, eliminated risk of malignant transformation, prolonged circulation times, and increased stability.[1] In particular, mesenchymal stem cell (MSC)-derived EVs have demonstrated great potential as therapeutic agents in various disease models. For example, ADSC-EVs have been shown to induce anti-inflammatory, anti-oxidant, anti-apoptotic, and angiogenic effects in neurological, genitourinary, organ-specific, and endocrine disorders, as well plastic surgery and regenerative applications.[7] Preclinical EV studies have primarily
focused on laboratory expanded MSCs, which have limited scalability for human use. Therefore, few clinical trials have been performed, with the exception of four clinical trials for treatment of conditions, such as type 1 diabetes mellitus, chronic kidney disease, macular degeneration, and ischemic stroke.\[9\]

Notably, noncell culture-derived EVs and other biogenic nanoparticles (BiNPs) have been overlooked as therapeutic agents. In this study, an innovative approach based on BiNPs obtained directly from adipose tissue is described. Adipose tissue is an easily accessible, abundant source of biological material with known immunosuppressive components.\[9–11\] BiNPs were obtained from lipoaspirate (Lipo-NP) using a minimally manipulated approach that is faster, cheaper, and produces higher yields compared to cell culture-based BiNP isolation. Moreover, the absence of cell culture media increases biocompatibility and reduces contamination risk. BiNPs obtained directly from a native heterogeneous environment could potentially have unique properties that are not expressed in a homogenous in vitro expanded environment.

The clinical translation of EVs and other BiNPs has also been hindered due to inefficient and nonscalable methods of isolation,\[12–14\] such as ultracentrifugation, which is the most commonly used method.\[15\] To overcome the issues faced by conventional isolation techniques, a highly robust, pure, and scalable tangential flow filtration (TFF)-based method for BiNP isolation was employed.\[16\] The size, morphology, zeta potential, and protein content of patient-matched ADSC-EVs and Lipo-NPs were assessed. Moreover, protein markers, lipid classes, and RNA classes were also identified. Protective effects of patient-matched Lipo-NPs and ADSC-EVs in macrophages exposed to chemical and oxidative stress were evaluated. Additionally, the dual use of Lipo-NPs as endogenous anti-inflammatory agents and drug delivery vehicles was explored. Encapsulation of small molecule drugs in nanocarriers can improve circulation times, site-specific delivery, and reduce side effects, and several synthetic NPs have gained clinical approval.\[17–21\] The use of BiNPs for drug delivery may have unique advantages compared to synthetic NPs due to complex surface structures that promote biologically relevant interactions, such as tissue tropism.\[22\] In this study, guanabenz (GBZ), a clinically approved drug with known anti-inflammatory properties,\[24\] was loaded into Lipo-NPs to further suppress inflammation.

2. Results and Discussion

2.1. Processing and Characterization of Adipose Tissue

ADSC-EVs have previously been shown to possess immunomodulatory properties, including induction of peripheral tolerance and mitigation of inflammatory responses.\[22\] In this study, the physicochemical characteristics and anti-inflammatory properties of patient-matched ADSC-EVs and Lipo-NPs were compared. Lipoaspirate was collected from three patients undergoing liposuction for orthopedic purposes (nonobese), and BiNPs were isolated from the cell-depleted fraction (Figure 1A). The cell fraction was cultured to obtain patient-matched ADSCs that displayed plastic adherence (Figure S1, Supporting Information) and a high percentage (>95%) of mesenchymal stem cell markers (CD73, CD90, and CD105), as determined by flow cytometry (Figure S2, Supporting Information). The conditioned media from ADSCs was processed to obtain EVs.

2.2. Characterization of Lipo-NPs and ADSC-EVs

Conditioned media from ADSCs and cell-depleted lipoaspirate were processed with TFF to obtain ADSC-EVs and Lipo-NPs, respectively. TFF is based on the use of membranes with specific pore dimensions to obtain size-based concentration and diafiltration of nanosized components. The fluid flows tangentially to the membranes, reducing pore clogging, and leading to more effective separation when compared to conventional (dead-end) filtration. Notably, TFF is very effective at separating nanosized components from larger (micrometer-sized and above) and smaller (angstrom-sized) components,\[16\] but is unable to separate different types of nanoparticles (e.g., EVs and lipoproteins). However, one of the main advantages of TFF is that large volumes (>1 L) can be processed for scalable manufacturing, whereas other EV isolation techniques, such as size-exclusion chromatography (SEC), are limited by much smaller input volumes.\[26–28\] TFF also results in higher batch-to-batch consistency compared to conventional methods, such as ultracentrifugation.\[16\] Moreover, standard testing for bacteria, mycoplasma, and endotoxin has previously demonstrated that sterility can be maintained throughout the process (from lipoaspiration to obtaining the final product with TFF)\[16\] which is an important aspect for clinical translation. Therefore, in comparison to other isolation methods TFF is more suitable for clinical use, despite not being able to separate different types of biological nanoparticles, resulting in a heterogeneous product. In fact, from a regulatory perspective, the batch-to-batch consistency of the product is more important than the heterogeneity.\[29\]

ADSC-EVs and Lipo-NPs were characterized to identify potential differences in physicochemical properties.\[30\] Cryogenic transmission electron microscopy (cryo-TEM) demonstrated that both ADSC-EV and Lipo-NP samples contained spherical structures with unilamellar and multilamellar lipid bilayers, resembling that of EVs (Figure 1B), as previously shown.\[31\] Additionally, lipoprotein-like spherical structures could be seen in the Lipo-NP samples (Figure 1B). Both samples displayed a multimodal size distribution ranging from 40–400 nm with the majority of BiNPs falling within the 80–250 nm range (Figure 1C). NP tracking analysis revealed that ADSC-EVs and Lipo-NPs had a mean size of 196.4 and 153.9 nm, respectively (Figure 1D). This size range is comparable to other studies examining the same class of EVs.\[32–34\] The yield of BiNPs from the same amount of starting material was approximately 30-fold higher for lipoaspirate compared to conditioned media (Figure 1E). Both Lipo-NPs and ADSC-EVs displayed similar negative zeta potential values, except for one of the three patients, where ADSC-EVs had a significantly lower zeta potential (Figure 2A), indicating patient heterogeneity. Notably, the above-mentioned properties (concentration, size distribution, and zeta potential) did not differ when measured in water or phosphate buffered saline (Figure S3, Supporting Information). ADSC-EVs had a substantially higher protein content.
content normalized to the amount of BiNPs compared to Lipo-NPs (Figure 2B). Moreover, Western blot analysis demonstrated the presence of EV-enriched markers, including CD63 and annexin V in both ADSC-EVs and Lipo-NPs (Figure 2C). ADSC-EVs displayed a higher content of CD63, and CD9 (same BiNP amount), while Lipo-NPs displayed a higher content of annexin V, when using the same BiNP amount. None of the BiNP samples were positive for calnexin, which is an intracellular contaminant marker. All three Lipo-NP samples expressed APOE, while none of the ADSC-EVs had this marker (Figure 2C). In accordance with the 2018 minimal information for studies of EV (MISEV) guidelines, at least one protein from each of the following criteria was demonstrated: the presence of transmembrane or lipid-bound extracellular proteins (such as CD9 and CD63), the presence of cytosolic proteins (such as annexin V), and the absence of intracellular proteins (such as calnexin). Other studies on this class of EVs have examined similar proteins within the recommended criteria for characterization.

Figure 1. Isolation and characterization of lipoaspirate nanoparticles (Lipo-NPs) and adipose-derived mesenchymal stem cell extracellular vesicles (ADSC-EVs). A) Schematic representation of the major steps involved in isolation of biogenic NPs from adipose tissue. B) Evaluation of morphology by cryogenic transmission electron microscopy (cryo-TEM) (LP: lipoprotein). Scale bar, 100 nm. C) Size distribution, D) mean size, and E) yield determined by nanoparticle tracking analysis (NTA). D,E) Data is presented as mean ± SD of biological triplicates. Statistics by Student’s t-test. **, P < 0.01. TFF: tangential flow filtration.
In conclusion, BiNP characterization was successfully performed in accordance with the 2018 minimal information for studies of EV (MISEV) guidelines,[30] revealing marked differences in yield, protein concentration, and protein markers between ADSC-EVs and Lipo-NPs. Various lipoprotein-based BiNPs can be found in the body, including chylomicrons (200–600 nm), very low-density lipoproteins (VLDL; 30–90 nm), low-density lipoprotein (LDL; 21–27 nm), and high-density lipoproteins (HDL; 7–13 nm).[35] As isolation is based on size-dependent separation of components, it is likely that Lipo-NPs are a mixture of both EVs and lipoproteins, as indicated by cryo-TEM and lipoprotein markers.

2.3. Analysis of Lipid Classes in Lipo-NPs and ADSC-EVs

The lipid composition of BiNPs plays an important role in biological function. Lipidomic analysis was performed to identify major lipid classes in patient-matched Lipo-NP and ADSC-EV samples. Lipid classes included glycerolipids consisting of diglyceride (DG) and triglyceride (TG), glycerophospholipids consisting of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS), and sphingolipids consisting of ceramide (Cer) and sphingomyelin (SM). Fatty acyls were the major lipid class in both Lipo-NPs and ADSC-EVs, while glycerolipids (triglycerides in particular) were enriched in Lipo-NPs, although to a different extent, highlighting patient variability (Figure 3A). These differences in lipid composition could stem from the presence of lipoproteins in Lipo-NPs, as triglycerides are associated with lipoproteins in the body.[36] However, other studies have shown that cancer cell-derived EVs contain high amounts of glycerolipids, making it difficult to draw any conclusions.[37]

2.4. Relative Composition of RNA Classes in Lipo-NPs and ADSC-EVs

BiNPs can impact cellular function through transfer of bioactive RNA. Although the importance of RNA involvement in BiNP function is largely undisputed, RNA composition cannot yet be used for identification of EVs.[30] The relative composition of RNA classes in one patient-matched Lipo-NP and ADSC-EV sample was determined (Figure 3B). Lipo-NPs had a larger amount of microRNAs (miRNAs), which may be due to lipoprotein binding,[38] and could have unique functional attributes. Table S1 in the Supporting information shows the 25 most differentially expressed known miRNAs.

2.5. Protective Effects of Lipo-NPs and ADSC-EVs in Macrophages

Previous studies have demonstrated that MSC-EVs contain biocommunicar cargo with anti-apoptotic and anti-oxidant effects. For example, miR-21[33] and miR-126[39] were shown to decrease cellular apoptosis through, e.g., the phosphatase and tensin homolog (PTEN)-protein kinase B (AKT) pathway. Moreover, glutathione peroxidase in MSC EVs was shown to decrease oxidative stress in cells.[40] In this study, the potential protective effects of BiNPs against apoptotic (ethanol) and oxidative...
stress-inducing (hydrogen peroxide) agents were assessed. Macrophages were chosen as an in vitro model of cell stress as the largest portion of intravenously administered nanoparticles (synthetic or biological) are internalized by circulating or resident macrophages.\textsuperscript{1,41–44} Moreover, macrophages play a major role in tissue regeneration and several studies have demonstrated that the depletion of these cells causes impaired tissue repair.\textsuperscript{45,46} Cell viability studies demonstrated that both Lipo-NPs and ADSC-EVs protected macrophages from hydrogen peroxide (Figure 4A) and ethanol-induced stress (Figure 4B) to a similar extent.

In addition to evaluating the protective effects of EVs, anti-inflammatory effects were assessed. Specifically, lipopolysaccharide (LPS) was used to activate the toll-like receptor 4 (TLR4) pathway, which plays a critical role in multiple disease conditions, including ischemia-reperfusion injury,\textsuperscript{47–49} neonatal hyperoxia-induced kidney injury,\textsuperscript{50} and liver injury.\textsuperscript{51,52} In both Raw 264.7 macrophages and primary human macrophages treatment with Lipo-NPs and ADSC-EVs led to a reduction in the levels of LPS-induced interleukin 6 (IL-6) (Figure 4C,D), a cytokine that plays a major role in inflammation.\textsuperscript{53} At lower concentrations of Lipo-NPs (10^7 and 10^8 mL\(^{-1}\)), IL-6 remained unchanged (Figure S4, Supporting Information). ADSC-EVs have previously been shown to contain miR-let7b,\textsuperscript{54} which is a known suppressor of TLR4-induced inflammation.\textsuperscript{85} It is likely that various miRNAs in both ADSC-EVs and Lipo-NPs are involved in suppression of TLR4-induced inflammation. In summary, Lipo-NPs and ADSC-EVs have similar protective and anti-inflammatory effects, although the former is cheaper and faster to obtain. However, as Lipo-NPs are taken up by cells to a greater extent than ADSC-EVs, Lipo-NPs may be a class of material with much lower anti-inflammatory potency once inside the cell.

2.6. Properties of the EV Fraction of Lipo-NP

To further study the specific role of EVs as one of the components of Lipo-NPs, SEC was used to separate EVs from lipoproteins (Figure 5A). Previous studies have shown that 95% of HDL can be removed from samples with SEC.\textsuperscript{55} The results demonstrate that 43% (±7.1%) of Lipo-NPs consist of EVs (Figure 5B), although this number may be higher due to some loss of EVs in the isolation process. The EV population displayed a slightly narrower size distribution and a marginally lower zeta potential than Lipo-NPs (Figure 5C). Western blot analysis verified that the lipoprotein marker APOE was removed after sample processing through SEC, while the EV-enriched marker annexin V was present (Figure 5D). Moreover, comparison of anti-inflammatory properties revealed that Lipo-NPs and the EV fraction of Lipo-NPs caused a similar reduction of IL-6 in LPS stimulated RAW 264.7 cells when normalized for particle number (Figure S5, Supporting Information).

2.7. Cellular Uptake of BiNPs in Hepatocytes and Macrophages

One of the primary mechanisms by which BiNPs affect target cells is through receptor-ligand interactions between membrane
surfaces, which triggers internalization and subsequent cargo release.\(^{[57]}\) To assess BiNP cellular uptake, ADSC-EVs, Lipo-NPs, and the EV fraction of Lipo-NPs were labeled with DiI. DiI bound to a similar extent to Lipo-NPs and EVs (Figure S6, Supporting Information), as assessed by measuring the fluorescence intensity after removal of the free dye. Moreover, the fluorescent intensity did not change over a 24-hour incubation period in FluoroBrite media at 37°C (Figure S6, Supporting Information). The DiI labeled BiNPs were incubated with HepaRG, RAW 264.7, and Kupffer cells for 24 h. Flow cytometry revealed that Lipo-NPs were taken up in higher amounts by HepaRG, RAW 264.7, and Kupffer cells for 24 h. Flow cytometry revealed that Lipo-NPs were taken up in higher amounts by HepaRG, RAW 264.7, and Kupffer cells compared to ADSC-EVs (Figure 6A). Similarly, the mixed population of Lipo-NPs was taken up to a greater extent by HepaRG cells than the EV fraction of Lipo-NPs (Figure 6A). This could potentially be due to scavenger receptor class B type I-mediated uptake of lipoproteins.\(^{[58,59]}\) Immunofluorescence images demonstrated that BiNPs were clustered in perinuclear regions after 24 h of incubation (Figure 6B).

2.8. GBZ-Loaded Lipo-NPs

Lipo-NPs were further optimized as anti-inflammatory agents, as they were deemed superior to ADSC-EVs in regard to manufacturing costs and time-efficiency, while displaying comparable anti-inflammatory effects. Specifically, GBZ, a small molecule therapeutic agent was incorporated into Lipo-NPs. GBZ is a clinically approved \(\alpha\)-2 adrenergic agonist for treatment of hypertension,\(^{[23]}\) and has also been found to lower LPS-induced inflammatory cascades,\(^{[24]}\) which was confirmed in this study (Figure S7, Supporting Information). GBZ exerts anti-inflammatory effects in macrophages through two mechanisms: i) eukaryotic initiation factor 2 (eIF2\(\alpha\))-dependent signaling, which downregulates IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), and cyclooxygenase 2 (COX-2), and ii) eIF2\(\alpha\)-independent signaling, which downregulates interleukin 1 beta (IL1\(\beta\)) and tumor necrosis factor (TNF\(\alpha\)).\(^{[24]}\) Therefore, we hypothesized that loading Lipo-NPs with GBZ could further suppress TLR4-induced inflammatory cytokines. Additionally, there are several advantages of NP-encapsulation as opposed to co-administration of a free drug, including prolonged circulation times, enhanced site-specific delivery, reduced side effects, and simultaneous delivery with other NP cargo.\(^{[17-19]}\)

Previously, coumarin 6 (similar molecular weight as GBZ) has been used as a model hydrophobic small molecule drug to assess drug loading and release through fluorescence intensity measurements.\(^{[60,61]}\) In this study, coumarin 6 was successfully loaded into Lipo-NPs using a previously described passive loading method.\(^{[62]}\) The encapsulation efficiency for coumarin 6 was 65.4% ± 2.6%. Similarly, the encapsulation efficiency for
GBZ using absorbance measurement was 44%, resulting in a final GBZ concentration of 0.0489 µmol GBZ in 10^9 Lipo-NPs. In simulated physiological conditions (10% FBS, 37 °C, magnetic stirring), less than 8% of coumarin 6 was released from Lipo-NPs in the first 8 h and 24% was released after 72 h (Figure 7A). Although the loading of therapeutic agents, such as RNAs and proteins, in BiNPs is usually highly inefficient,63 this study demonstrates that high levels of hydrophobic small molecules can be loaded in BiNPs. However, it is unclear whether this dose of GBZ would be sufficient for in vivo use, although previous studies have demonstrated that nanoparticle loading of therapeutic drugs improves circulation times and site-specific delivery, reducing the required dose.17–21

2.9. Anti-Inflammatory Effects of GBZ-Loaded Lipo-NPs in Macrophages

Inflammatory pathways were activated in Raw264.7 macrophage cells through LPS (TLR-4 ligand) exposure, and the anti-inflammatory potential of Lipo-NPs and GBZ-loaded Lipo-NPs was compared. Western blot analysis and enzyme linked immunosorbent assays (ELISA) were performed, as many inflammatory mediators, such as cytokines, have unstable intracellular mRNAs due to their AU-rich elements (AREs).64 A 50 × 10⁻⁶ m GBZ dose was used, as this was the highest dose that did not reduce cell viability (Figure 7B). This dose of GBZ is also higher than what has previously been shown to suppress inflammatory molecules in RAW264.7 cells (IC50 = 15.9 × 10⁻⁶ m for leukotriene C4 and 28.4 × 10⁻⁶ m

![Figure 5](image)

Figure 5. Properties of the EV fraction of Lipo-NPs. A) Schematic illustrating isolation methods used to obtain Lipo-NPs and the EV fraction of Lipo-NPs. B) Schematic and percentage of Lipo-NPs and the EV fraction of Lipo-NPs. C) Size distribution (NTA) and zeta potential measurements. D) Western blot analysis of APOE and annexin V.

![Figure 6](image)

Figure 6. Cellular uptake of patient-matched Dil-labeled ADSC-EVs, Lipo-NPs, and the EV fraction of Lipo-NPs after 24 h. A) Median fluorescence intensity of Dil in Hoechst+ subsets of HepaRG hepatocyte, RAW 264.7 macrophages, and Kupffer cells measured by flow cytometry. Experiments were performed in triplicates and results are presented as mean ± SD. Statistics by Student’s t-test. ***, P < 0.001. B) Immunofluorescence images of HepaRG incubated with Lipo-NPs or ADSC-EVs. Scale bar, 100 µm.
for prostaglandin E2). One of the main consequences of TLR4 activation is upregulation of inflammatory mediators COX-2 and inducible nitric oxide synthase (iNOS). Western blot results demonstrated that Lipo-NPs were unable to lower COX2 and iNOS levels, while GBZ-loaded Lipo-NPs caused suppression of the aforementioned inflammatory mediators (Figure 7C). However, Lipo-NPs caused substantial reduction in inflammatory cytokines, including interleukin 1 alpha (IL1α), IL-6, and TNF-α, which was further enhanced by GBZ loading (Figure 7D). Similar reductions in inflammatory cytokines have previously been noted in response to ADSC EVs, particularly with TNF-α and IL-6.[33] Lipo-NPs alone did not reduce the levels of GM-CSF, but Lipo-NPs loaded with GBZ did (Figure 7D). It is possible that additional changes to cytokine levels would be detectable on the mRNA level, as highly sensitive method for mRNA detection are available.[64] It is worth noting that free GBZ (Figure S7, Supporting Information) outperforms Lipo-NP encapsulated GBZ, which is expected, as small molecules in dimethyl sulfoxide (DMSO) rapidly diffuse across the cell membrane and can immediately interact with intracellular targets. On the contrary, NPs take much longer to enter cells through endocytosis and encapsulated drugs need to be released in the intracellular space prior to interacting with target molecules.[68,69] However, in vivo, small molecules undergo rapid renal clearance, which is prevented with nanoparticle encapsulation. Therefore, nanoparticle-encapsulated drugs usually outperform their free counterparts in vivo due to size-mediated prolonged circulation times and also improved targeted delivery.[17–21]

In the body, the aforementioned cytokines and inflammatory molecules can activate immune cells, cause acute phase reactions, and hematopoietic responses.[70] IL-6 inhibitors are approved for the treatment of rheumatoid arthritis,[71] while TNF-α inhibitors are approved for rheumatoid arthritis, psoriatic arthritis, Crohn’s disease, ulcerative colitis, psoriasis, and ankylosing spondylitis.[72] Similarly, COX-2 inhibitors are approved for conditions such as rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis.[73] Therefore, Lipo-NPs and GBZ-loaded Lipo-NPs are promising therapies for various inflammatory diseases. A major advantage of Lipo-NPs over the above-mentioned clinically approved small molecule therapies is suppression of multiple molecules involved in macrophage-driven inflammation. Moreover Lipo-NPs ensure simultaneous delivery of therapeutic cargo to cells, which is challenging to achieve with small molecule combination therapy, as pharmacokinetic profiles of molecules differ.

3. Conclusion

EV-based therapies are emerging as promising alternatives to cell-based therapies. Although ADSCs and ADSC-derived EVs have demonstrated immunomodulatory properties,[74] studies on adipose tissue-derived BiNPs are lacking. Direct isolation of BiNPs from adipose tissue takes less time, costs less, and results in higher yields compared to isolation of cell culture-derived EVs. This approach based on limited manipulation could also preserve unique properties that are not present in
an in vitro expanded environment. In fact, previous studies have shown that the secretome of cultured intact adipose tissue has superior anti-inflammatory properties compared to the secretome of cultured stromal vascular fraction (SVF), which has been enzymatically digested and lacks certain cell types, such as adipocytes.[73] These results indicate that the secretome may be more functional when derived from an intact adipose environment compared to adipose tissue that has been digested and cultured. In this study, the physicochemical and anti-inflammatory properties of patient lipoaspirate-derived BiNPs were studied. BiNPs were isolated using a TFF-based scalable and sterile technique, which results in high yields, batch-to-batch consistency, purity, and time-efficiency. Patient-matched ADSC-EVs were also obtained for comparison.

The results from this study indicate that Lipo-NPs, consisting of a combination of EVs and lipoproteins, have similar anti-inflammatory and protective functions as patient-matched ADSC-EVs. These therapeutic properties may stem from both EVs and lipoproteins, as the latter have also previously been shown to display anti-apoptotic and anti-inflammatory potential in cardiovascular,[74] liver,[75] and neurodegenerative diseases.[76] Additionally, Lipo-NPs potentially have dual use as endogenous anti-inflammatory agents and exogenous (drug) delivery vehicles. Encapsulation of therapeutic agents in NPs has several advantages, such as prolonged circulation times, enhanced site-specific delivery, reduced toxicity, and co-delivery with other cargo.[17,18] In this study, GBZ loading was shown to further promote anti-inflammatory effects, suggesting a promising future combination therapy strategy for inflammatory conditions. Further studies are necessary to determine effects of GBZ-loaded Lipo-NPs in vivo. Lipo-NPs are envisioned for future autologous applications, but could also have potential allologenic uses considering that BiNPs are frequently exchanged between individuals in the clinical setting. For example, plasma transfusions seldom cause adverse reactions,[79] although this biological fluid contains a large amount of BiNPs from various cells.[80] Moreover, it is possible that Lipo-NPs could be lyophilized to aid in storage for clinical applications. Indeed, studies have shown that EVs retain function after lyophilization.[81,82] Although less clinically feasible, Lipo-NPs could alternatively be stored at ~80 °C (for up to two years), as studies have shown that both EVs and lipoproteins remain stable under such conditions.[83,84]

4. Experimental Section

Lipoaspirate Processing: Approval from the Mayo Clinic Biospecimens Review Group (ID: 17-010290) was obtained for collection of de-identified residual material (considered nonhuman subjects research) from patients that had undergone a liposuction procedure for orthopedic purposes (nonobese). Lipospiration was performed through a small (1.5 cm) incision, and ~60 mL of subcutaneous fat was removed from under the skin using a tumescent lipospiration technique. Briefly, a standard wetting solution (1 L Lactated Ringers, 50 mL of 1% lidocaine, and 1 cm³ of 1:1000 epinephrines) was infused through the incisions in the abdomen or flank using a standard multi-hole infusion device. A small (1.5 cm) incision, and ~60 mL of subcutaneous fat was transferred to sterile tubes and centrifuged (800 × g; 30 min; Sorval ST 16R centrifuge, Thermo Fisher Scientific) to remove large debris. BiNPs were isolated using a KrosFlo Research 2i Tangential Flow Filtration System (Spectrum Labs, Los Angeles, CA, USA) as previously described.[85] TFF and SEC. The ADSC culture media or content of the Lipogems waste bag was transferred to sterile tubes and centrifuged (800 × g; 30 min; Sorval ST 16R centrifuge, Thermo Scientific) to remove large debris. BiNPs were isolated using a KrosFlo Research 2i Tangential Flow Filtration System (Spectrum Labs, Los Angeles, CA, USA) as previously described.[85] Briefly, the cell culture media or waste bag supernatant was filtered through sterile hollow fiber polyethersulfone membranes with 0.65 µm pores. The permeate was then filtered through hollow fiber polysulfone membranes with 500 kD molecular weight cutoff pores.
The final retentate was dialyzed six times and concentrated (5–8 mL) in a cryoprotective sucrose buffer (5% sucrose, 50 × 10^{-3} to Tris, and 2 × 10^{-3} MgCl₂). Samples were stored at −80 °C until further analysis. For some studies, SEC was used to further purify the product. Specifically, qEV columns (Izon Science) were used to separate EVs from Lipo-NPs. Briefly, the column was equilibrated with sucrose buffer, Lipo-NPs (200 µL) were loaded into the column, and five EV-enriched fractions (7–11) were collected for further analysis.

**Nanoparticle Tracking Analysis (NTA):** The size distribution of isolated BiNPs was determined with NTA. BiNPs were diluted (1:100) in high-performance liquid chromatography (HPLC)-grade water or phosphate buffered saline and analyzed with a Nanosight NS300 (Malvern Panalytical, Westborough, MA, USA). The measurement time was set to 60 s with three replicates.

**Zeta Potential:** Zeta potential measurements were performed as previously described.[88] Briefly, BiNPs were diluted in HPLC-grade water (10^6 BiNPs mL⁻¹) or phosphate buffered saline and placed in folded capillary cells (Malvern, USA). A Zetasizer Nano ZS (Malvern, USA) was used to measure the zeta potential by laser Doppler micro-electrophoresis (Smoluchowski’s theory). Five measurements with ten runs each were recorded.

**Western Blot Analysis of BiNPs:** The protein content of BiNPs was quantified using a Bradford assay (Thermo Fisher Scientific, USA) and mixed with 6x reducing sodium dodecyl sulfate (SDS)-sample loading buffer (Boston Bioproducts, USA) and boiled at 95 °C for 10 min. ADSCs were lysed with radioimmunoprecipitation assay (RIPA, Thermo Fisher Scientific, USA) buffer and used as a control. Proteins were separated on 4–12% Bis-Tris Plus gels (Invitrogen, USA), transferred to nitrocellulose membranes (Abcam, USA), and incubated with the following primary antibodies: anti-C9 mouse monoclonal (1:1000; #ab22123, Abcam, USA) or goat polyclonal monoclonal (1:1000; #ab134045, Abcam, USA); anti-annexin V rabbit monoclonal (1:1000; #ab14196, Abcam, USA); anti-calnexin rabbit monoclonal (1:1000; #ab22595, Abcam, USA), and anti-apolipoprotein E (APOE) goat monoclonal (1:1000; #K74180B-0.5, Meridian Life Science, USA). Rabbit polyclonal secondary antibody to mouse, goat polyclonal secondary antibody to rabbit, and donkey polyclonal secondary antibody to goat (all horseradish peroxidase (HRP)-conjugated) used at 1:5000 (Cell Signaling Technology, USA) was detected by chemiluminescence (SuperSignal West Femto, Thermo Scientific, USA) buffer and used as a control. Proteins were separated on an ultra-high-performance liquid chromatography (UHPLC) system (Shimadzu, USA). For lipid separation, the lipid extract was injected onto a 1.8 µm particle diameter, 50 × 2.1 mm id Waters Acuity HSS T3 column (Waters, Milford, MA). Elution was performed using acetonitrile/water (40:60, v/v) with 10 × 10^{-3} u ammonium acetate as solvent A and acetonitrile/water/isopropanol (10: 5: 85 v/v) with 10 × 10^{-3} u ammonium acetate as solvent B and subjected to LC-MS.

**Immunofluorescence:** One patient-matched Lipo-NP and ADSC-EV sample was sent to QIAGEN Genomic Services for analysis of RNA via next-generation sequencing (NGS). All procedures were conducted at QIAGEN Genomic Services. The miRNAsey Serum/Plasma Kit was used to isolate RNA according to the manufacturer’s instructions. The library preparation was done using the QIAseq miRNA Library Kit (QIAGEN). A total of 5 µL total RNA was converted into miRNA NGS libraries. Adapters containing unique molecular identifiers (UMIs) were ligated to the RNA. Then RNA was converted to cDNA. The cDNA was amplified using polymerase chain reaction (PCR) (22 cycles) and during the PCR indices were added. The samples were purified after PCR. Library preparation QC was performed using either Bioanalyzer 2100 (Agilent) or TapeStation 4200 (Agilent). Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar ratios. The library pool(s) were quantified using qPCR. The library pools were then sequenced on a NextSeq500 sequencing instrument according to the manufacturer’s instructions. Reads were categorized as outmapped for example polyA and polyC homopolymers as well as abundant ribosomal RNA (rRNA) and mitochondrial (mtRNA) sequences, unmapped (no alignment to reference genome possible, genome (aligning to reference genome), and bi-map (to smallRNA or miRNA), miRNA (maps to used version of mmBase), and smallRNA (<200 nucleotides) (maps to smallRNA database compiled by QIAGEN Genomic Services).

**Lipidomics:** For lipid analysis, lipids were extracted from Lipo-NPs and ADSC-EVs using the Bligh-Dyer method. The extraction was carried out by 2:2:2 (v/v/v) water/methanol/dichloromethane at RT after stripping internal standards (Table S2). The organic layer was collected and dried completely under a stream of nitrogen. Dried extracts were resuspended in 100 µL of solvent B and subjected to liquid chromatography–mass spectrometry (LC-MS). Chromatographic separation was performed on an ultra-high-performance liquid chromatography (UHPLC) system (Shimadzu, USA). For lipid separation, the lipid extract was injected onto a 1.8 µm particle diameter, 50 × 2.1 mm id Waters Acuity HSS T3 column (Waters, Milford, MA). Elution was performed using acetonitrile/water (40:60, v/v) with 10 × 10^{-3} u ammonium acetate as solvent A and acetonitrile/water/isopropanol (10: 5: 85 v/v) with 10 × 10^{-3} u ammonium acetate as solvent B and subjected to LC-MS. For chromatographic elution a linear gradient beginning with 60% solvent A and 40% Solvent B was used. The gradient was ramped in a linear fashion to 98% solvent B over the first 10 min and was then held at 98% B for 7 min. The composition was then returned to 40% solvent B and 60% solvent A and held for 3 min. The flow rate used for these experiments was 0.4 mL min⁻¹ and the injection volume was 5 µL. The column was equilibrated for 3 min before the next injection and run at a flow rate of 0.400 mL min⁻¹ for a total run time of 20 min. For lipid classification, including glycerolipids, glycerophospholipids, sphingolipids, glycosphingolipids, and fatty acyls.

**Drug Loading:** Lipo-NPs (10^9) were labeled with Dl (1:200 dilution; #V22885, Thermo Fisher Scientific, USA) by incubating for 20 min before being passed over an exosome spin column (Thermo Fisher Scientific, USA) to remove excess fluorophore, as previously described.[89] The fluorescence intensity of labeled BiNPs was measured with a microplate reader (Synergy HT, Biotek, Winooski, VT, USA). Prior to performing cell culture uptake studies, the stability of the labeled BiNPs in FluoroBrite media (Thermo Fisher Scientific, USA) at 37 °C for 24 h was assessed by re-measuring the fluorescence intensity. Raw 264.7, Kupffer, and HepaRG cells (seeded 24 h prior at 2 × 10^5 cells per well in 6-well plates) were incubated with BiNPs (10^9 mL⁻¹) for 1 h with Exo-free FBS (System Biosciences, USA) and Hoechst 33 342 (2 µg mL⁻¹) for 15 min prior to analysis. Cells were trypanotized, washed, resuspended in PBS (200 µL), and analyzed by flow cytometry (Attune NxT flow cytometer; Thermo Fisher Scientific, USA). The number of cells showing fluorescence for both Dil and Hoechst was recorded for 3 × 10^4 gated events (Figure S8, Supporting Information).

**Immunofluorescence:** For BiNp uptake studies, Dil labeled ADSC-EVs or Lipo-NPs were incubated with HepaRG cells (seeded 24 h prior at 2 × 10^5 cells per well in 24-well plates) at 37 °C for 24 h, fixed with 4% paraformaldehyde for 30 min at room temperature (RT), and then permeabilized with 0.1% Triton X-100 for 5 min at RT. Cells were stained at RT with Alexa Fluor 488 phalloidin (Thermo Fisher Scientific, USA) which was a high-affinity filamentous actin (F-actin) probe conjugated to green-fluorescent Alexa Fluor 488 dye. Hoechst 33 342 (2 µg mL⁻¹) was incubated with cells for 15 min at RT for nuclear staining. Cells were washed and visualized with a fluorescent microscope (IX71 Olympus, Japan).

**Lipidomics:** For lipid analysis, lipids were extracted from Lipo-NPs and ADSC-EVs using the Bligh-Dyer method. The extraction was carried out by 2:2:2 (v/v/v) water/methanol/dichloromethane at RT after stripping internal standards (Table S2). The organic layer was collected and dried completely under a stream of nitrogen. Dried extracts were resuspended in 100 µL of solvent B and subjected to liquid chromatography–mass spectrometry (LC-MS). Chromatographic separation was performed on an ultra-high-performance liquid chromatography (UHPLC) system (Shimadzu, USA). For lipid separation, the lipid extract was injected onto a 1.8 µm particle diameter, 50 × 2.1 mm id Waters Acuity HSS T3 column (Waters, Milford, MA). Elution was performed using acetonitrile/water (40:60, v/v) with 10 × 10^{-3} u ammonium acetate as solvent A and acetonitrile/water/isopropanol (10: 5: 85 v/v) with 10 × 10^{-3} u ammonium acetate as solvent B and subjected to LC-MS. For chromatographic elution a linear gradient beginning with 60% solvent A and 40% Solvent B was used. The gradient was ramped in a linear fashion to 98% solvent B over the first 10 min and was then held at 98% B for 7 min. The composition was then returned to 40% solvent B and 60% solvent A and held for 3 min. The flow rate used for these experiments was 0.4 mL min⁻¹ and the injection volume was 5 µL. The column was equilibrated for 3 min before the next injection and run at a flow rate of 0.400 mL min⁻¹ for a total run time of 20 min. For lipid classification, including glycerolipids, glycerophospholipids, sphingolipids, glycosphingolipids, and fatty acyls.
for 1 h and then over an exosome spin column (Thermo Fisher Scientific, USA) to remove excess drug. Absorbance (GBZ: 5260 nm) or fluorescence (coumarin 6; λex: 457 nm/λem: 505 nm) was measured with a plate reader (Synergy HT; Biotek, Winooski, VT, USA) before and after removal of free drugs in order to calculate the encapsulation efficiency based on a standard curve.

Drug Release: After successful loading of Lipo-NPs with coumarin 6, 10% FBS with 0.5% Tween 80 in PBS was used to assess drug release as previously described. Briefly, coumarin 6-loaded Lipo-NPs were placed in a Float-A-Lyzer (Thermo Fisher Scientific, USA) with cellulose ester membranes that have a molecular weight cutoff of 50 kD, allowing free coumarin 6 to pass through. The membrane was then placed into 10% FBS with 0.5% Tween 80 in PBS (with 1% antibiotics to prevent bacterial growth). A heated (37 °C) magnetic stirrer was used to simulate physiological conditions. At set time points, 1 mL of solution was removed for analysis and replaced with fresh solution. The amount of released drug was measured as described above using a standard curve.

Protective and Anti-Inflammatory Effects: Ethanol and hydrogen peroxide (H2O2) were used as cell stressors to evaluate the protective role of Lipo-NPs on Raw 264.7 and Kupffer cells. Cells (seeded 24 h prior at 5 × 105 cells per well in 96-well plates) were exposed to B/NPs and ethanol or H2O2. After 48 h, cell viability was measured using an MTS assay (Promega, USA). For assessment of anti-inflammatory effects, Raw 264.7 and Kupffer cells (seeded 24 h prior at 105 cells per well in 96-well plates), as well as primary human macrophages (seeded 24 h prior at 106 or 107 cells per well in 96-well plates) were treated with 10−7, 10−8, or 10−9 mL Lipo-NPs or ADSC-EVs for 6 h, followed by exposure to LPS (1 μg mL−1) for 24 h. Levels of IL-6 in the cell culture media were measured with a Quantikine ELISA Kit (R & D Systems, USA) according to the manufacturer’s instructions. To determine the optimal dose of GBZ for treatment of Raw 264.7 cells (seeded 24 h prior at 104 cells per well in 96-well plates), various doses of GBZ were assessed, and cell viability was measured using an MTS assay (Promega, USA) after 24 h. The anti-inflammatory effects of GBZ-loaded Lipo-NPs (50 × 10−6 μg MBZ) was assessed in Raw 264.7 cells (seeded 24 h prior at 104 cells per well in 96-well plates) simultaneously exposed to LPS (0.5 μg mL−1) for 24 h. Levels of various cytokines in cell culture media were measured with a multianalyte ELISAarray kit (Qiagen, USA) according to the manufacturer’s instructions, which also states that the standard curves for all cytokines were virtually superimposable, indicating that the 12 assays provide similar linearity and sensitivity under the same standardized conditions and incubation times. Additionally, cell lysates from Raw 264.7 cells were detected by Western Blot with the following primary antibodies: anti-iNOS rabbit monoclonal (1:1000; #ab178945, Abcam, USA), COX-2 rabbit monoclonal (1:1000; #133 466, Abcam, USA), and anti-β-actin rabbit monoclonal (1:1000; #ab8226, Abcam, USA). Experiments were repeated three times.

Statistical Analysis: Experimental data was presented as mean ± SD and analyzed by GraphPad Prism software. The statistical significance between two samples or more was assessed by Student’s t-test or one-way analysis of variance (ANOVA), respectively.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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anti-inflammation, exosome, extracellular vesicles, inflammation, lipoprasite, microvesicle

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