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Short and Long Term, In Vitro and In Vivo Correlates of Cellular and Tissue Response to Mesoporous Silicon Nanovectors**

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Abstract

The characterization of nanomaterials and their influence and interactions on the biology of cells and tissues are still partially unknown. Multistage nanovectors based on mesoporous silicon have been extensively studied for drug delivery, thermal heating and improved diagnostic imaging. Here we analyzed the short and long-term changes occurring in human cells upon the internalization of mesoporous silicon nanovectors (MSV). Using qualitative and quantitative techniques as well as in vitro and in vivo biochemical, cellular and functional assays, we demonstrated that MSV did not cause any significant acute or chronic effects on cells and tissues.

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We analyzed in vitro cell toxicity and viability, the maintenance of cell phase cycling, and the architecture upon the internalization of MSV. In addition, we evaluated if MSV produced any pro-inflammatory response and studied its biocompatibility in vivo. We followed the biodistribution of MSV using longitudinal in vivo imaging and assessed organ accumulation using quantitative elemental and fluorescent techniques. Finally, a thorough pathological analysis of collected tissues demonstrated a mild transient systemic response in the liver that dissipated upon clearance of particles. In conclusion, with this study we propose that future endeavors aimed at understanding the toxicology of naked drug carriers should be designed to address their impact using in vitro and in vivo short and long-term evaluations of systemic response.

Keywords

Cancer Therapy; Drug Delivery; Mesoporous Materials; Nanomaterials; Toxicity

1. Introduction

Over the course of the last two decades, nanotechnology has grown to include an array of materials, structures, and solutions that have significantly impacted a variety of biomedical applications. These not only include cancer therapies,^[1] but also tissue engineering,^[2] neurodegenerative disease,^[3] cardiovascular,^[4] and many others.^[5-7] A critical aspect that needs to be addressed is the potential impact on healthy tissues (both in the short and long-term) as a consequence of the exposure to, contact with and internalization of nanomaterials. The basis of nanomaterial toxicity is dependent on multiple parameters, such as elemental composition, physical size and shape,^[8] surface and core chemistry, biodegradation,^[9] and tendency to aggregate.^[10] While the chemical and physical manipulation of particles (e.g., particle diameter, aspect ratio, porosity, rigidity, and surface topography^[11, 12]) allow the control of blood circulation kinetics and tumor accumulation rates, these properties might also promote potential toxicity by altering the cells' ability to clear the particles from the system.^[8] In addition, as new synthetic methods and bulk elements for the production and modification of nanomaterials are discovered, the potential permutations and combinations of nanoparticle properties, features and structures significantly increase.^[13, 14]

Despite multiple individual studies focusing on the toxicity and biocompatibility of nanoparticles, a comprehensive approach to address acute and chronic responses to nanomaterials both in vitro and in vivo is a formidable task, and a complete approach has not yet been defined effectively in the scientific community.^[15] For an adequate analysis of nanomaterial interaction with biological systems, parameters at the cellular and organ system levels must be investigated. For instance, particle distribution should be described both in terms of intracellular trafficking as well as biodistribution within all major body tissues. To garner a better understanding of in vitro cytotoxicity, substances such as (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are commonly used to assess the cytotoxic range of a compound and to quantify the proliferation rates of exposed cells.^[16, 17] However, for a complete picture of biocompatibility and toxicity, a multifaceted strategy is essential. For example, detailed information regarding the intracellular distribution and trafficking of particles can be obtained from in vitro studies via specialized stains of cytoskeletal elements such as actin and microtubules. These findings complement in vivo studies that identify whole body biodistribution and tissue accumulation of particles in living animals.

In this study, we put these correlates to the test using the porous silicon particles developed by our group. Silicon was selected as core material because of its extensive use in biomedical^[19] and industrial applications.^[20, 21] Its unique physicochemical properties

enable the production of precise nanopatterned structures with controlled features,^[11] high surface area ^[22, 23] and loading capacity,^[24, 25] tunable surface chemistries^[26] and release properties.^[27-29] In the last five years we developed a mesoporous silicon nanovector (MSV)^[30] core whose fabrication process not only imparted a high degree of biodegradability,^[31, 32] but due to the flexibility of the lithographic approach and of the electrochemical etching parameters, also allowed for the production of particles with enhanced cell surface adhesion along with superior blood margination rates.^[33, 34]

In this article, we introduce a comprehensive strategy to compare several in vivo and in vitro assays to examine the impact of MSV on intracellular and systemic distribution, cellular and tissue structure, cellular proliferation and toxicity, cell cycle analysis, and apoptotic induction. The results showed negligible cytotoxic effects in all assays supporting the use of MSV for biomedical applications.^[35]

2. Results

2.1 Particle Fabrication

Hemispherical MSV were fabricated by combination of standard photolithography and electrochemical porosification of silicon, allowing for accurate control over particle size and shape. Figure 1 illustrates the typical fabrication process of an hemispherical MSV by patterning an array of circles on a dielectric layer (silicon nitride) deposited on the silicon wafer, followed by a two-step electrochemical etch in a HF solution (Figure 1A-D). Fabricated MSV have aligned cylindrical nanopores at the center with a radial distribution of pores around the rings. Their geometry and porous structure were verified using SEM microscopy. Hemispherical MSV of 3.2 μm diameters (Figure 1E) containing small (SP) and large pores (LP) of 6 and 21 nm respectfully, were used in this study (Figure 1F,G).

2.2 Particle Internalization

Understanding the initial cellular interactions and distribution of MSV is critical for the assessment of acute responses due to the introduction of MSV in the system. Human umbilical vein endothelial cells (HUVEC) were exposed to different concentrations of MSV (Cell to MSV ratio equal to 1:5, 1:10, 1:20) for various incubation times (1 hour, 2 hour, and continuous) and measured fluorescence intensity using flow cytometry at 24, 48, and 72 hours (Figure 2A). Previous observations from our group have established that when exposed at a minimum ratio of 1:5 nearly 100% of HUVEC had internalized at least one particle. Thus, as the ratio of exposed MSV increased, a significant increase in cell fluorescence was observed indicating that the intracellular concentrations of MSV were increasing. The difference between incubation times did not appear to provide any significant advantage in the internalization rates suggesting that, even at the higher ratios, a majority of MSV were internalized within the first hour.^[14, 17, 36, 37]

Furthermore, the distribution of internalized MSV between daughter cells was investigated to determine the partitioning of MSV during cell proliferation. The dilution of MSV in the cell population over time was studied using flow cytometry and fluorescence microscopy. Inspecting the distribution at 24, 48 and 72 hours for all ratios studied we showed a minor decrease in overall fluorescence between 24 and 48 hours while a distinct decrease between 24 and 72 hours was observed (Figure 2B). This inverse relationship suggested that HUVEC were dividing and distributed MSV across their progeny while diluting the overall number of MSV per cell. Fluorescence microscopy analysis demonstrated an equivalent amount of fluorescent signal per field of view at different times (Figure 2C), indicating that the total number of MSV remained consistent and evenly distributed across the HUVEC progeny.

2.3 Cellular Proliferation and Toxicity

Although MSV were efficiently internalized and evenly distributed during cell division, no true indication of viability or response to internalization could be drawn from these experiments. Therefore, cells were analyzed with MTT and lactate dehydrogenase (LDH) assays to determine the potential cytotoxic response resulting from the internalization of MSV. Specifically, we investigated if HUVEC could proliferate (MTT) and if any abnormal increase in membrane damage could be detected (LDH). HUVEC were exposed from low (1:5, 1:10, 1:20) to high (1:50, 1:100) ratios of MSV and assayed for the incorporation and subsequent reduction of the MTT dye for 72 hours. Both low (Figure 2D) and high ratios (Figure 2E) exhibited minimal effects on the proliferation rate of HUVEC, compared to control cells. Surprisingly even at the 1:100 ratio, HUVEC demonstrated the unique capacity to maintain their ability to survive and divide. These results indicated that HUVEC could tolerate high volumes of MSV while retaining their ability to internalize and proliferate. HUVEC have an average volume of $2200 \mu\text{m}^3$ ^[38] while hemispherical MSV have a theoretical volume of $8.6 \mu\text{m}^3$. Hence, HUVEC can maintain their proliferative capacity despite a 40% increase in the total volume by MSV.

LDH was chosen as an in vitro marker for cell response to a cytotoxic damage. Typically, LDH is used as a clinical diagnostic tool to identify tissue breakdown and to assess general tissue damage.^[37] We investigated the effect of MSV at different ratios (1:1, 1:5, 1:10) and surface chemistries (hydroxyl-terminated, negative charge; amino-terminated, positive charge) on the release of LDH from HUVEC. After internalization, both hydroxyl-terminated (oxidized, Figure 2F) and amino-terminated (3-aminopropyl triethoxysilane or APTES, Figure 2G) MSV produced insignificant release of LDH compared to control HUVEC at all studied ratios. While the minimal increase of LDH release was due to the growth of cells in a highly confluent setting (see materials and methods), the comparable values registered in all tested groups suggested that MSV had a minimal impact on membrane integrity after internalization.

2.4 Cellular Integrity

While measuring the metabolic activity or the release of specific enzymes can be useful in understanding the overall effect of nanoparticles on cellular response, it fails to provide any detailed and real-time information on the specific events that occur within cells during MSV internalization. For example, the expression or translocation of phospholipids and the distribution of phases within the cell cycle provided more detailed information regarding cellular response to internalized MSV. During apoptosis, cells signal for their removal by phagocytic cells by translocating cytosolic phosphatidylserine (PS) lipids to the extracellular surface of cellular membranes. Annexin V a high affinity binding protein for PS was used to identify apoptotic cells. HUVEC were exposed for 1 hour, 4 hours, or continuously to different amounts of particles and analyzed using flow cytometry at 24, 48 and 72 hours (Figure 2H). The percentages of annexin V (i.e., apoptotic) events for all ratios and exposures at any given time were almost indistinguishable from control. As time progressed, a minor increase (~10%) was observed in annexin V positive events from 24 to 72 hours in all tested conditions thus confirming that MSV did not induce any significant increases in apoptotic events after internalization.

HUVEC were also analyzed to understand the distribution of the cell cycle phases upon internalization of MSV with different surface chemistry and structure (Figure 3). All cells analyzed demonstrated similar distribution in each phase after 72 hours. A minor discrepancy was observed at 12 hours between HUVEC with internalized MSV and control cells, where MSV treated cells demonstrated increased concentration in the sub G_0 (i.e., apoptotic/inactive cell phase). This difference was attributed to the different confluency of

control cells (70%), compared to MSV, which were treated at a confluency of 95% to ensure the attainment of the intended cell to particle ratios. In all later time points (> 24 hours) as control cells became more confluent the amount of cells detected in the sub G₀ and G₂/M (i.e., active/mitotic) phases were similar to those containing MSV. Further proof of this claim was observed by comparing the effect of MSV internalization to that of cis-platinum treatment. When this molecule was introduced to HUVEC, inducing apoptosis via DNA crosslinking, a significant increase was observed in the sub G₀ phase and a simultaneous decrease in the G₂/M phase. Due to the fast induction of an apoptotic fate in HUVEC, no cells were available at 48 and 72 hours (missing data points in Figure 3). Comparison of cis-platinum and MSV treated cells revealed a clear distinction suggesting that MSV did not induce an apoptotic-like reaction within HUVEC. In addition, comparable amounts of cells were identified in G₀/G₁ and S phases between control and MSV treated HUVEC after 24 hours, indicative for resting/checkpoint and DNA replication, respectfully. Additionally, no significant reduction or increase in cell cycle phases was observed when comparing the surface chemistry of hydroxyl or amino MSV (oxidized vs. APTES) or for different pore structure (SP vs. LP). Thus after internalization of MSV, cells were able to progress through the different cellular phases without altering DNA synthesis and allowed for their continued cellular division.

2.5 Cellular Architecture

Investigation of the cytoskeleton and intracellular distribution of particles was performed to demonstrate the influence of MSV on cellular architecture. HUVEC were exposed to MSV at 1:20 and cultured for one month. Samples were acquired one and thirty days post internalization. HUVEC were stained using common immunocytochemistry (ICC) techniques for f-actin (microfilaments, red) and alpha tubulin (microtubules, green), two critical cytoskeletal components indicative of the rearrangements that occur upon internalization. HUVEC containing internalized MSV displayed short and long-term stability of cytoskeletal filaments (**Figure 4A**). HUVEC retained a normal cytoskeleton organization with parallel and well-organized microfilaments (red) throughout the cell body and microtubules (green) originating from the peri-nuclear area and radiating throughout the cytoplasm. Microtubule staining at Day 1 in MSV treated cells is not prominent due to the dominant intensity of MSV, but when oversaturated the microtubule staining resembles that of control cells. However at Day 30, the microtubule staining is visible due to the decreased signal attributed to MSV (white arrow) due to its progressive surface degradation. A bright field image confirms the presence of MSV (Supplementary Figure 1).

Previous investigations demonstrated that MSV were internalized via the endolysosomal pathway [14, 17]. After internalization, MSV were transported to the peri-nuclear region of cells (Figure 4B) regardless to the number internalized. In addition, features of the nucleus such as its size and shape appeared to be unaffected by the internalization of MSV, preserving traits similar to those of control cells and providing further confirmation of the lack of cytotoxicity in HUVEC.

2.6 In vivo Cytokine Expression

In order to provide insight into the organ response and immunoreactivity upon administration of MSV, a profile of anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-6 and TNF- α) were collected before the establishment of a tumor, 15 days after tumor implantation, and 3 and 10 days after the treatment of the mice with MSV or saline (control). As shown in Figure 5, the induction patterns of selected cytokines appeared to differ, such that IL-1 β and IL-6 reached maximum levels earlier compared to IL-1 α and TNF- α . Differences between MSV treated and control groups across different time points stayed comparatively the same for certain cytokines. For example,

MSV treated group showed no significant difference in the expression of IL-1 β (Figure 5C), IL-7 (Figure 5E), and IL-10 (Figure 5F) compared to the control group. However, MSV appeared to induce pro-inflammatory cytokines in a time-dependent fashion up to 10 days after treatment. In this study, we observed that levels of TNF- α , IL-1 α , and IL-6 increased initially after injection (Day 3) yet were observed to be similar to levels of control by Day 10, suggesting a temporary and reversible misbalance in cytokine production.

2.7 Biodistribution

A fundamental understanding of the systemic distribution of MSV was critical to assess in what organs and tissues potential toxicities could occur. In order to investigate the biodistribution of MSV, near infrared (NIR) imaging and elemental analysis were used to visualize and quantify MSV accumulation in real-time.

2.7.1 Live Animal Longitudinal Imaging—MSV labeled with a NIR fluorescent dyes retained similar fluorescence over several days (Figure 6A) and displayed increasing fluorescence as the particle concentration increased (Figure 6B,C). Mice were intravenously administered with NIR labeled MSV and imaged longitudinally for fluorescent signal at 0, 0.5, 2, 24, 48, and 168 (1 week) hours (Figure 6D). Mice were sacrificed at 2, 4 and 168 hours and organs were collected and imaged (Figure 6E). The distribution of fluorescent signals was measured by creating regions of interest (ROI) around the abdomen, bladder, and tumor and then plotted against the time points collected (Figure 6F). Minimal autofluorescence from the abdomen was observed in mice at 0 hr. Upon administration, MSV were quickly concentrated in the abdomen and remained at relatively high concentrations up to 48 hours. The weak fluorescent signal registered in the bladder throughout the duration of the experiment indicated good retention of the NIR dye on the surface of the MSV. On the other hand, tumor accumulation increased in a linear fashion up to 24 hours when it reached a maximum and then plateaued between 48 and 168 hours.

2.7.2 Quantitative Biodistribution—Tissues were collected, washed with PBS and imaged for fluorescent intensity and quantified by creating ROI around each organ. Ex vivo quantitation of organs significantly reduces interferences from light scattering allowing for enhanced particle distribution analysis. The quantitation of fluorescent signal is shown in Figure 7A. Here we observed a significant increase of signal accumulation in the liver at the early time points (doubled between 2 and 4 hours) with a relatively constant amount recorded between 4 and 168 hours (1 week, 5% decrease). Other organs (lung, spleen and heart) were characterized by reduced intensities with maximum accumulation at 4 hours. Tumor tissue displayed the same trend but compared with 2 hours, the intensity at 4 hours increased by more than 3 times. Signal from the intestines was minimal at all times collected and thus not included. The same organs were then analyzed for elemental analysis of silicon as shown in Figure 7B. This type of analysis demonstrated almost equivalent concentrations of MSV in lung and liver with minor comparative decreases in the spleen and heart at 2 hours. At 4 hours, both the lungs and liver experienced substantial increases (4.5, 8 μg of silicon g^{-1} of tissue respectfully) while spleen and heart only increased a relatively small amount (1.75, 1.8 μg of silicon g^{-1} of tissue respectfully). At one week, traces of silicon were found within the lungs and heart, and increases of 5.4 and 2 μg of silicon g^{-1} of tissue within the spleen liver, respectfully. The tumor experienced a 200% increase (1.41 to 4.5 μg of silicon g^{-1} of tissue) from 2 to 4 hours, and a minimal decrease (0.7 μg of silicon g^{-1} of tissue) after one week. Overall, both quantitative methods provided valuable insight into the biodistribution of MSV confirming the NIR imaging data.

These results established that after an initial accumulation in many vital organs (heart, lung, spleen, liver), the biodistribution of MSV reached a long-term accumulation in the liver and the spleen.

2.8 Histological Analysis

The histological evaluation that included: Hematoxylin and Eosin, Ki-67 and TUNEL staining was performed to provide a deeper insight into the coordinated tissue response to MSV. Tissues were collected at 4 hours, 48 hours, 7 days, 2 months, 3 months, and 6 months to look for both acute and chronic responses. The results are presented in the sections below.

2.8.1 Hematoxylin and Eosin—Harvested Tissues were stained with hematoxylin and eosin (H&E) to understand the cytological and structural impact of MSV on tissues. The lungs exhibited alterations of normal tissue structure (Figure 8). This fact is particularly evident at 4 hours, where a prominent neutrophil infiltration of the lung, coupled with a collapse of the alveoli was observed. In addition, no sign of pulmonary edema or any other pathologic sign of importance was detected. After 48 hours (7 days, 2, 3, and 6 months) no evidence of any damage or other anomaly within the lung was observed. Examination of liver tissue revealed a complex pattern of pathological manifestations that deviated from normal tissue morphology. Beginning at 2 hours, liver tissue displayed an alteration of its normal architecture in the form of sinusoidal dilatation with a subsequent loss of sinusoid structure observed at 4 hours (Figure 8). At 48 hours, we detected a re-normalization of hepatic tissue with a return to normal sinusoid structure, that were similar to control pictures, but contained minor increases in the number of well limited small circular vesicles within the hepatocytes, suggestive of microvesicular steatosis.^[39] At 7 days we discovered improvements in the morphology, as the cytoplasm presented no vesicles. However, at 2 months we observed a mild alteration characterized by a decrease in sinusoid size and diffuse inclusions eosinophilic in nature scattered in a pattern resembling ground glass hepatocytes.^[40] At 3 months we noticed a clear regression of the previous pattern resulting in a return towards normal morphology with regular histological findings clearly distinguishable at 6 months (Figure 8). Heart tissue (Supplementary Information Figure 2) maintained myofibril organization and intercalary disks were intact while kidney (Supplementary Information Figure 2) showed typical glomerular structure across all time points. Within the spleen tissue, a slight increase of macrophages was observed in each field of view but did not contribute to any significant alteration of tissue morphology.

2.8.2 TUNEL—Terminal deoxynucleotidyl transferase dUTP nick end labeling, commonly known as “TUNEL staining”, is a common histological stain used to visualize apoptotic events. TUNEL targets DNA fragments and binds to the 3'-hydroxyl termini of DNA ends. In light microscopy, a positive TUNEL assay is visualized by a dark brown pigmentation indicating that cells have undergone apoptosis. If the sample is negative, the tissue will be absent of this dark brown pigment and remain unstained.^[41] Within the samples evaluated for this study, the TUNEL staining (Figure 9) was minimal for all tissues (lung, liver, spleen, heart and kidney) examined across all time points indicating an absence of any significant apoptotic cell death response.

2.8.3 Ki-67—In order to evaluate the proliferation of cells, tissues were stained with a Ki-67, a protein associated with cellular proliferation and closely related with the transcription of ribosomal RNA.^[42] In histological preparation, a positive Ki-67 staining is observed within the nucleus and results in a dark brown appearance. In this experiment, Ki-67 was used to determine if there was any regeneration of hepatocytes in response to liver tissue aggression. All liver samples were found to be negative for Ki-67 staining for all

times collected indicating the absence of liver regeneration response upon exposure to MSVs further suggesting the lack of MSV induced tissue toxicity (Figure 10).

3. Discussion

MSV represents a promising drug delivery platform capable of providing enhanced imaging and therapeutic applications upon the loading of nanoparticles.^[32, 43-45] In this study, the cytotoxicity due to MSV internalization was assessed in vitro using human primary endothelial cells (MTT, LDH, annexin V, cell cycle, ICC) and confirmed in vivo (cytokine expression, biodistribution, IHC). Though the primary use of MSV has been limited to cancer applications focused on the delivery of cytotoxic payloads, the results showcased here encourage its use for biomedical applications aimed at non-destructive treatments (i.e., tissue engineering, metabolic disorders, degenerative pathologies, etc.).

All in vitro assays performed, confirmed minimal detrimental effects upon the internalization of MSV. As a matter of fact, upon exposure with a MSV ratio of 100:1, HUVEC maintained their ability to divide effectively adjusting for the 40% volume displacement attributed to MSV. Furthermore, LDH analysis revealed a negligible release of this enzyme when compared to control levels indicating only minor tissue breakdown upon exposure to MSV. To look for any underlying damage of HUVEC, annexin v staining and cell cycle analysis was performed to evaluate apoptotic induction. Annexin V staining demonstrated a minimal expression of PS on HUVEC surface after 24, 48, and 72 hours with internalized MSV. Cell cycle analysis indicated a similar result, were only minor deviations were detected in the resting synthetic and mitotic phases. ICC on cytoskeletal confirmed preserved cellular architecture after short (day 1) and long-term (day 30) internalization of MSV. Taken together, these results established that MSV did not induce any appreciable increase in apoptosis, maintained normal cell division and DNA synthesis, and conserved cellular structure after internalization within HUVEC in the short and long-term.

The noninvasive NIR imaging of MSV biodistribution, showed MSV accumulating within the liver, lungs, spleen, and heart at early time points (Figure 6). After seven days, MSV were cleared from the lungs and heart but remained within liver and spleen. Cytokine evaluation indicated transient signs of a mild inflammatory response at early time points (Figure 5). The minor difference in TNF- α levels (4 pg mL^{-1}) between MSV treated and control levels were also found to be consistent with a mild response.^[46] IL-1 α , IL-1 β , IL-6 and TNF- α , all recognized to play a important role in the pathogenesis of chronic inflammatory lung disease,^[35, 47, 48] returned to basal levels by day 10, indicating a mild transitory response rather than a chronic pathology.

Liver tissues showed a complex pattern of progression that initially indicated a response characterized by dilation of sinusoid spaces and loss of tissue organization, signs of reversible liver damage.^[16] The transitory nature of this reaction is corroborated by the microvesicular steatosis^[39] that eventually disappeared by day 7 (Figure 8). Furthermore the characteristic eosinophilic “ground glass” hepatocytes, similar to those found in mild dose-dependent drug-induced liver toxicity,^[40] demonstrated a sub-chronic and reversible alteration of the liver possibly due to an initial interaction with particles or by the creation of byproducts from the breakdown of MSV that give way to a benign inflammatory response. This response was a transient and reversible state as the MSV degraded and eventually cleared from the tissues leaving them unaltered. These results could be observed only due to the comparison between explants at short (4 hours, 48 hours, and 7 days) and long-term (2, 3, and 6 months) time points thus demonstrating the importance of short and long-term correlations for a better understanding of the impact of systemically administered agents.

The current paradigm of toxicity assessment of novel biomedical materials is based on cell assays that only explore cellular viability and toxicity as a general feature of the whole cell population. In this paper we proposed to investigate the toxicity of MSVs, employing an in-vitro/in-vivo approach analyzing and correlating toxicity through multiple cellular and tissue assays. This type of analysis was directed beyond single cell response and towards a more holistic approach that looks at the complex relationships of coordinated organ response (liver, spleen, lung). Our study also highlights the coordinated studies involving live animal longitudinal imaging, cytokine expression and pathological evaluation aimed at better understanding the interaction of injected particles with the body and to facilitate their translation to later stages of research.

4. Conclusion

The low toxicity and multifunctional nature of MSV suggests that their use could be expanded into different clinical applications beyond the realm of cancer therapy into the fields of tissue regeneration and for the treatment of metabolic disorders. For these types of applications, it is crucial for the delivery vector to induce minimal toxicity in order to avoid potential hindrances to the therapeutic treatment. The assessment of MSV toxicity based on the combined use of in vitro and in vivo assays demonstrated that by comparing key information on cellular architecture, proliferation and apoptosis at short and long-term time points, it was possible to achieve a more coherent and insightful analysis of the impact of MSV upon systemic administration.

5. Experimental Section

Particle fabrication

Fabrication of MSV was performed in the Microelectronics Research Center at The University of Texas at Austin by modification of previously described protocols.^[11] Briefly, starting with a heavily doped p-type silicon wafer with resistivity of 0.005 ohm-cm (Silicon Quest, Inc., Santa Clara, CA) as the substrate, a layer of 100 nm low-stress silicon nitride was deposited on the wafer in a Low Pressure Chemical Vapor Deposition (LPCVD) furnace (Figure 1A). Standard photolithography was then performed to pattern arrays of 2 μm circles over the wafer by using a contact aligner (SUSS MA6) and AZ5209 photoresist. Patterned silicon nitride was selectively removed by CF_4 Reactive Ion Etching (RIE) (Figure 1B). SF_6 based RIE was followed to etch 600 nm deep trenches into particle patterns (Figure 1C). The silicon nitride on the backside of the wafer was also removed by RIE. After the photoresist was stripped in a piranha solution, the wafer was assembled in a Teflon cell for the two-step electrochemical etching (Figure 1D). SP MSV were formed in a mixture of HF (49%) and ethanol (1:1 v/v) by applying a current density of 2mA cm^{-2} for 120s. A high porosity release layer was followed by applying the current density of 110mA cm^{-2} for 6 s in a 2:5 v/v mixture of HF (49%) and ethanol. LP MSV were formed in a mixture of hydrofluoric acid (HF) and ethanol (3:7 v/v) by applying a current density of 20mA cm^{-2} for 40 s. Finally, the nitride layer was removed in HF, and porous silicon particles were released by ultrasound in isopropyl alcohol (IPA) for 1 min. The morphology of the particles was examined by SEM imaging.

Particle modification

MSV were modified with hydroxyl groups via piranha oxidation and amino terminated via APTES, as previously demonstrated.^[30, 49] Briefly, MSV were re-suspended in a 3:1 solution of concentrated sulfuric acid to hydrogen peroxide (30%) and reacted for 2 hours. MSV were allowed to cool down, diluted with Millipore water, centrifuged at $4000 \times g$ for 10 minutes. Piranha solution was removed and replaced with water and re-centrifuged. This

process (i.e., washing) was repeated three times in water, followed by three times in IPA. Oxidized MSV were modified with APTES by reacting 1×10^8 MSV in an APTES (2%) solution in IPA for 2 hours at 35 °C with constant mixing. APTES modified MSV were washed three times in IPA and dried under vacuum overnight. APTES modified MSV were modified with fluorescent dyes for flow cytometry and live animal imaging using DyLight® 488 and 800, respectfully. This dye conjugation was accomplished by reacting 50 µg of dye with 1×10^8 MSV in dimethyl sulfoxide (DMSO) for 2 hours at room temperature under rotation. After incubation, MSV were washed three times in DMSO and IPA and stored at 4°C under needed for experiments.

Cell culture

HUVEC were purchased from LONZA and maintained using endothelial basal media-2 supplemented with EGM-2 SingleQuots (LONZA). HUVEC were sub-cultured, or passaged, near 80-90% and used for experiments between passages 3-10. Unless specified, cells pre-treated with MSV were prepared in by seeding 75,000 cells cm^{-2} HUVEC into culture treated vessels and allowed to adhere for 4 -10 hours to achieve a highly confluent (>95%) monolayer of cells. Thereafter, MSV were added at the desired ratio and were incubated for at least 4 hours, unless otherwise noted.

Internalization of MSV

DyLight® 488 MSV at varying ratios (1:5, 1:10, 1:20) were exposed to HUVEC for 1 hour, 4 hours, or continuously and collected at 24, 48 and 72 hours to understand internalization using a FACSCalibur (BD), as previously demonstrated [36]. Internalization was assessed by analyzing the fluorescence collected using FL1 and graphed by comparing the Mean fluorescence at each time point.

Annexin V staining

HUVEC were exposed to MSV at varying ratios (1:5, 1:10, 1:20) for 1 hour, 4 hours, or continuously and collected at 24, 48 and 72 hours, including cellular debris. Collected cells were stained with Alexa Fluor 555 annexin V (Invitrogen), and analyzed following manufactures recommendations. Briefly, HUVEC were harvested and washed with ice cold PBS. An annexin-binding buffer was prepared by combining HEPES (10 mM, Invitrogen), NaCl (140 mM, Sigma Aldrich), CaCl_2 (2.5 mM, Sigma Aldrich), and adjusted to pH 7.4. Recovered cells were re-suspended in annexin-binding buffer (100 µL). Annexin V (5 µL) was added to cell suspensions (100 µL) and incubated at room temperature for 15 minutes. Annexin-binding buffer (400 µL) was carefully added and HUVEC were gently mixed and kept on ice until analysis could be performed on a FACSCalibur.

MTT Assay

Cells were examined for continued proliferation through the active incorporation of 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide (MTT) dye (Invitrogen) in dividing cells. Briefly, cells (5,000) per well were seeded into a 96 well plate. At pre-determined times, cells were treated with 0.5 mg mL^{-1} of MTT (200 µL) in complete media for two hours. After which, the media was completely removed and incubated with DMSO (200 µL) for 30 minutes. Plates were then measured on a Synergy H4 plate reader at OD of 570 nm.

LDH Assay

A lactate dehydrogenase (LDH) assay (Abcam) was used to study the in vitro release of LDH of cells after internalization of PSP, following the instructions from the manufacturer. Briefly, cells were allowed to internalize PSP overnight, and were counted to next day to be seeded in 96 well plates (5,000 per well). At each time-point, cellular media (100 µL) was

removed and mixed with the LDH Assay mix (100 μ L) for 30 minutes at room temperature. After incubation with LDH reagents, 96 well plates were measured on a Synergy H4 (BioTek) plate reader at OD of 450 nm.

Cell Cycle Analysis

HUVEC treated with PSP (or 50 μ g mL⁻¹ of cisplatin) were analyzed at pre-determined times to understand their effect on cell cycle using established methods.^[17] Briefly, cells were collected and fixed in ethanol (70%) at 4°C for 10 minutes and subsequently stored at -20°C for at least 30 minutes. A 50 μ g mL⁻¹ propidium iodide (PI) solution was prepared in a Tris buffer (10 mM) at pH 7.3 containing MgCl₂ (5 mM), sterile filtered, and stored at 4°C. For every 10⁶ cells, one mL of PI solution was slowly added under vigorous manual tapping followed by 50 μ L of a 1.5 mg mL⁻¹ solution of RNase I in distilled water. Cells were incubated at 37°C for one hour, followed by three washes in ice cold PBS. Cells were then re-suspended in PBS (200 μ L) and analyzed using a Becton Dickinson FACSCalibur.

Immunocytochemistry (ICC)

HUVEC were stained for actin and tubulin to further assess the effect of internalization of PSP on cytoskeletal elements. HUVEC were seeded into chambered glass slides and stained for actin and tubulin using Alexa Fluor-555 Phalloidin (Invitrogen) and a FITC mouse monoclonal antibody to alpha tubulin (Abcam), respectfully. Cells were prepared for staining based on manufacturer's recommendation. Briefly, cells were fixed with in paraformaldehyde (4%) in PBS for 10 minutes, followed by permeabilization with Triton X-100 (0.2%, Sigma) in PBS for 10 minutes. Cells were then blocked for 30 min at room temperature in bovine serum albumin (1%, Sigma Aldrich) in PBS containing Tween-20 (0.05%, Sigma Aldrich), followed by hour incubation with the alpha tubulin antibody at room temperature. Cells stained with phalloidin were fixed and permeabilized as described earlier and blocked in bovine serum albumin (1%) in PBS for 10 minutes at room temperature followed by an 20 minute incubation with phalloidin (2.5%) in PBS. Cells were then mounted using Prolong Gold (Invitrogen) with DAPI and allowed to dry overnight before examination with a Nikon Eclipse 80i equipped with an Andor monochrome camera.

Animal Care

Animal studies were performed in accordance with the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals based on approved protocols by The University of Texas M.D. Anderson Cancer Center's Institutional Animal Care and Use Committee. Female athymic nude mice (NCR-Fox1^{nu}; 4-6 week old) were purchased from Charles Rivers Laboratories and maintained as previously described.^[43] When used with tumors, mice used for experiments were carefully implanted with orthotopic breast cancer models by injecting 4T1 (5 $\times$ 10⁵) mouse adenocarcinoma cells. Mice were either treated with DyLight® 800 MSV (5 $\times$ 10⁷) in sterile PBS (100 μ L) or PBS (100 μ L).

Cytokine Analysis

The cytokine activity was measured to understand any potential toxic side effects. Mice were randomly separated into either MSV or PBS (control) treated groups ($n=6$). Blood was collected from mice via retro-orbital bleeding prior to tumor implantation, after tumor implantation, three and ten days after injection of MSV or PBS, and stored in heparin-coated tubes at -80°C. IL-1a, IL-1b, IL-6, IL-7, IL-10 and TNF- α were determined using the MILLIPLEX MAP Mouse Cytokine/Chemokine premixed Immunoassay plate (Millipore, MPXMCYTO70KPMX, Temecula, CA, www.millipore.com).

Live animal imaging and quantification

For biodistribution studies, nude mice ($n=4$) with 4T1 tumors were randomly divided into groups for sacrifice at 2 hours, 4 hours and 1 week. Each mouse was injected with MSV (5×10^7) modified with DyLight 800, as previously described.^[50] At pre-determined times, mice were imaged for distribution of MSV using an IVIS Lumina (Caliper Life Sciences) equipped with the indocyanine green excitation and emission filters. Mice were sacrificed at 2 hours, 4 hours and one week. Organs were harvested, washed in PBS, imaged, and then weighed. Images and data were then analyzed using the Living Image software.

Inductively Coupled Plasma – Atomic Emission Spectroscopy

Weighed organs were frozen until ready for elemental analysis of Silicon using ICP-AES, as previously described.^[50, 51] Organs were homogenized in ethanol (20%, 3 mL) in NaOH (1 M) for 48 hours at room temperature under rotation. Tissues were then centrifuged at $4000 \times g$ for 30 minutes and supernatant (1.0 mL) was collected and diluted with Millipore water (4.0 mL) for elemental analysis. ICP-AES was performed using a Varian Vista-Pro housed within Rice University's Geochemistry Laboratory.

Immunohistochemistry (IHC)

Mice were intravenously injected with 5×10^7 MSV (or PBS) in sterile PBS (100 μ L). Mice were sacrificed at 4 hours, 48 hours, 7 days, 2 months, 3 months, and 5.5 months and tissues were collected for IHC.

H&E and Ki-67

Excised tissues were fixed in formalin (10%) overnight and then embedded into paraffin. Sections intended for H&E, Ki-67, and CD204 (i.e., macrophage) were deparaffinized with xylene, followed by re-hydration with decreasing concentrations of alcohol, and then briefly washed in water. Sections were separated for H&E, Ki-67, and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection.

Ki-67 staining

Tissues were stained with antibodies for Ki-67 (Abcam) and processed with STAT-Q IHC staining system (Innovex Biosciences) with DAB and counterstained with hemotoxylin, as described within the manufactures instructions. Briefly, target retrieval was accomplished by incubating cells in citrate buffer for 30 minutes, followed by a 10 minute treatment with hydrogen peroxide (3%). Tissues were then washed with PBS and incubated for 20 minutes at room temperature, followed by incubation with primary antibody for one hour at room temperature. Tissues were washed thrice with PBS and then incubated with biotinylated secondary antibody for 10 minutes, followed by incubation with streptavidin label for 10 minutes. Lastly, tissues were incubated with DAB/substrate solution for 5 min, counterstained with hemotoxylin, and mounted with a glass coverslip.

TUNEL

Tissues were processed for apoptosis detection using the TUNEL method via the TdT-FragEl DNA fragmentation detection kit (Oncogene Research Products) as described within the manufactures instructions. Briefly, deparaffinized tissues were permeabilized with Proteinase K (1:100) for 20 minutes at room temperature, followed by washing and incubation with 3% hydrogen peroxide for 5 min. Tissues were then incubated with TdT equilibration buffer for 10 minutes prior to labeling with TdT for 90 minutes at 37 °C. After the allotted time, labeling was stopped by quickly rinsing the slide with TBS and incubated with the stop solution for 5 minutes at room temperature followed by wash. Tissues were then blocked using the included blocking buffer for 10 min, followed by a 30 minute

incubation with the conjugate (1x) at room temperature. Lastly, tissues were rinsed with TBS, incubated with DAB solution for 15 minutes, and mounted with a glass coverslip.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MSV Fabrication. Schematic of MSV fabrication is demonstrated here. A) Deposition of a low-stress silicon nitride film (brown). B) Photolithographic patterning of arrays of circles and Reactive Ion Etching through nitride film. C) Etching trenches into silicon to tune the shape of porous silicon particles. D) Two step electrochemical etching in HF based solution to make arrays of porous silicon particles. E) SEM image of a cluster of 3.2 μm hemispherical porous silicon particles. F) SEM close-up view of porous structure of SP particles. G) SEM close-up view of porous structure of LP particles.

Figure 2.

In vitro assessment of MSV exposure to HUVEC. A) Internalization of MSV was assessed using flow cytometry, comparing mean fluorescence of HUVEC at various ratios and incubation times at 24, 48, and 72 hours. B) Dilution of MSV at 24, 48, 72 hours for various ratios which demonstrated decreased fluorescence with time. C) Fluorescent images of HUVEC with internalized MSV at 24, 48, and 72 hours displaying similar fluorescence across all fields indicating dilution of MSV to daughter cells. D) Proliferation of HUVEC was examined at low (left) and high (right) concentrations of MSV by examining the enzymatic reduction of the MTT dye by HUVEC. E) Cellular toxicity was detected using an LDH assay to compare the impact of various ratios and surface chemistry. Oxidized/negative (left) and APTES/positive MSV (right) were evaluated for LDH. F) Annexin V staining of HUVEC with internalized MSV at 24, 48, and 72 hours demonstrating minor increases over time, consistent with control cells indicating minimal induction of apoptosis.

Figure 3.

Cell cycle analysis of MSV impact. HUVEC were exposed to MSV and samples were taken at 12, 24, 48 and 72 hours for cell cycle analysis. Untreated and cis-platinum-treated HUVEC were used to compare the effect of treating cells with MSV with varying surface chemistry (oxidized and APTES) and pore sizes (SP vs LP).

Figure 4.

Cellular architecture and intracellular distribution of MSV. A) The cytoskeleton of HUVEC were stained for microfilaments (f-actin, red), microtubules (α -tubulin, green), and nucleus (blue) to understand the effect of MSV (labeled in green) incorporation. Images were acquired after one and thirty days, which indicated preserved cellular structure after a month of internalization. White arrow identifies MSV at Day 30. B) Intracellular distribution and cell status were confirmed upon inspection of nuclear (blue) stain and labeling of MSV (green). MSV were found to accumulate in the peri-nuclear region while maintaining the integrity and features of normal nuclei. Scale bar = 5 μ m.

Figure 5. Blood Cytokine Analysis

Blood was collected from mice via retro-orbital bleeding for various time points (Pre-tumor, Post-tumor, 3 days, 10 days), various cytokines were analyzed: A) TNF- α , B) IL-1 α , C) IL-1 β , D) IL-6, E) IL-7, F) IL-10.

Figure 6.

Noninvasive live animal imaging of MSV distribution. A) MSV were labeled with a NIR dye and B) showed increasing fluorescent signal during serial dilution. C) Left axis, green: MSV demonstrated intensified signal as the concentration increased; Right axis, white: the supernatants collected after washing demonstrated decreases in dye concentration per MSV wash. D) Longitudinal imaging of mice using noninvasive NIR optical imaging. E) Organs (from top: Liver, Spleen, Heart, Lung, and Tumor) were harvested, imaged and quantified using NIR imaging. F) Quantification of the abdomen, bladder and tumor were collected in real-time by creating ROI around the specific regions within the mice and intensities were graphed against time.

Figure 7.

Quantification of MSV biodistribution. A) The percent-injected dose detected per organ was determined by quantification using imaging software from harvested organs at 2 hours, 4 hours and 7 days (168 hours). Intensities from organs matched that observed in mice during real-time. B) ICP-AES was used to quantitate the amount of elemental silicon detected in organs collected above. Values were expressed as, μg of silicon per g of tissue and were normalized to control mice that were not treated with MSV.

Figure 8.

Histological evaluation of tissues using H&E staining. Lung, liver, and spleen were stained with H&E. Images in the lung revealed neutrophil infiltration at 48 hours (arrows) present in the lung tissue. Liver tissue showed microvesicular steatosis at 48 hours (circle) and evidence of “ground-glass hepatocytes” at 2 months (square) with normal hepacyte structure by 5.5 months. Spleen exhibited no pathological findings of interest at all time points collected.

Figure 9.

Apoptotic events in tissues. : Lung, liver, and spleen were processed for apoptosis detection using the TUNEL detection method. Tissue staining was negative for lung and liver. Spleen sections displayed minor staining but were within normal range for this tissue.

Figure 10.

Liver tissue proliferation. Liver sections were collected at short (2 and 4 hours) and long-term (2, 3, and 5.5 months) time points and stained for Ki-67 at several time points. At all time points, Liver tissue exhibited minimal staining for Ki-67 of hepatocytes, ruling out the possibility of a regenerative response upon the administration of MSV.