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Bioactivity and Cell-Mediated Targeting of Multistage Nanoporous Silicon Particles

Jonathan O. Martinez

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BIOACTIVITY AND CELL-MEDIATED TARGETING OF MULTISTAGE NANOPOROUS SILICON PARTICLES

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BIOACTIVITY AND CELL-MEDIATED TARGETING OF MULTISTAGE NANOPOROUS SILICON PARTICLES

A DISSERTATION

Presented to the Faculty of
The University of Texas
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in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by
Jonathan Otto Martinez, B.S.
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May, 2014
Dedication

To my uncle, Orlando Gomez
To my grandfather, Otoneil Gomez
To my mother, Esperanza
    To my father, Raul
    To my brother, Daniel
Acknowledgments

I would like to begin by thanking my principal off-site advisors Dr. Mauro Ferrari and Dr. Ennio Tasciotti for their support and mentorship since joining the lab in May 2008. I have felt extremely privileged to be under their tutelage as they have guided and framed my way of thinking and becoming the scientist I am today. In particular, with their assistance, I have made great strides in significantly improving my presentation and writing skills, which are essential to achieve long-term success as a scientist. In addition, I would like to thank Dr. David Gorenstein for agreeing to serve as my on-site advisor at UT Health and providing me with the resources and assistance necessary to complete my Ph.D. training at UTHSC.

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Amy Wright, Ashley Torregrossa, James Gu, Aaron Mack, Vivek Kuran, Evan Shegog, Abe Wang, and Ari Faust.
Progress in drug delivery approaches have not adequately translated into clinical advances in the diagnosis or treatment of inflammatory disorders (e.g., cancer). This disconnect is rooted in the inefficient delivery of imaging and therapeutic agents to the inflamed site upon systemic delivery. A multitude of biological barriers pose insurmountable obstacles limiting the ability of the agent to effectively reach and accumulate at the target site. Nanoparticles (NP) surfaced as potential vectors to encapsulate and deliver biological agents. However, even after surface decoration, NP have failed to evade biological barriers (i.e., MPS) and to accumulate at the tumor site at therapeutic dosages, resulting in toxicity to healthy organs and other side effects.

The objective of these studies was to characterize multistage nanoporous silicon particles (NSP) for the loading and release of NP, degradation, and biocompatibility. Furthermore the development of novel solutions to redirect the biodistribution of NSP by using antibodies for vascular endothelial growth factor receptor 2 (VEGFR2) to enhance vascular targeting and relying on the natural tumor tropism of mesenchymal stem cells (MSC) to achieve dynamic targeting were investigated. Briefly, successful modulation of the release kinetics was achieved by adjusting the pore size of NSP regulated by the degree of
penetration. In addition, by adjusting the pore size the degradation rates of NSP were significantly increased and removal of the nucleation layer triggered premature collapse of the pores resulting in a less stable NSP. NSP failed to alter advanced cellular functions (e.g., tube formation, multi-potent differentiation) and systemic administration did not any significant trigger immune response. Functionalization with α-VEGFR2 endowed NSP with a 5-fold increase in the specificity of targeting VEGFR2 allowing for enhanced docking and stable adhesion to tumor vasculature. Incorporation of NSP within MSC conserved their innate functions, including differentiation, interaction with inflamed endothelia, and homing towards cancer and inflammatory sites. In addition, NSP loaded with nanoparticles permitted MSC to be activated remotely and carry a toxic formulation while incorporation within MSC avoided sequestration by the MPS. These delivery strategy aims to direct therapy specifically to the tumor, thereby limiting harmful exposure to healthy tissues.
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Abbreviations

APTES – 3'-aminopropyltriethoxysilane
ADMSC – Adipose derived Mesenchymal Stem Cell
BLI – Bioluminescent Imaging
DOX – Doxorubicin
EPR – Enhanced Permeability and Retention
FACS – Fluorescence Activated Cell Sorting
FSC – Forward Scatter
FTIR – Fourier Transform Infrared Spectroscopy
GFP – Green Fluorescent Protein
H&E – Hematoxylin and Eosin
HAuNS – Hollow Gold Nano Shells
HUVEC – Human Umbilical Vein Endothelial Cell
ICP-AES – Inductively Coupled Plasma – Atomic Emission Spectroscopy
ID – Injected Dose
IHC – Immunohistochemistry
IVM – Intravital Microscopy
LDH – Lactate Dehydrogenase
M-DOX – Micelle Doxorubicin
MPS – Mononuclear Phagocyte System
MPVEC – Mouse Pulmonary Vein Endothelial Cell
MSC – Mesenchymal Stem Cell
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIR – Near Infrared
NP - Nanoparticles
NSP - Multistage Nanoporous Silicon Particles
PAEC – Porcine Aortic Endothelial Cell
PB – Phosphate Buffer
PBS – Phosphate Buffered Saline
PS – Phosphatidylserine
pSi – Porous Silicon
QD – quantum dot
ROI – Region of Interest
SEM – Scanning Electron Microscopy
SMCC – Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SSC – Side Scatter
TEM – Transmission Electron Microscopy
TNFα – Tumor Necrosis Factor alpha
TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGFR – vascular endothelial growth factor receptor
α-VEGFR – anti VEGFR
YFP – Yellow Fluorescent Protein
Chapter I: Introduction

1.1 Current cancer therapy

In 2005 cancer surpassed heart disease as the leading cause of death in the United States in the population of 85 years and younger [1] and second overall accounting for 1 of every 4 deaths [2]. It is associated with a lifetime risk of 1 to 2 in men and 1 to 3 in women [2]. For 2014, over 230,000 new cases of breast cancer and approximately 40,000 deaths are expected [3, 4]. This ranks breast cancer first in new cases accounting for approximately 29% of new cases and the second leading cause of mortality in females, corresponding to an expected 15% of total deaths for 2014 [2, 4] (Fig 1.1). Progress in cancer treatment and management has been hindered by the inability of current chemotherapeutic agents to accumulate at the tumor site at therapeutically relevant dosages. In general, cancer treatments have relied heavily on non-targeted agents that have resulted in minimal clinical successes, largely due to the limited concentrations that amass in tumors and the undesired side effects on healthy normal tissues [5]. Breast cancer is a remarkable exception, showing promise in therapies targeted at factors over-expressed at the tumor site, and is commonly treated with hormone and biological (i.e., monoclonal antibodies) therapies [3]. Equally important to therapy is the timely and early detection of tumors, which is crucial to prevent the progression of advanced cancers and to diagnose cancer relapse post-therapy. Current noninvasive imaging relies on the use of contrast agents that take advantage of increased metabolic and amino acid metabolism within tumors, but these are limited by background noise and nonspecific uptake [6]. Thus, the development of novel targeted approaches with the potential of delivering therapeutic and diagnostic agents (i.e. theranostic modalities) directly to the tumor site are urgently needed.
1.2 Inflammation

Inflammation is a physiological response to foreign and harmful stimuli, such as infections, toxins and tissue injury, inducing a complex signaling cascade. Typically these foreign bodies are removed or surrounding tissues are repaired. However if the stimulus persists, this irritation can result in a variety of pathological disorders such as arthritis, heart disease, and cancer progression. The delivery of active agents directly to sites of inflammation has the potential to provide effective treatment and diagnosis of the disease. In general, few tumors are influenced solely by cytotoxic or mitogenic mechanisms but rather are largely dependent on mechanisms of inflammation to drive clonal expansion [7]. Furthermore, inflammation plays an essential role following injury and during the process of repair [8]. The inflammatory process is often regarded as the first of several ordered processes that must occur for timely wound repair. To re-achieve tissue integrity, the process to achieve proficient healing can be separated into three critical phases: inflammation, tissue formation, and tissue remodeling [8]. The inflammation phase is characterized by platelet aggregation resulting in infiltration and extravasation of leukocytes into the wound site. This infiltration of immune cells plays an instrumental role by interacting with extracellular matrix molecules, soluble mediators, and various resident cells for initiating the remodeling and deposition of structural components required for the continuation in healing the wound [8].

Similar to cancer, wound repair has suffered from the timely and spatially delivery of agents. The focus of wound repair has recently seen a shift, and the evaluation of factors that enhance the recruitment of vascular and stromal cells during the inflammatory process has stimulated a wide interest in the scientific community [9-12]. Neovascularization plays a critical role in the process of wound healing. Vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) play essential roles
for the timely and complete repair of the wounded site and aid in the progression of the healing process [8, 13]. Thus, for effective cancer and tissue injury therapy there is a huge demand for the development of novel targeted approaches with the potential of delivering therapeutic and diagnostic agents directly to the intended site in a timely fashion.
1.3 Nanotechnology

Nanoparticles have emerged as promising platforms capable of delivering cytotoxic and imaging agents to tumor sites at efficacious doses, all the while minimizing adverse side effects [14-16]. However, to reach the tumor site nanoparticles must be endowed with the proper tools to bypass the natural defenses of the body, often referred to as biological barriers (e.g., mononuclear phagocyte system (MPS), osmotic pressure, macrophage uptake, and the endothelial barrier) [17, 18] (Fig 1.2). Due to their shape, surface charge, and inadvertent environmental activation, nanoparticles fail to reach the tumor site, impeded by the several biological barriers encountered [18, 19]. Advances in nanoparticle synthesis and conjugation schemes has opened new avenues that impart properties on nanoparticles to partially circumnavigate biological barriers by functionalizing their surface with polyethylene glycol and adjusting their size to exploit the enhanced permeability and retention (EPR) effect [5, 15]. The EPR effect allows nanoparticles to passively accumulate in tumors by taking advantage of the fenestrations occurring in tumor-associated vessels [19]. On the other hand, active targeting can be achieved by coating the surface with targeting moieties such as antibodies, peptides, aptamers, and phages [20]. In spite of active targeting, nanoparticles have failed to accumulate at tumor sites at therapeutically relevant concentrations.

Metal-based nanoparticles offer a unique advantage for cancer therapy. Advances in technology have bestowed unique properties onto metal particles in the form of noninvasive hyperthermal therapy in addition to conventional drug delivery and imaging applications [21]. For cancer therapy, heat has been used dating back several hundreds of years ago [22, 23]. As a matter of fact, tumors typically display a higher sensitivity towards changes in temperature compared to normal tissue [24]. Metal-based nanoparticles have spurred interest because of their ability to provide site-specific thermal therapy upon excitation with near infrared (NIR) light [25-28], magnetic field [29], and radiofrequency [30, 31]. The NIR
window (680-900 nm) (Fig 1.3) represents a unique portion of the electromagnetic spectrum that allows for deeper tissue penetration due to the reduction of the absorption and scattering of photons by biological molecules [32, 33]. This distinct feature enables in vivo diagnostic and therapeutic applications with minimally invasive procedures. Of the various available agents that take advantage of NIR, gold (Au) nanoshells are being tested in clinical trials for the treatment of primary and metastatic lung tumors [26, 34-37]. Au nanoshell-based thermal therapy in combination with radiation proved to have a synergist effect on human breast tumors yielding a pronounced ablation of the cancer stem cell sub-population [38]. The success of these gold nanoshell platforms prompted the development of smaller nanoparticles (< 50 nm) with a hollow core, or hollow gold nanoshells (HAuNS) [27, 28, 39].

Hence, for nanoparticles to evolve as effective solutions for systemic administration, they must overcome these biological barriers, all the while retaining, their selectivity and functionality for the tumor site. The effective treatment of patients whose tumor has metastasized and spread to distant sites continues to be associated with poor prognosis and lower percentages of survival. Advances in nanotechnology, drug delivery, cancer cell biology, and drug development have not produced specific tools capable of selectively treating metastasis while ignoring normal tissue. Thus, given the complexity and the sequential and synergistic mode of action of biological barriers, it is reasonable to believe that bestowing a single NP with all the necessary tools to achieve its intended goal is practically impossible [40].
**Figure 1.2 Biological barriers that impede the delivery of nanoparticles.** Several barriers stand in the way of nanoparticles from reaching their intended site. This schematic illustrates several barriers, including MPS, endothelial and nuclear membranes, ionic pumps, abnormal blood flow, and pressure gradients. Reproduced with permission from Nature Publishing Group, taken from Sanhai, W.R., J.H. Sakamoto, R. Canady, and M. Ferrari, *Seven challenges for nanomedicine.* Nat Nanotechnol, 2008. **3**(5): p. 242-4.
Figure 1.3 NIR window for imaging and therapy. The window between 650 and 900 nm is optimal for imaging and therapy as the light absorption by water and hemoglobin is minimal. Reproduced with the permission from Nature Publishing Group, taken from Weissleder, R., *A clearer vision for in vivo imaging*. Nat Biotechnol, 2001. **19**(4): p. 316-7.
1.4 Multistage delivery strategy

The challenge is to develop novel vectors capable of protecting and transporting therapeutic and diagnostic agents to the tumor site. Using advanced mathematical modeling of microvasculature and blood flow dynamics [41-44], our laboratory rationally designed and fabricated a novel class of particles developed to successfully protect and ferry nanoparticles, upon where they will recognize and dock at the tumor vasculature [45, 46], as illustrated in Figure 1.4. The objective of the particles was to decouple the multitude of tasks that are required of single agents to negotiate biological barriers and distribute them among multiple stages [47]. Within this scheme, the first-stage is responsible for carrying, distributing, and protecting subsequent stages; navigating the blood system while shielding themselves from MPS sequestration. Ultimately, the first stage will recognize and anchor at the tumor site [47]. Embedded within the first-stage are second-stage NP, which once released at the tumor site can penetrate and diffuse through the fenestrations of blood vessels [47]. Due to its biocompatibility [48-52], degradability [53-56], and the ability to adapt their size, shape, porosity, pore size, and surface properties [57-59] (Fig 1.5), porous silicon (pSi) was chosen as the material for the first stage. These multistage nanoporous silicon particles (NSP) are capable of loading and releasing a diverse set of nanoparticles including, but certainly not limited to, quantum dots (QD) [46], superparamagnetic iron oxide [60, 61], carbon nanotubes [46, 62], fullerenes [62], liposomes [63] and gold nanoparticles [64]. Other studies have demonstrated the ability of porous silicon to load chemotherapeutic molecules [56, 65, 66], diagnostic agents [67, 68], anti-inflammatory drugs [69], steroids [70] and proteins [71]. Moreover, by taking advantage of the tunable porosity and surface properties of porous silicon, the embedded payload can be tailored to effectively control its release [66] and tune the degradation of silicon particles from hours to days [72]. However, NSP have not reached the tumor site in sufficient concentration. The incorporation of a NP payload within the NSP enables the emergence of unique therapeutic and diagnostic
attributes, such as prolonged gene silencing [73-75], enhanced magnetic relaxivity [76], and increased hyperthermal therapy [77]. However, to overcome the sequestration by MPS and promote the selective accumulation at primary and metastatic sites, novel strategies are urgently needed to redirect NSP therapeutics upon systemic administration.
Figure 1.5 Versatile nature of NSP. The flexibility of NSP allow for simple adjustments in fabrication parameters to generate particles with various size/shapes and porosity/pore size and whose surface can be modified with a myriad of recognition and fluorescent molecules. These parameters facilitate the loading of numerous nanoparticles by manipulating their features. Reproduced with permission from American Chemical Society, taken from Godin, B., E. Tasciotti, X. Liu, R.E. Serda, and M. Ferrari, *Multistage nanovectors: from concept to novel imaging contrast agents and therapeutics*. Acc Chem Res, 2011. **44**(10): p. 979-89.
1.5 Mesenchymal stem cells

Mesenchymal stem cells (MSC) have spurred interest for their ability to differentiate into bone, cartilage, and fat (Fig 1.6), as well as for their ability to migrate and accumulate at tumors [78-81]. MSC display the unique ability of migrating to pathological sites (e.g., ischemia, injury, and cancer) through the active sensing of the gradients of inflammatory cytokines typically released by these sites [82]. In particular interest they have been shown to preferentially migrate towards local and disseminated malignant cancers: breast [83], ovarian [84], glioma [85]. Furthermore, MSC can be isolated and derived in large quantities from several sources (e.g, adipose tissue, bone marrow) and safely used in transplant applications after ex vivo manipulation [86]. Due to their hypo-immunogenic nature and isolation from autologous sources, MSC has been deemed the cell source of election for several clinical applications [87]. As a matter of fact analysis of the clinical trials involving stem cells in 2012, revealed that bone marrow was the source for nearly 40% of all the stem cell related clinical trials in the world conducted in 2012 for several indications (e.g., cancer, cardiovascular, liver and eye diseases, and diabetes) [88].

Several studies have demonstrated the ability of MSC to act as vehicles for the delivery of diagnostic agents (e.g., QD [89], iron oxide nanoparticles [90]) and gene therapy [83, 91]. Moreover, recent studies have established the role of inflammatory signals and growth factors in the attraction of stromal cells to tumor vasculature [92-94]. In particular, the platelet derived growth factor (PDGF) family was shown to play a significant role in the recruitment of MSC to breast cancers, including enhanced tropism towards breast cancer stem cells [95]. However MSC’s pro-angiogenic and immunomodulatory qualities may in fact favor tumor progression if viable cells remain within the tumor mass after therapy [87]. Hence, a method to selectively eliminate BMSC and avoid these potential detrimental effects is highly desirable. Thus, if used in the correct setting MSC have the potential be an extremely efficient and powerful delivery vehicle for therapeutic and diagnostic therapy.
**Figure 1.6 Multipotent nature of MSC.** Schematic showing the ability of MSC to self renew and differentiate into other specialized cells within the mesoderm lineage (straight arrows). Other cells within ectoderm and endoderm are also possible. Reproduced with permission from Nature Publishing Group, taken from Uccelli, A., L. Moretta, and V. Pistoia, *Mesenchymal stem cells in health and disease*. Nat Rev Immunol, 2008. 8(9): p. 726-36.


Chapter II: Psychochemical features, loading, and release of NSP


2.1 Abstract

Nanovectors hold substantial promise in abating the off-target effects of therapeutics by providing a means to selectively accumulate payloads at the target lesion, resulting in an increase in the therapeutic index. A sophisticated understanding of the factors that govern the release dynamics of these nanovectors is imperative to achieve these ambitious goals. In this work, we elucidate the relationship that exists between variations in pore size and the impact loading and release of multistage nanovectors. Larger pored vectors displayed higher loading of nanoparticles, while exhibiting the slowest release rate. Empirical loading and release studies of nanoparticles (e.g., quantum dots) along with diffusion modeling revealed this prolonged release was modulated by the penetration within the porous core of the vectors regulated by their pore size.
2.2 Introduction

Porous silicon (pSi) is a biomaterial that is non-cytotoxic [96], degradable [97] and photoluminescent [98]. The ability to control its fabrication, surface, loading and release of imaging and therapeutic moieties transformed pSi into an extremely powerful and versatile material for biomedical applications [57, 99]. As a result, pSi finds applications ranging from tissue engineering [50, 100], to optoelectronics [101, 102], to biomedical devices [103], and brachytherapy [104].

Recently, significant research efforts have been devoted to pSi platforms for the delivery of drugs [69, 70, 105] and proteins [106, 107]. The use of mesoporous (i.e., pores ranging from 2 – 50 nm) silicon allows for the targeting and delivery of payloads (drugs, biologics, nanoparticles, etc.) to diseased sites, while providing controlled release over the embedded agents and effectively resulting in their confinement, protection, and entrapment along the journey [108]. Nanoparticles have emerged with the potential to target and deliver immense payloads to the site of action, maximizing efficacy while limiting adverse side effects [15, 40]. However, due to limitations regarding their shape, size, surface charge, and inadvertent environmental activation, they present themselves imperfectly leading to their sequestration by biological barriers [18].

Thus by using advanced modeling of the microvasculature, blood flow dynamics, mechanisms of endothelial cells endocytosis and mononuclear phagocyte system (MPS) sequestration [41, 42, 109-111], our group engineered multi-stage nanoporous silicon particles (NSP) designed specifically to overcome biological barriers and deliver therapeutic and diagnostic agents to the target site [46, 74, 76, 112]. This approach decouples the multitude of tasks typically required by nanoparticles and distributes them onto multiple stages. The first stage is responsible for the storage and protection of the nanoparticles (i.e., second stage) upon systemic administration. This stage is based on nanoporous silicon
particles of defined size and shape with tunable pore size (5-150 nm) and porosities (30-90%) [43].

In this work, we aimed to understand the effect of modifications in the engineering (e.g., pore size or porosity) of NSP has on their release kinetics. Here, we investigated the loading and release of model payloads (e.g., quantum dots (QD), micelles, and liposomes) from NSP with pore sizes ranging from 10-50 nm using flow cytometry. The release data from QD was interpreted using a continuum diffusion model to elucidate the contribution of pore size to release.

2.3 Experimental

2.3.1 NSP fabrication

NSP were microfabricated according to published protocols [43] at the Microelectronics Research Center at The University of Texas at Austin. We formed arrays of 2µm diameter, 300nm deep cylindrical trenches into SiN masked, 0.005 W-cm, p-type silicon wafer by UV photolithography and reactive ion etch. We selectively porosified the trenches by electrochemical etch of the patterned substrate in HF ethanoic solution, first applying a porosification current profile and then increasing the current to form a highly porous layer at the particle/substrate interface to allow particle release. The solution composition and porosification current density depends on the target pore size and porosity for every specific particle and is detailed in our previous publication [43]. Higher current or higher ethanol to HF ratio leads to higher porosity and larger pores. Nonporous particles were fabricated by forming an array of 2µm disks in a 330nm thick layer of polycrystalline Si (PolySi) grown over 800nm of LPCVD oxide. NSP were detached from the substrate by lift-off through buffered oxide etch of the LTO sacrificial layer. All particles were thoroughly rinsed in DI water multiple times to ensure removal of processing reagents.
2.3.2 Porous Structure Characterization

The pore size distribution and porosity of the particles were characterized by N\textsubscript{2} adsorption/desorption isotherms according to the Barret-Joyner-Halenda and the Brunauer-Emmett-Teller models respectively. A collection of oxidized NSP from 10 independent fabrication processes were mixed, centrifuged to form a pellet, the supernatant was removed and the pellet was transferred to a sample cell and allowed to dry at 80°C overnight in a vacuum oven. The sample was degassed at 200°C for 12 h and the isotherms were measured at 77K in a Quantachrom Autosorb-3B.

2.3.3 Surface Modification of NSP

Surface modification of NSP was achieved using established protocols [46]. Briefly, NSP were oxidized using a piranha etch treatment (1 volume H\textsubscript{2}O\textsubscript{2} and 2 volumes of H\textsubscript{2}SO\textsubscript{4}) for 2 hours followed by extensive washings in water. NSP were modified 3-aminopropyl triethoxysilane (APTES; Sigma Aldrich, St. Louis MO) using a 2% (v/v) solution in IPA for 2 hours at 35°C with mixing.

2.3.4 Scanning Electron Microscopy (SEM)

NSP were imaged with SEM using an FEI Quanta 400 equipped with SE, backscatter, and EDS detectors made available through Rice University’s Shared Equipment Authority. NSP samples were spotted onto an aluminum mount (Ted Pella) and allowed to dry overnight in a vacuum desiccator. Samples were then imaged in high vacuum with a working distance of 10 mm and voltage of 30 kV.

2.3.5 Micelles containing doxorubicin synthesis
A solvent evaporation method was used to encapsulate doxorubicin (DOX) into 5k-x-5k PEG-PCL (Sigma Aldrich) micelles, as previously described [113].

**Generation of Hydrophobic DOX:** DOX-HCl (Adriamycin) was made hydrophobic based on established protocol [47]. Briefly, 49 mg of DOX-HCL was stirred overnight in a solution containing 12.5% (v/v) methanol in chloroform and 0.4% triethylamine. The solution was filtered with a 0.02 µm syringe filter (VWR), covered in aluminum foil, and allowed to evaporate in the fume hood for 48-72 hours. The weight of the hydrophobic DOX was assessed and re-suspended in tetrahydrofuran at a concentration of 3 mg/mL and stored at -20°C.

**Synthesis of M-DOX:** PEG-PCL (9 mg) was dissolved in tetrahydrofuran containing 1 mg of DOX and was added to water dropwise under sonication using a Misonix Sonicator 3000 and then allowed to evaporate overnight with gentle agitation. The micelles containing DOX (MDOX) were then filtered using a 0.45 µm nylon filter (VWR), free drug was removed using 100K Amicon Ultra Centrifugal Filter Units (Millipore), and then stored at 4°C.

**2.3.6 Characterization of micelles and liposomes**

A mixture of phosphatidylcholine, phosphatidylethanolamine, and cholesterol were used to synthesize liposomes by Dr. Nicoletta Quattrocchi.

The zeta potential of micelles and liposomes were measured by suspending particles in 1.5 mL of water and measured using a ZetaPALS analyzer (Brookhaven Instruments). Each sample was analyzed over four times with a cycle of 30 runs. Dynamic light scattering was used to measure the size of liposomes (ZetaPALS analyzer) and micelles (Malvern Zetasizer Nano ZS).

**2.3.7 Loading of micelles and liposomes**
Micelles and liposomes were loaded passively into NSP taking advantage of electrostatics. Briefly, oxidized and APTES modified NSP were incubated with particles for 30 minutes in the dark. NSP were washed twice in water to remove unloaded particles and used immediately. Loading was characterized using flow cytometry, UV-Vis, and fluorescence microscopy.

2.3.8 Loading and Release of Quantum Dots

NSP were loaded with QD following established protocols [43, 46]. Briefly, NSP were exposed to 525-carboxyl QD (Invitrogen) in 200 mM Tris-HCl (Sigma-Aldrich) for 15 minutes under rotation. NSP were centrifuged and washed in water to remove unloaded QD and a small aliquot was collected for initial analysis. QD-loaded NSP were suspended in PBS containing 0.025% Triton X-100 and placed on a tube rotator (10 r.p.m.) incubated at 37 °C. At pre-determined times, aliquots were removed and centrifuged at 3500 x g, supernatants were discarded, and NSP were stored at 4 °C until analysis could be performed. Fluorescent signal associated with NSP was acquired using a BD FACS Fortessa (BD Biosciences) equipped with a 488 nm excitation source and a forward scatter photomultiplier tube housed within Houston Methodist Research Institute’s Flow Cytometry Core.

2.3.9 Diffusion Modeling

A continuum diffusion model was created using a discretized continuum Finite Element (FE) method [114, 115] by Dr. Ziemys and Dr. Kojic. In brief, the fundamental relation in the continuum description of diffusion is Fick’s law:

\[ J = -D \nabla c \]  

(1)

where \( J \) is the mass flux and \( c \) is the concentration gradient. The governing mass balance equation can be written as
\[-\frac{\partial c}{\partial t} + \frac{\partial}{\partial x_i} \left( D \frac{\partial c}{\partial x_i} \right) + q = 0 \]  \hspace{1cm} (2)

where \( q \) is a source term, and summation is implied on the repeated index \( i=1,2,3 \). This equation is further transformed into a balance equation of a single finite element by using a Galerkin procedure. Since the diffusion coefficient \( D \) is a function of concentration \( c \), an incremental-iterative scheme is employed within the implicit solution algorithm (governing equations are satisfied at the end of the time step) that suppresses error propagation. This FE model is incorporated into the FE software PAK [116] used for linear and nonlinear analysis of solids, fluids, field and coupled problems, and in bioengineering [114, 115].

Pore models were constructed as cylinders with 700 nm length and 15, 26 and 51 nm diameters, having one end closed. Based on geometrical considerations, the maximum concentration of 10 nm QD was calculated to be 3.2mM if pores are filled 100%. First, 51 nm XLP pores were assumed to be filled 100%. Then diffusion coefficient \( D \) was predicted by matching experimental release profile of QD release, where \( D \) was found \( 8 \cdot 10^{-6} \) \( \mu \text{m}^2/\text{s} \). Next, using derived \( D \), MP and LP experimental release profiles were matched by adjusting pore filling. In our approach, \( D \) serves as a transport coefficient that integrates restrained QD diffusion inside nanoscale confined spaces [117-119] and silica matrix degradation effects on release.

2.3.10 Statistical Analysis

All the data are the result of samples measured in triplicates. Statistics were calculated with Prism GraphPad software. Loading results were tested for significance using a one-way ANOVA followed by a Tukey post-test to compare all pairs of columns.
2.4 Results

2.4.1 NSP Characteristics

Figure 2.1 displayed the procedures by which NSP were fabricated resulting in uniform populations of particles that can be imparted with varying pore sizes (Fig. 2.1C1&C2). In this study, five NSP types were investigated: nonporous (NP); small pores (SP); medium pores (MP), large pores (LP) and extra-large pores (XLP). NP were non-porous and as previously reported [43], SP had a 10 nm pore diameter and 46.3% porosity, MP 15 nm pores and 51.1% porosity, LP 26 nm pores and 66.1% porosity, and XLP 51.3 nm pores and 82% porosity (Table 2.1). The volume dispersed as measured by coulter counter and the mass of each NSP was compared to all NSP investigated (Fig. 2.2A). As expected, decreased volumes per NSP were observed as pore size increased, as did the mass of the individual NSP ranging from 3 to 9 pg/particle for XLP to SP respectfully. NP does not conform to this rule as they were fabricated using silicon of a different density and were smaller than SP-XLP NSP. Furthermore, zeta potential measurements (Fig. 2.2B) revealed that the surface for both oxidized and APTES modified NSP was equivalent.

Table 2.1: NSP Characteristics

<table>
<thead>
<tr>
<th>Name of NSP</th>
<th>Porosity (%)</th>
<th>Pore size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Porous (NP)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Small Pore (SP)</td>
<td>46</td>
<td>10.1</td>
</tr>
<tr>
<td>Medium Pore (MP)</td>
<td>51</td>
<td>15.2</td>
</tr>
<tr>
<td>Large Pore (LP)</td>
<td>66</td>
<td>26.3</td>
</tr>
<tr>
<td>Extra Large Pore (XLP)</td>
<td>82</td>
<td>51.3</td>
</tr>
</tbody>
</table>
Figure 2.1 Schematic representation of the NSP fabrication process. A) Fabrication began with a silicon rich silicon nitride layer. B) Patterning in CF$_4$ yielded an array of 2 µm diameter holes with pitch of 2 µm. C) A reactive ion etch formed pores corresponding to photolithographic holes from the previous step. Graphs on right show the effect of current applied over time for SP to LP (top) and XLP (bottom) with SEM images depicting porosity. D) The optional removal of the nucleation layer was performed in a reactive ion etch with CF$_4$. E) NSP particles were released after etch with CF$_4$. 
Figure 2.2 NSP volume, mass, and charge. A) Bar graph comparing volume (left axis) and mass (right axis) of various NSP. B) Bar graph comparing the surface charges of NSP after oxidization (stripped) and APTES (solid).
2.4.2 Characterization & Loading of Nanoparticles

The results of the zeta potential and dynamic light scattering analysis of both micelles and liposomes are shown in Table 2.2. Micelles are characterized with a smaller size and higher (i.e., less negative) zeta potential compared to liposomes. Both nanoparticles are less than 50 nm and are negatively charged and thus based on electrostatics should favor loading into positively charged NSP (APTES). Size and zeta potential of DOX reveal a negligible size that was undetectable and positive charge.

Table 2.2: Nanoparticle Characteristics

<table>
<thead>
<tr>
<th>Payload</th>
<th>Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-PCL Micelle</td>
<td>21</td>
<td>-7</td>
</tr>
<tr>
<td>PC-PE Liposome</td>
<td>44</td>
<td>-23</td>
</tr>
<tr>
<td>DOX</td>
<td>---</td>
<td>+7</td>
</tr>
</tbody>
</table>

Loading into NSP was investigated by comparing the retention of these nanoparticles into oxidized (negatively charged) and APTES (positively charged) NSP. Fluorescence microscopy revealed that both micelles (Figure 2.3A) and liposomes (Figure 2.3B) preferred loading into APTES modified NSP confirming our suspicions about loading being favored by optimal electrostatics. Further validation of this loading into APTES NSP was performed using flow cytometry, demonstrating substantial increases in NSP fluorescence upon loading with micelles (Figure 2.3C) or liposomes (Figure 2.3D). Lastly, quantification of M-DOX incorporation into NSP was investigated using UV-Vis measuring the absorbance at 480 nm and collecting the washes. Figure 2.3E displays that approximately 1 µg of DOX was successfully incorporated into 1x10^7 NSP.
Figure 2.3 Loading of micelles and liposomes into NSP. A,B) Fluorescence images of oxidized and APTES NSP loaded with micelles (A) and fluorescently labeled liposomes (B). C,D) Flow cytometry analysis of micelles (C) and liposomes (D). Black graph represents control NSP, while colors represent NSP loaded with nanoparticles. E) UV-Vis analysis of concentration of M-DOX within NSP along with the amount found in the washes.
2.4.3 Loading of QD into NSP

The retention of 10-15 nm QD was used to understand the effect porosity has on the loading and release kinetics of nanoparticle payloads within NSP. Opposing electrostatic charges between QD and NSP, specifically using carboxyl and amino terminal groups on their surface respectfully, was used to enhance loading, as previously demonstrated [46]. The retention, distribution, and depth of penetration of QD within NSP porous matrix were investigated using scanning transmission electron microscopy on embedded ultrathin sections of loaded particles (Fig 2.4A&B). High magnification of images in part A for MP NSP (Fig 2.4B) exhibited a large extent of size exclusion towards QD showing high concentrations of nanoparticles at the surface (red arrows, Fig 2.4B) with minimal penetration. On the other hand, NSP with larger pores demonstrated substantial QD penetration within their porous matrix (red arrows, Fig 2.4B) with XLP NSP displaying uniform distribution throughout the entire pore. Fluorescent quantification of QD loading using flow cytometry confirmed that NSP exhibit an extremely significant size-dependent correlation between the pore size of NSP and the diameter of QD (Fig 2.4C). As the pore size increased from 10 nm (SP) to >50 nm (XLP), the median fluorescence associated with the NSP progressively escalated from 12,000 to 65,000 indicating increased loading in NSP with larger pores.
**Figure 2.4 Loading of QD into NSP of varying pore sizes.** A) Low magnification of scanning transmission electron microscopy images of NSP loaded with QD. B) High magnification images of the inset within A (red box), confirming the accumulation of QD (red arrows) at the surface (MP) or penetration within the porous matrix (LP, XLP). C) QD loading quantification within the different NSP tested. Each NSP demonstrated a highly significantly different ability to load QD (** = p < 0.001). Electron microscopy images were taken by Ciro Chiappini.
2.4.4 Release of QD from NSP

Following the loading, the release dynamics of QD from NSP were investigated from MP, LP, and XLP (SP was not followed as negligible QD penetration was observed) using flow cytometry. The percentage released at various times of each NSP was fitted with a one-phase association exponential equation using constraints of $Y_0$ equal to 0.0 and the plateau at 100 (Figure 2.5A). We observed that NSP with larger pore sizes, and containing higher concentrations of QD, released at a slower rate (i.e., smaller rate constant) compared to those with smaller pore sizes. For example, at 2 hours NSP released 75%, 63%, and 38% of the QD payload for MP, LP, and XLP respectively. While at 24 hours, MP and LP had released 97% and nearly its entire payload while XLP only had released 83% of its initial payload within the same time period. These values then corresponded to distinct, highly significant rate constants that decreased as the pore size of NSP increased, ranging from 0.66 to 0.18 h$^{-1}$ for MP to XLP respectfully. Similar to the analysis for degradation, Figure 2.5B plotted the pore size (P, nm) versus the time (t, hours) estimated to achieve 50% and 95% release of embed payloads (Table 2.3). However, in this case, a positive correlation was achieved for this pairing and enables future investigators to tune their release rate by adjusting the pore size of NSP. A linear fit through these points, gave us equations to determine the time in order to achieve 50% (equation 3) and 95% (equation 4) release:

\[
t_{50\%} = 0.079 \times P - 0.234 \\
t_{95\%} = 0.334 \times P - 1.010
\]

Table 2.3: NSP release kinetics

<table>
<thead>
<tr>
<th>NSP</th>
<th>Pore Size (nm)</th>
<th>K (h$^{-1}$)</th>
<th>50% (h)</th>
<th>95% (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>15.2</td>
<td>0.6641</td>
<td>1.076</td>
<td>4.651</td>
</tr>
<tr>
<td>LP</td>
<td>26.3</td>
<td>0.4160</td>
<td>1.667</td>
<td>7.201</td>
</tr>
<tr>
<td>XLP</td>
<td>51.3</td>
<td>0.1801</td>
<td>3.850</td>
<td>16.64</td>
</tr>
</tbody>
</table>
Figure 2.5 Release of QD from NSP. A) The release of QD was investigated and plotted with an exponential fit producing distinct release rates for each NSP. B) Values extrapolated from the exponential fit to determine 50% and 95% release were plotted as pore size versus time. A linear straight fit was then applied to give an equation relating the two variables at each percentage.
2.4.5 Interplay between Pore Size and QD Release

Tuning the pore size of NSP functioned as an effective method to regulate the loading and release of nanoparticles. QD loading in NSP was confirmed using high-resolution electron microscopy and flow cytometry demonstrating QD infiltrating pores of NSP large enough to accommodate their size (10-15 nm) and highly significant increased loading in NSP with larger pores. We postulated that this occurred due to the higher penetration of QD within the porous matrix of NSP with larger pore sizes. Monitoring the gradual release over time of QD from NSP permitted the investigation of this assumption. A diffusion model was developed to calculate the mass release of QD for MP, LP and XLP using a recently developed numerical technique [114] under the assumption of varying penetration of QD attributed to potential obstruction by pore confinement. Hence on these observations, we assumed QD-induced stabilization of NSP and XLP were fully loaded with QD throughout the 700 nm pore length (Fig. 2.6A). The first assumption was justified due to the prolonged release not consistent with degradation kinetics that exhibited more than 80% silicon dissolution within the first ten hours. The second assumption relied on the large XLP and QD pore size to diameter ratio, where QD penetration is least obstructed by pore confinement. While the pores of XLP may be loaded 100%, the model analysis showed that experimental release may be matched only if LP and MP are filled to a penetration of 300 nm (40%) and 130 nm (18%), respectively (Fig. 2.6B). This finding concurs with our previous experimental and theoretical work, where we have shown that nanoparticle penetration into nanochannels was controlled by varying the channel height [120].
Figure 2.6 Modeling release based on payload penetration. B) A diagram depicting the parameters and assumptions used to develop the diffusion model. The diagram shows that QD (green spheres) penetrate deeper in XLP and that the concentration of QD decreases as the approach the pore opening. C) The resulting data from the model is plotted against the actual values corresponding to their pore size and pore penetration of 18%, 40% and 100% for MP (15 nm), LP (26 nm), and XLP (51 nm) respectfully.

Although common convention predicts that the degradation rate controls the release of embedded payloads, it fails to account for the transport of QD diffusion inside nanoscale-confined space and the effect of payload on the degradation of the silicon matrix. Previous reports have demonstrated that nanochannels can experience significant pH shifts due to properties of within the loading milieu [121, 122]. In our case, the acidic nature of the carboxyl groups on QD can reduce the local pH within the pores resulting in slower dissolution of NSP, as observed in other pSi materials [123]. Hence in our experiments the impact of NSP degradation on the payload release was minimal and largely dependent on the payload penetration within the porous matrix imparted by the pore size of NSP.

In summary, tuning the pore size of NSP revealed variations in their overall degradation and loading/release dynamics. Engineered NSP with larger pores exhibited
accelerated degradation kinetics, higher loading concentrations of nanoparticles, and prolonged release of payloads attributed to deeper pore penetration. Thus tailoring the pore size of NSP has substantial repercussions on several of their functions, including degradation and release.

2.5 Conclusion

The effective and timely delivery of drugs is the ultimate goal of any delivery system. Adjusting the pore size of NSP prompted unique release profiles corresponding to the degree of payload penetration, a correlation that could potentially give rise to tailored drug delivery vectors. These types of nanovectors allow for the engineering of particles whose pore size can control the release profile of a drug or nanoparticles nested within the pores. Thus, by tuning the pore size of NSP one can control and optimize the rate of payload release from nanovectors to tailor the pharmacokinetics of the agent within.
Chapter III: Degradation of NSP


3.1 Abstract

The careful scrutiny of drug delivery systems is essential to evaluate and justify their potential for the clinic. Among the various studies necessary for pre-clinical testing, the impact of degradation is commonly overlooked. Here, the effect of fabrication (porosity and nucleation layer) and environment (buffer and pH) factors on the degradation kinetics NSP was investigated. Increases in porosity resulted in accelerated degradation exhibiting smaller sized particles at comparable times. Removal of the nucleation layer (thin layer of small pores formed during the initial steps of etching) triggered a premature collapse of the entire central porous region of NSP. Variations in buffers prompted a faster degradation rate yielding smaller NSP within faster time frames while increases in pH stimulated erosion of NSP and thus faster degradation. The degradation of these particles was characterized to occur in a multi-step progression where they initially decreased in size leaving the porous core isolated, while the pores gradually increased in size.

3.2 Introduction

In the past decade, porous silicon (pSi) has gained significant momentum within the biomedical field due to desirable traits such as favorable degradability[55],
biocompatibility[124], and photoluminescence.[125] These unique set of qualities allowed pSi to be applied to applications ranging from optics[126] and biosensors[127] to microelectronics[128] and tissue engineering.[129, 130] Moreover, the flexible nature of pSi permits the adjustment of fabrication parameters to modify precise characteristics (e.g., size, shape, surface, pore size) and has made pSi particularly favorable for drug delivery applications. For example, by leveraging the increased surface area and pore volume of pSi vectors, the secure attachment and incorporation of various molecules, therapeutic moieties, and nanoparticles was successfully achieved.[131-133] This inherent versatility allowed pSi vectors to increase the bioavailability, drug solubility, and provide prolonged release of various payloads.[106]

Previous research into the degradation of pSi has shown that their byproducts are released in the form of monomeric orthosilicic acid, a highly abundant trace element in organisms vital for normal bone homeostasis [123]. Furthermore, research efforts demonstrated that slight increases in orthosilicic acid in the bloodstream were well-maintained and excreted in an efficient and timely manner [134]. Additional endeavors employing inductively coupled plasma-atomic emission spectroscopy (ICP-AES), showed that the dissolution kinetics of pSi films could be tuned through the control of pH and temperatures [123]. These results yielded interest in the development of injectable pSi nanovectors that degrade in a controlled spatial and temporal manner. The complete degradation of these materials can generally span from minutes to hours to days and is significantly shorter than polymeric particles (weeks-months) [15]. These many advantages provided by pSi by-products allow pSi to be a suitable material for drug delivery applications.

Thus by taking advantage of the numerous features of pSi, our group successfully engineered[135] and tested[74, 76, 136] NSP. NSP can be fabricated to enable the creation of pore sizes ranging from 3-100 nm in diameter to accommodate the loading of various sized payloads.[135] Additionally, to ensure increased retention within the pores, a
nucleation layer is incorporated to prevent payloads from passing freely through the pores. Although both properties serve to enable the greatest efficacy of a payload-loaded NSP, the characteristics that regulate their degradation have yet to be fully investigated.

In addition to efficiency and safety, a major consideration for drug delivery systems is their ability to be compatible within the body’s environmental conditions and maintain stability for systemic administration. While previous work examined the degradation effect of pegylation[137] and biofluids[138] on pSi, this work aims to investigate the degradation dynamics of NSP to understand their stability at different porosities, removal of the nucleation layer, and impact of buffer and pH. Furthermore, the consequences of NSP degradation by-products on cellular architecture, proliferation, and cell cycle were explored. Herein, we monitored the impact of fabrication (porosity/pore size, nucleation layer) and environmental (buffer, pH) parameters on the degradation of NSP using ICP-AES, scanning electron microscopy (SEM), flow cytometry, and size and zeta potential analysis. In addition, the cyto-compatibility of NSP was assessed using primary cells to assess detrimental effects on cell structure, proliferation, and cell cycle.

3.3 Experimental Materials & Methods

3.3.1 Experimental procedure for degradation

Phosphate Buffered Saline (PBS; Invitrogen; Carlsbad, California), without CaCl₂ and MgCl₂ at pH 7.2, was used to investigate the degradation of NSP. 1 x 10⁸ NSP were equally split into three low-binding micro-centrifuge tubes (VMR; West Chester, PA) and diluted to a concentration of 1.515 x 10⁷ particles per mL with 0.025% Triton X-100 (Sigma-Aldrich) in PBS. Samples were rotated in triplicate on a LabQuake® tube rotator (Thermo Fischer Scientific; Waltham, MA) at 10 rpm for 72 hours at 37°C.
Effect due to fabrication: Aliquots were stored at 4 °C until analysis could be performed. NP, SP, MP, LP, and XLP were used to study the effect of porosity and MP was selected to study the contribution of a nucleation layer.

Effect due to environment: Buffer: Tris-HCl 20 mM pH 7.3 + 0.9% NaCl (Sigma-Aldrich) as the ‘saline’ solution and Dulbecco’s modified eagle medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) as the ‘cell culture media’ (CCM) were used. SP and LP NSP were selected to have a range of NSP porosities tested. $1.2 \times 10^7$ NSP (SP & LP) were suspended in 1.5 mL of saline or CCM solution, and measured in triplicate. pH: The effect attributed to pH was investigated by adjusting 20 mM Tris-HCl to various pH levels. $1.5 \times 10^6$ LP NSP were suspended in 1.5 mL of each pH and rotated at 37°C.

### 3.3.2 Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES)

At pre-determined times, 100µL aliquots were removed, placed in nylon centrifugal 0.45 µm filter tubes (VWR), and centrifuged for 10 minutes at 4200 rpm to remove any particle debris from the sample. The flow through was collected and stored at 4°C for further analysis. Samples were diluted 1:100 in water containing 1 ppm of yttrium and measured using a Varian Vista Pro Simultaneous Axial Inductively Coupled Plasma Atomic Emission Spectrometer (Varian; Palo Alto, CA) housed at Rice University’s Geochemistry Laboratory, as previously described [76].

### 3.3.3 Scanning Electron Microscope (SEM)

Samples were washed thrice in deionized water to remove salt, placed on aluminum mounts (Ted Pella; Redding, CA), and left in a vacuum desiccator to dry overnight. Samples were analyzed in a Zeiss Neon 40 microscope equipped with an in-lens detector at an acceleration voltage between 2 and 5keV at a working distance of approximately 4mm.
3.3.4 Flow cytometry

Aliquots from each specified time point were spun down at 4200 rpm for 10 min and the supernatant was discarded. The pellet was re-suspended in 150µL of DI water and changes in side and forward scatter were measured using a BD FACSCalibur™ system (BD Biosciences; San Jose, CA) analyzed with CellQuest. The instrument’s detectors (FSC & SSC) were calibrated and adjusted for particle recognition as previously described [46], in order to accurately evaluate intact and non-degraded particles. Bivariate counter plots and three-dimensional plots were generated, that displayed SSC versus FSC to evaluate the changes in shape and size of the particles over time. The overall shapes and distributions of particles were observed and the geometric means of the FSC and SSC were recorded.

3.3.5 Z2 size & distribution

NSP size distribution and number were examined using the Z2™ COULTER COUNTER® Cell and Particle Counter outfitted with a 50 µm ampoule aperture tube (Beckman Coulter; Fullerton, CA). Samples were diluted into 1.0 mL of ISOTON and counted using limits of 1.0 to 2.8 µm, with the resulting data exported to Excel.

3.3.6 Zeta Potential

Zeta potential measurements were resuspended in 1.5 mL of filtered monobasic PB (10 mM, pH 7.4) and measured using a ZetaPALS analyzer (Brookhaven Instruments Corporation; Holtsville, NY).

3.3.7 Statistical Analysis

All the data are the result of samples measured in triplicates. Statistics were calculated with Prism GraphPad software. Linear regression analysis was performed and significance was calculated by testing whether slopes. Nonlinear regression analysis was
performed using a one-phase association fit and constraints of $Y_0$ at 0.0 and plateau equal to 100. The rate constants were then compared to each other using an extra sum of squares F test. Zeta Potential values were tested using a Two-Way ANOVA followed by a Bonferroni post-test to compare NP to SP and MP. In all cases: * was used for p-values between 0.01 to 0.05, ** for values between 0.001 to 0.01, and *** for values < 0.001.
3.4 Results

3.4.1 Impact of porosity on NSP degradation

The flexible nature of NSP allows for tunable porosity to accommodate a variety of payloads and thus can have critical implications for drug delivery. This impact was studied by comparing five different conditions (Table 2.1): NP, SP, MP, LP, and XLP. Differences in degradation profiles were investigated using SEM, flow cytometry, ICP-AES, changes in size/volume distribution, and zeta potential analysis at several pre-determined times.

3.4.1.1 SEM analysis

Upon inspection of Figure 3.1, we observed a progression in the manner and time-scale of degradation that highlighted a correlation between porosity and NSP degradation rate. SEM images demonstrated that NSP initially had consistent shapes consisting of a central region with vertically aligned pores of uniform size, surrounded by a porous ring with angled pores constituting the corona. This corona is less prominent on XLP and not present in NP due to the different fabrication strategy. As time progressed, NP preserved their overall size and shape displaying minimal signs of degradation over 72 hours. On the other hand, NSP experienced a significant effect on their size and shape during degradation due to their porous nature. As a matter of fact, as the porosity increased (SP to XLP) we observed a shift in their overall size with time resulting in smaller sized NSP. From these observations, certain windows could be delineated where NSP were no longer present or had completely degraded: SP, after 48 hours; MP, after 24 hours; LP, after 18 hours; and XLP, after 12 hours.
**Figure 3.1 NSP degrade in distinct times after pore size adjustment.** SEM micrographs of NSP at various times emphasizing the impact pore size has on the overall changes in shape and size of NSP, scale bar = 1 um. Contour and 3D plots of FSC versus SSC (z-axis is counts) acquired from flow cytometry confirm the observed changes in NSP size and shape over time. Black arrows indicate the peak of intact, or non-degraded, NSP while red arrows are used to specify where the degradation by-products accumulate.
Further inspection of magnified SEM images displayed a distinctive pattern common to all NSP undergoing degradation (Figure 3.2A). As time increased, the corona (or porous ring) is observed as the first major feature to experience the effects of degradation. Consequently, NSP experienced a significant decrease in size as the corona eroded away leaving the highly porous central core occupied predominantly with vertically aligned pores. At the same time, the pores contained within the core were also subjected to degradation. However compared with the size of NSP, the pores on the backside and front-side of NSP became larger with time thus increasing the effective porosity of NSP (Figure 3.2B). When comparing the pores on the front-side of different pore sized NSP (SP & XLP), we observed the same phenomenon but over distinct time frames.
Figure 3.2 SEM images of degradation impact on NSP. A) SEM micrographs depict the degradation experienced by NSP over time on both their frontside and backside. Images were split to show the effect on the particle’s shape and size (left, scale bar = 1 µm) and on its pores (right, scale bar = 100 nm). B) High magnification SEM images of NSP pores comparing SP & XLP at different points during degradation, scale bar = 100 nm.
3.4.1.2 Flow Cytometry analysis

Flow cytometry was performed to confirm and quantitate the shape and size changes of NSP over time. Figure 3.1 shows bivariate contour plots and 3D plots at each time, for the different NSP analyzed. Contour plots were used to examine the size (FSC) and shape (SSC) uniformity of NSP. While 3D plots were useful in determining the size and shape distribution with respect to the number of events/counts (z-axis). Contour plots illustrated that NSP began with compact and uniform distributions displaying consistent size and shape throughout the events acquired prior to the start of degradation. However, given sufficient time the size and shape distribution progressively became decentralized, displaying wide variations and gradually drifting towards the origin (lower left) of plots (indicative of dramatic changes in their structure). The progression of this distribution followed a similar porosity-dependent trend where those with low porosity (SP) retained structure stability for longer periods of time compared with higher porosity (LP, XLP) NSP. SP predominately began accumulating at the origin at 48 hours, MP between 18-24 hours, LP at 18 hours, and XLP at 12 hours. Comparably the 3D plots originated with a centralized peak (black arrow-heads) and with time, a shift towards the origin was similarly seen (indicated by red arrows). This shift indicated a larger concentration of NSP was undergoing degradation with increasing events acquired with smaller sizes and shapes. In addition, equivalent patterns were observed after plotting the geometric means of FSC and SSC (Fig 3.3) over time. The data confirmed that NSP degraded in distinct windows such that the higher pore sized particles were completely degraded (i.e., reached zero) at different times.
Figure 3.3 FSC and SSC quantification over time for various pore sizes. A) The geometric means of forward scatter (FSC) and B) side scatter (SSC) were normalized to 1.0 and plotted over time comparing NP, SP, MP and LP MSV. In both graphs, NP remained near 1.0 throughout the study indicating minimal degradation and substantial changes in size (FSC) or shape (SSC). As the pore size of MSV increased SP to LP, a discernable trend was observed such that the FSC and SSC values approach zero within distinct time frames. As we established using ICP-AES and SEM, this trend correlated with the pore size such that larger pore sizes reached zero at earlier times.
3.4.1.3 Determining degradation kinetics

ICP-AES was used to measure the dissolution of silicon that accumulated in solution during NSP degradation. Figure 3.4A plotted the rates observed in different NSP of varying porosity by plotting time versus the percentage of total silicon detected in solution and fitting the values with a one-phase association exponential equation with $Y_0$ and plateau set at 0 and 100, respectively. We observed highly significant ($p < 0.0001$) and distinct rate constants for each NSP analyzed. In addition, using simple linear regression analysis to test if the slopes were significantly different revealed that the slopes governing the rate of degradation of NSP was very significant throughout the entire data set and extremely significant within the first 24 hours with 0.12% and 0.012% (respectively) chance of randomly choosing data with slopes this different. The exponential fitting of the points provided specific rate constants for each NSP such that we could extrapolate values where the particles have degraded 50% and 95% (Table 3.1). As expected, the rate constants for each NSP increased from NP to XLP ranging from 0.0034 to 0.1711 h$^{-1}$. Figure 3.4B plotted the pore size ($P$, nm) versus the time ($t$, hours) needed to achieve 50% and 95% degradation revealing a negative correlation between this pairing. This type of analysis permits one to engineer NSP such that they degrade at a given percentage within a specified time frame (i.e., degradation index) based on tailoring its pore size. A linear fit gave us equations to determine the time in order to achieve 50% (equation 5) and 95% (equation 6) degradation:

\[ t_{50\%} = -0.239 \times P + 15.86 \]  \hspace{1cm} (5)

\[ t_{95\%} = -1.031 \times P + 68.53 \]  \hspace{1cm} (6)
Table 3.1: MSV degradation kinetics

<table>
<thead>
<tr>
<th>MSV</th>
<th>Pore Size (nm)</th>
<th>K (h⁻¹)</th>
<th>50% (h)</th>
<th>95% (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>0</td>
<td>0.0034</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SP</td>
<td>10.1</td>
<td>0.0490</td>
<td>14.15</td>
<td>61.15</td>
</tr>
<tr>
<td>MP</td>
<td>15.2</td>
<td>0.0569</td>
<td>12.18</td>
<td>52.64</td>
</tr>
<tr>
<td>LP</td>
<td>26.3</td>
<td>0.0816</td>
<td>8.50</td>
<td>36.73</td>
</tr>
<tr>
<td>XLP</td>
<td>51.3</td>
<td>0.1711</td>
<td>4.05</td>
<td>17.51</td>
</tr>
</tbody>
</table>
Figure 3.4 Quantitative validation of degradation impact on NSP. A) ICP-AES quantification of the concentration of silicon deposited in solution for each NSP. Values were then fitted with an exponential equation resulting in distinct degradation rates. B) Values extrapolated from the exponential fit to determine 50% and 95% degradation were plotted as pore size versus time. A linear straight fit was then applied to give an equation relating the two variables at each percentage of degradation.
3.4.1.4 Changes in volume distribution

Figure 3.5A compared the volume distributions of various NSP at different time points. The distributions were offset on the y-axis for clarity and to compare the changes in size at different times. The initial peak at 1 fL was the detection limit of the instrument and was common to all NSP examined. As NSP degraded, a shift in their volume towards the detection peak is apparent for all NSP with the exception of NP. These shifts appeared earlier for NSP with higher porosities and whose peaks progressed closer to the detection limit at a faster rate. Figure 3.5B substantiates this claim by normalizing to zero hours and plotting the modes (e.g., highest occurring number) collected during volume analysis. As time increased, we observed the same trend as the volume graphs where the more porous NSP displayed an overall smaller size at each time point while NP experienced minimal changes in size. For example, at 24 hours the size of NSP had decreased by 37%, 56%, 62%, and 70%, respectively, for SP, MP, LP, and XLP.

3.4.1.5 Surface charge analysis

Zeta potential measurements were performed to determine the surface charge of NSP as they degraded. Figure 3.5C diagramed the changes in surface charge of NP, SP, and MP NSP during degradation. Due to their APTES modification, NSP were initially imparted with a positive charge (+10-15 mV) and with time NSP became highly negative (~25-30 mV). At eight hours, NSP reached a plateau of negative charge of ~40mV and ~35mV for NP and SP/MP, respectively. By 24 hours, the surface charges of SP and MP were neutral while NP remained negative (significantly different from SP and MP). Hence as the porosity of NSP increased, an equivalent increase in degradation rate was observed. Surface charge measurements revealed that the APTES surface modification quickly degraded and exhibited a pattern such that the highest value was achieved at eight hours potentially indicating the point where the highest surface area existed. On the other hand,
the surface charge measurements of NP NSP remained consistent confirming minimal degradation over time.

Figure 3.5 Changes in volume and surface charge. A) Volume distributions of NSP with varying porosities are plotted at various times comparing the shifts in size. Distributions are offset on the y-axis to highlight differences between time points. B) The mode, or the value that appears most frequent, at each time point is plotted and compared to other porosities of NSP. NP serves as control, as degradation of non-porous silicon particles is minimal and was used for comparison. C) Zeta potential measurements were used to provide insight on the surface charge of NP, SP, and MP NSP. The results are shown as mean ± SD. Asterisks denote data points where the difference was statistically significant compared to NP. * p < 0.05, ** p < 0.01, *** p < 0.001.
3.4.2 Absence of nucleation layer and effect on pore structure

A critical function for NSP is to load and retain a nanoparticle payload within its porous structure. The nucleation layer serves to prevent payloads from passing freely through NSP and is typically associated with pore sizes less than 2 nm. We investigated the impact on NSP degradation by comparing SEM, ICP-AES, flow cytometry, and size analysis. SEM images illustrated that NSP without a nucleation layer (NSPxNL) began with larger pores on the front-side while retaining the same overall shape compared to regular NSP (Fig 3.6A). The overall shape of NSP and NSPxNL remained similar over time. However, we observed that the porous structure of NSPxNL displayed significant remodeling such that by 18 hours considerably larger pores were observed. This resulted in premature pore instability ultimately triggering their complete collapse by 24 hours (Fig 3.6A).

As shown in Figure 3.6B, the size distribution of NSPxNL began with slightly smaller sizes consistent with the earlier results of higher porosity NSP. The shifts in size over time associated with NSPxNL were comparable to the variations observed in conventional NSP containing a nucleation layer. Flow cytometry (Fig 3.6C) confirmed that NSPxNL size (forward scatter, FSC) and shape (side scatter, SSC) degraded in a manner consistent with classical NSP. FSC and SSC contour and 3D plots illustrated similar patterns with analogous shifts accumulating at the origin of the graphs, indicative of NSP degradation between 24 and 48 hours. In addition, ICP-AES (Fig 3.6D) confirmed that NSPxNL had minimal effect on the overall degradation of NSP as no significant increase in Si was detected in solution determined using nonlinear regression analysis and comparing rate constants. The absence of a nucleation layer from NSP had a negligible effect on their overall degradation pattern resulting in comparable changes in size and shape. However, the porous structure of NSP experienced a remarkable difference in their degradation displaying increased instability generating increased porosity in the core of NSP resulting in
complete pore collapse by 24 hours. Although the absence of a nucleation layer may negatively impact payload loading or retention, it may be beneficial in order to achieve a quicker release rate attributed to the quicker pore break-down observed.

Figure 3.6 The impact of the nucleation layer on pore stability. A) NSP with and without a nucleation layer (NSPxNL) were compared at various times looking at the overall effect on size and shape (left) and their pores (right) on the front-side where the nucleation layer is visible. Scale bars, left for particle is 1 µm; right for pores is 50 nm. B) Volume distribution of NSP and NSPxNL showing smaller sized for NSPxNL but with comparable shifts in size with increased time. C) Flow cytometry verifying similar patterns for size (forward scatter) and shape (side scatter) of the two NSP. D) ICP-AES contrasting NSP versus NSPxNL to confirm minimal change in overall degradation of NSP without nucleation layer.
3.4.3 Degradation behavior of NSP in varying buffers

An understanding of the interplay between the local environment and the effect on the degradation of drug delivery vectors is vital to gauge its use for systemic administration. To this extent, we compared the degradation kinetics of NSP in two different types of buffers: DMEM with 10% FBS (CCM) and Tris-HCl + 0.9% NaCl (Saline), both at pH 7.3. SP and LP NSP were investigated to discover the impact of buffer on degradation using ICP-AES, SEM, and size analysis. Figure 3.7A demonstrated that when suspended in CCM, the overall degradation process was accelerated for both SP and LP. In CCM, SP reached a plateau of maximum Si deposition by 24 hours, while in saline this was not achieved till 48 hours (90% at 24 hours). Similarly, LP reached plateaus at 18 hours in CCM and 48 hours in saline (80% at 24 hours). Nonlinear regression analysis revealed that NSP in CCM experienced a significant acceleration in degradation rate, such that they degraded more than 3.5× faster in CCM compared to saline for both SP and LP.

Furthermore, SEM images (Fig 3.7B) confirmed that both SP and LP degraded quicker in CCM compared to saline. In CCM, NSP exhibited dramatic transformations in the overall appearance displaying smaller sized particles at 18 and 24 hours for SP and 12 versus 24 hours for LP. Figure 3.7C validated that dramatic shifts in size were observed for NSP in different buffers. Both SP and LP in CCM experienced a substantial shift within five hours resulting in acquisition of NSP predominantly at the lower detection limit, which was not the case for NSP in saline. Thus, NSP exposed to CCM revealed accelerated degradation kinetics resulting in substantial structural instability and shifts in size compared to NSP in saline buffers. The addition of amino acids and serum proteins commonly found in CCM[139] and FBS possibly contributed to increased degradation. The disparity between CCM and saline suggests that NSP will experience a significant increase in degradation upon encountering sites rich in proteins, amino acids, and salts such as the tissue and cellular microenvironment and during systemic blood circulation.
Figure 3.7 Influence of buffer in degradation of NSP. A) ICP-AES comparison of NSP, SP (left column) and LP (right column), degradation in CCM and saline solution. B) SEM images showing NSP degradation in CCM (left) and saline (right) solutions at various time points. C) Volume distributions of NSP in CCM (top) and saline (bottom) are plotted at
various time points relating the shifts in size. Distributions are offset on the y-axis to highlight differences between time points. The results are shown as mean ± SD. Asterisks denote where the difference was statistically significant compared to Saline. *** p < 0.001. Data was collected and kindly provided by Ennio Tasciotti.
3.4.4 pH-induced degradation of NSP

Comprehension of the degradation phenomenon governing NSP in varying pH environments is essential to ensure stability during systemic administration and upon interactions within cellular environments. The influence of pH on NSP degradation was investigated using a range from 9-12. In alkaline solutions, the adsorption of hydroxyl ions on Si produces its dissolution into orthosilicic acid as the concentration of hydroxyl ions are increased.[140] This favors the formation of a bioinert neutral monomer of silicate ions as equilibrium is reached that plays a beneficial role in the optimal growth of bone and collagen.[141] NSP degradation in alkaline conditions can be represented as shown in Figure 3.8A. In an aqueous solution, the hydroxyl layer of the NSP undergoes weak nucleophilic attack by the water molecules. Increasing the pH of the solution, therefore, provides a stronger nucleophilic attack that ultimately produces accelerated degradation of NSP into silicic acid. As shown in Figure 3.8B, the contour plots acquired by flow cytometry displayed progressive loss of organization of the overall shape and size of NSP as pH increased, indicated by the increase of particles located outside the gating box. The ROI box within the plots was used to monitor the extent of degradation, such that as NSP degrade their distribution will extend outside of the box and drift towards the origin where small fragments accumulate. NSP experienced substantial effects on the size and shape in 24 h dependent on the pH used. At pH 12, NSP began drifting at 2.5 hours while at pH 10 manifestations were not apparent until 20 hours. Next, we compared the total counts collected by the flow cytometer at various times (Figure 3.8C). This was performed by fitting the data using a plateau followed by a one-phase exponential decay constraining the plateau to 0 and \( Y_0 \) to 100. At increasing pH, NSP revealed an accelerated degradation with fewer NSP collected at increasing pH levels. We fitted the data using a plateau followed by a one-phase exponential decay constraining the plateau to 0 and \( Y_0 \) to 100. This resulted in distinct degradation rate constants of 0.053, 0.306, 0.638, 4.45 h\(^{-1}\) for pH 9, 10, 11, 12,
respectively. In addition, this model calculated delays in the initiation of degradation (X0) such that as the pH increased the decay began earlier with times ranging from 1.2 to 8.6 hours. Taken together, this data demonstrated that the increasing concentrations of hydroxide ions resulted in a rapid degradation on the surface of NSP producing considerable structural modifications. As the solution became more basic, the strength of the nucleophilic attack intensified resulting in an overall increase in exposed pSi surface area and ultimately yielding the final product of orthosilicic acid.

Figure 3.8 Degradation effect of NSP in varying pH environments. A) Schematic showing possible interactions between the NSP surface and hydroxyl groups. Hydroxyl ions of an alkaline solution weaken the surface of the NSP forming bioinert silicic acid. B) Flow cytometry exhibiting distinct patterns and changes in size (forward scatter) and shape (side scatter) exposed to the varying pH environments. C) Flow cytometer counts collected at various time points to show degradation rates of NSP at pHs between 9 and 12. Data was collected and kindly provided by Ennio Tasciotti.
3.4.5 Mechanism of NSP Degradation

Surveying the degradation results, we observed distinct time frames for each NSP investigated that displayed a consistent evaluation among the various techniques used (ICP-AES, flow cytometry and SEM). For example, NP showed minimal degradation and their shape remained conserved throughout the duration of the experiment, SP showed complete degradation within 48-60 hours, MP within 36 hours, and LP and XLP completely degraded within 24 and 18 hours respectively.

Taking this into consideration, we proposed a mechanism (Fig 3.9) that describes how NSP progressively degrade. Inspection of SEM images revealed that NSP degraded such that the highly porous corona was the first main feature to experience significant degradation and began eroding away, allowing for increased surface area and decreasing the amount of bulk silicon. While the corona of NSP degraded at a much quicker rate, the central highly porous region was isolated where the pores demonstrated an overall increase in their diameters. This increase was induced by the degradation of the pore wall resulting in decreased wall thickness and larger pore diameters ultimately resulting in unstable NSP whose pores collapsed due to insufficient structural integrity. Hence, the overall degradation of NSP occurred such that the outer envelop (corona) experienced faster degradation attributed to increased exposure of this region to the environment, compared to the core, resulting in smaller sizes over time. Upon eroding the corona, the pores contained within the core experienced a drastic enlargement due to the pore wall degradation process. The presence of larger pores accelerates this process as it results in premature core instability triggering their eventual collapse within a much faster time frame than NSP with smaller pores.

The degradation of NSP can be liken to the two main mechanisms that govern that of polymer-based material, specifically surface and bulk degradation [142]. Surface degradation was characterized as occurring only at the surface resulting in smaller sized
particulates, leaving the core of the material intact during degradation. On the other hand bulk degradation results in the uniform erosion throughout the material yielding fragments as byproducts [143, 144]. Hence, together, these two mechanisms can be used to describe the mechanism of degradation that is observed on the overall shape and pores of NSP, respectively. The initial erosion of the NSP corona left a circular region (or core) composed of pores intact a feature distinctive of surface degradation. While the pores exhibited a uniform increase in diameter resulting in pore fragment-like structures, consistent with bulk degradation.

Figure 3.9 Mechanism of NSP degradation. Schematic illustrating the proposed degradation mechanism of NSP.
3.5 Conclusions

In summary, the influence of both fabrication and environmental elements affect the degradation of NSP was demonstrated. This study can potentially serve as a model on how to characterize drug delivery vectors in order to achieve optimal degradation profiles while maintaining minimal impact on biological systems. Variations in NSP porosity resulted in substantial shifts in the size/shape producing faster degrading NSP with distinct degradation kinetics as the porosity increased. The removal of the nucleation layer greatly impacted the stability of pore structure generating a premature collapse of the pores. Environmental influences also dictated how NSP would behave such that buffers containing amino acids and proteins (e.g., cell culture media) and an increase in pH facilitated quicker degradation of NSP. The culmination of this work prompted the description of the overall mechanism for the degradation of NSP. The work presented here establishes a precedent for future studies to investigate the degradation of drug delivery vectors.
Chapter IV: Biocompatibility of NSP


4.1 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 effects that occur in primary human and mouse cells upon the internalization of NSP. Using qualitative and quantitative techniques as well as *in vitro* and *in vivo* biochemical, cellular and functional assays, we demonstrated that NSP did not cause any significant acute or chronic effects on cells and tissues. We analyzed *in vitro* cell toxicity and viability, the maintenance of cell phase cycling, the cellular and organelle architecture, the impact on advanced cellular functions (e.g., tube formation, differentiation) upon the internalization of NSP, and the effect of NSP degradation byproducts on cells. In addition, we evaluated if NSP produced any pro-inflammatory response and studied its biocompatibility *in vivo*. We followed the biodistribution of NSP 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 both in vitro and in vivo evaluations of systemic response as well as examining the impact on cellular functions and of their degradation byproducts.

4.2 Introduction

The emerging use and influence of nanotechnology in the biomedical field has driven investigators to create innovative solutions for the targeted treatment of pathological conditions such as atherosclerosis,[145] cancer,[146-148] diabetes,[149] traumatic brain and spinal cord injury,[150] and thrombosis[151, 152]. The rapid expansion in the use of nanotechnology-based products evoked concerns related to the possible adverse effects associated with nanomaterials. However, disagreements on methodology to evaluate toxicity levels and on the choice of nanoparticles to use as a standard have troubled this field of research from its inception[153].

The development of effective in vitro methods to understand the impact of nanomaterials on cells and tissues, must consider that exposure to toxic agents typically results in three possible outcomes: cells undergo a loss of membrane integrity resulting in rapid death (necrosis), or they become senescent and stop functioning and dividing, or they activate a sequence of proteolytic events resulting in a highly ordered and controlled cell death (apoptosis)[154, 155]. Necrosis is characterized by the rapid swelling of the cell resulting in declined metabolism and rapid expulsion of proteins, metabolites, and organelles into the surrounding environment. Necrosis typically occurs as a consequence of acute toxicity. Cellular senescence normally occurs in response to cellular aging, however, low concentrations of a cytotoxic compound could cause cells to be unable to perform a particular function or to divide. In contrast, apoptosis is distinguished by an extremely
ordered process, which produces a cascade of biochemical events resulting in specific morphological alterations that trigger the efficient removal of cells from tissues.

There are several accounts that demonstrate the capacity of nanoparticles to elicit the aforementioned cellular outcomes [156-158]. Metabolic activity of cells and their membrane integrity are commonly measured to evaluate the toxicity of nanoparticles. However, these approaches, even if tested in combination, often fail to reveal if the exposure to nanoparticles has affected or altered other cellular functions in cases of low, minimally acute toxicity [159]. Hence, appropriate end points must be chosen to evaluate if cell senescence has been induced, if apoptosis markers are expressed, or if biochemical pathways have been altered, in addition to determining if cells are damaged acutely [153]. Another concern is the selection of an assay that limits false positives, which can occur if the nanomaterials absorb or spontaneously interact with dyes [153, 160, 161].

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 [162]. 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 [163, 164]. 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 tested NSP using conventional assays to investigate their effect on key cell functions. In particular, we examined the consequences of NSP internalization on cell proliferation, cell cycle, induction of apoptosis, tube formation, differentiation, and migration of human umbilical vein endothelial cells (HUVEC) [165] and mouse adipose-derived mesenchymal stromal cells (ADMSC) [80, 166]. The endothelial model was chosen because the vasculature is the first tissue to be encountered by a circulating particle upon systemic administration. The choice of ADMSC was based on the propensity of these cells for multipotent differentiation and migration [80, 166] thus providing a representative experimental model to study the influence of NSP on these processes. The impact on the cytoskeleton and ultrastructure were also studied to interpret potential effects on the cellular architecture, which is essential for maintaining normal cellular shape and size, motility, and intracellular transport. In addition, assessment of the impact of NSP on triggering an immune reaction, biodistribution, and tissue structure was also investigated.

4.3 Experimental Materials & Methods

4.3.1 Cell Culture

HUVEC were purchased from LONZA and maintained using endothelial basal media-2 supplemented with EGM-2 SingleQuots (Lonza). ADMSC were isolated from fat, as previously described.[167, 168] Briefly, cells from isolated using a mixed population of subcutaneous and intraperitoneal white adipose tissue from C57BL/6 mice by enzymatic digestion. MDA-MB-231 were purchased from American Type Culture Collection. ADMSC and MDA cells were maintained using dulbecco’s modified eagle medium (DMEM) with high glucose, L-glutamine (Invitrogen, GIBCO) and supplemented with 10% fetal bovine serum.
4.3.2 Cellular toxicity and Proliferation

LDH (Lactate Dehydrogenase) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays were purchased from Abcam and Invitrogen, respectfully, and preformed simultaneously following manufacturers instructions. Briefly, cells were seeded into 96 well plates. At pre-determined times, the media was removed for LDH and adherent cells were treated with 0.5 mg/mL of MTT dye for two hours in the incubator. LDH samples were measured on a Synergy H4 (BioTek) plate reader for OD at 450 nm. After incubation with MTT dye, cells were solubilized with DMSO for 30 minutes and then OD measured at 570 nm.

4.3.3 Annexin V staining

HUVEC were exposed to NSP 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 10 mM HEPES (Invitrogen), 140 mM NaCl (Sigma Aldrich), 2.5 mM CaCl$_2$ (Sigma Aldrich), and adjusted to pH 7.4. Recovered cells were re-suspended in 100 µL of annexin-binding buffer. 5 µL of annexin V was added to each 100 µL of cell suspension and incubated at room temperature for 15 minutes. 400 µL of annexin-binding buffer was carefully added and HUVEC were gently mixed and kept on ice until analysis could be performed on a FACSCalibur.

4.3.4 Cell Cycle Analysis

HUVEC treated with NSP (or 50 µg mL$^{-1}$ of cisplatin) were analyzed at pre-determined times to understand their effect on cell cycle using established methods, [164].
Briefly, cells were collected and fixed in 70% ethanol at 4°C for 10 minutes and subsequently stored at -20°C for at least 30 minutes. A 50 µg mL\(^{-1}\) propidium iodide (PI) solution was prepared in 10 mM Tris buffer at pH 7.3 containing 5 mM MgCl\(_2\), sterile filtered, and stored at 4°C. For every 10\(^6\) cells, one mL of PI solution was slowly added under vigorous manual tapping followed by 50 µL 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 200 µL of PBS and analyzed using a Becton Dickinson FACSCalibur.

4.3.5 Cytoskeleton

HUVEC and ADMSC were stained for actin and tubulin to further assess the effect of internalization of NSP on cytoskeletal elements. Cells 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 4% paraformaldehyde in PBS for 10 minutes, followed by permeabilization with 0.2% Triton X-100 (Sigma) in PBS for 10 minutes. Cells were then blocked for 30 min at room temperature in 1% bovine serum albumin (BSA; Sigma Aldrich) in PBS containing 0.05% Tween-20 (PBS-T; Sigma Aldrich), followed by an hour incubation with the alpha tubulin antibody at room temperature. Cells stained with phalloidin were fixed and permeabilized as described earlier and blocked in 1% BSA in PBS for 10 minutes at room temperature followed by an 20 minute incubation with phalloidin at 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.
4.3.6 Transmission Electron Microscopy (TEM)

Cells were grown to 90% confluency in six well plates. Cells were then treated with NSP overnight in incubator and then fixed in a 2% paraformaldehyde (Electron Microscopy Sciences) and 3% glutaraldehyde (Sigma-Aldrich) in PBS. After fixation, the samples were washed and treated with 0.1 % Millipore-filtered cacodylate buffered tannic acid, postfixed with 1 % buffered osmium tetroxide for 1 hr, and stained in bloc with 1 % Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in Spurr’s low viscosity medium. The samples were polymerized in a 70°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL) stained with uranyl acetate and lead citrate in a Leica EM stainer and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

4.3.7 Tube Formation

8-chambered slides (LabTek) were pre-coated with 100 µL of Matrigel (BD Biosciences) at 37 C for 30-45 minutes. Upon uniform coverage of well, 40,000 HUVEC were added to each well. Images were taken at 0.5, 1, 2, 3, 4, 6, and 24 hours using a Nikon TS100 equipped with a DS-Fi1. 24-hour images at 4x were sent to Wimasis for image analysis. In addition, HUVEC were labeled with Cell Tracker® Red (Invitrogen) and imaged at 24 hours for tube formation using a Nikon A-1 confocal microscope.

4.3.8 ADMSC Differentiation

80,000 ADMSC were seeded in 24 well plates and allowed to adhere overnight. The following day, plates were divided for osteogenic and adipogenic differentiation and media was replaced with their respected differentiation media. Media was replaced every 3 days
for 3 weeks, after cells were stained with toluidine blue, oil red o, von kossa, and ALP. Furthermore, a quantitative calcium assay (QuantiChrom) was used to compare the production of calcium in the mineral produced in the cells. Solubilized cells were measured for OD at 610 nm on a plate reader.

4.3.9 ADMSC Migration:

**Transwell:** Thincerts™ (Greiner Bio-One) in 24 well plates assessed the migration affinity of ADMSC towards breast cancer cells. Transwell were established by seeding 20,000 cancer cells in complete media on the bottom of the well, inserted a 8.0 pore transwell, and seeded 25,000 ADMSC (with and without NSP) in serum-free media within the transwell. ADMSC were allowed to migrate for 48 hours after which transwells were stained with crystal violet. Inserts were transferred to a new plate and measured on a plate reader for OD at 560 nm.

**Adapted Wound-healing Assay:** Ibidi® culture inserts within an ibidi® µ-Dish (35 mm, high) were used to investigate the 2D migration affinity of ADMSC towards breast cancer cells. Here, 10,000 ADMSC were seeded into one compartment while 30,000 cancer cells were seeded in the other. The following day, inserts were removed and migration was assessed using time-lapse imaging. Images were acquired in four z-planes every 4 minutes using an DSU IX81 Olympus microscope with a 20x objective for 18 continuous hours. Still shots were taken with a 10x objective before and after acquisition of time-lapse and used within the figure, while movies were composed of projection images of all captured z-planes per time.

4.3.10 Impact of degradation byproducts:

**MTT:** HUVEC were seeded in 96 well plates at 5,000 cells per plate. Degradation byproducts collected from the supernatant of completely degraded NSP were added to each
well. At pre-determined times, wells were incubated with MTT at 0.5 mg/mL in complete media for two hours at 37 °C, 5% CO₂ followed by an 30 minute incubation with DMSO and measured for absorbance at 570 nm on a BioTek plate reader.

**Cell Cycle:** HUVEC were treated with SP or LP NSP degraded by-products by completely degrading NSP and removing samples from the degraded supernatant portion (i.e., not partially degraded NSP). At 48 hours, cells were fixed and prepared for cell cycle analysis as described above.

**Cytoskeleton:** HUVEC were seeded into a chamber slide (LabTek) at 75,000 cells per well and allowed to adhere overnight. The following day, HUVEC were treated with DyLight® 555 NSP at a ratio of 1:10 and an equivalent amount of NSP fragments (i.e., partially degraded NSP) for 24 hours. HUVEC were then stained with AlexaFluor 488 Phalloidin (Invitrogen), mounted with Pro Long Gold containing DAPI, and imaged using an inverted Nikon Eclipse Ti microscope.

### 4.3.11 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-Fox1nu; 4-6 week old) were purchased from Charles Rivers Laboratories and maintained as previously described [169]. When used with tumors, mice used for experiments were carefully implanted with orthotopic breast cancer models by injecting 5x10⁵ 4T1 mouse adenocarcinoma cells. Mice were either treated with 5x10⁷ DyLight® 800 NSP in 100 µL of sterile PBS or 100 µL of PBS.
4.3.12 Cytokine Analysis

The cytokine activity was measured to understand any potential toxic side effects. Mice were randomly separated into either NSP 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 NSP 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).

4.3.13 Biodistribution

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 5x10⁷ NSP modified with DyLight 800, as previously described [170]. At pre-determined times, mice were imaged for distribution of NSP 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 (ICP-AES): Weighed organs were frozen until ready for elemental analysis of Silicon using ICP-AES, as previously described [137, 170]. Organs were homogenized in 3 mL of 20% ethanol in 1 N NaOH for 48 hours at room temperature under rotation. Tissues were then centrifuged at 4000 x g for 30 minutes and 1.0 mL of supernatant was collected and diluted with 4.0 mL of Millipore water for elemental analysis. ICP-AES was performed using a Varian Vista-Pro housed within Rice University’s Geochemistry Laboratory.
4.3.14 Immunohistochemistry (IHC):

Mice were intravenously injected with 5x10^7 NSP (or PBS) in 100 µL of sterile PBS. 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 10% formalin 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 accomplish by incubating cells in citrate buffer for 30 minutes, followed by a 10 minute treatment with 3% hydrogen peroxide. 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.

4.3.15 Statistical Analysis

For MTT, linear regression analysis was performed and significance was determined by testing whether the slopes and intercepts were significantly different (F test). In all cases: * was used for p-values between 0.01 to 0.05, ** for values between 0.001 to 0.01, and *** for values < 0.001.
4.4 Results

4.4.1 LDH and MTT

Previous investigations demonstrated that HUVEC, when seeded as a monolayer, quickly captured and internalized NSP by endocytosis[171] and resulted in partitioning of NSP into daughter cells upon division.[64] Cells (HUVEC, ADMSC) were seeded at high confluence and treated with NSP (Cell:NSP ratio of 1:1, 1:5, and 1:10) resulting in nearly 100% of cells containing at least one NSP per cell. Conventional cellular viability (MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and toxicity (LDH, lactate dehydrogenase) assays were conducted on both cell lines comparing different ratios in order to investigate if there was a dose dependent effect of NSP on cells. Both cell types showed a linear increase in MTT incorporation over 72 hours with minor difference between treated and control cells (Fig 4.1). This result indicated that the exposure of NSP at all concentrations did not significantly alter the proliferation of cells and that NSP internalization appears to be well tolerated by both cell types. Also the release of LDH was followed in both cell types, and it confirmed the lack of a cellular cytotoxic stress due to NSP internalization.
Figure 4.1 Cellular proliferation and toxicity of NSP. A) MTT assay was used to determine the effect NSP internalization on proliferation of HUVEC (top) and ADMSC (bottom). B) LDH assay was used to assess cellular toxicity and membrane damage that could have developed within HUVEC (top) and ADMSC (bottom) after the internalization of NSP.
4.4.2 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 NSP 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 NSP. 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 (Fig 4.2). 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 NSP did not induce any significant increases in apoptotic events after internalization.

![Figure 4.2 Apoptotic induction upon NSP internalization](image.png)

Figure 4.2 Apoptotic induction upon NSP internalization. Annexin V staining of HUVEC with internalized NSP at 24, 48, and 72 hours demonstrating minor increases over time, consistent with control cells indicating minimal induction of apoptosis.
HUVEC were also analyzed to understand the distribution of the cell cycle phases upon internalization of NSP with different surface chemistry and structure (Fig 4.3). All cells analyzed demonstrated similar distribution in each phase after 72 hours. A minor discrepancy was observed at 12 hours between HUVEC with internalized NSP and control cells, where NSP treated cells demonstrated increased concentration in the sub G₀ (i.e., apoptotic/inactive cell phase). This difference was attributed to the different confluency of control cells (70%), compared to NSP, 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 NSP. Further proof of this claim was observed by comparing the effect of NSP 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 NSP treated cells revealed a clear distinction suggesting that NSP 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 NSP 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 NSP (oxidized vs. APTES) or for different pore structure (SP vs. LP). Thus after internalization of NSP, cells were able to progress through the different cellular phases without altering DNA synthesis and allowed for their continued cellular division.
Figure 4.3 Cell cycle analysis of NSP impact. HUVEC were exposed to NSP 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 NSP with varying surface chemistry (oxidized and APTES) and pore sizes (SP vs LP). Data collected by Ennio Tasciotti.
4.4.3 Cellular Architecture

To understand the effect of NSP internalization on cell architecture, the cytoskeletal arrangement and the ultrastructure of the cell were investigated. HUVEC (Fig 4.4A) and ADMSC (Fig 4.4B) were exposed at several ratios and stained with fluorescent markers to label f-actin microfilaments (red), alpha tubulin microtubules (green), and nuclei (blue). Previous results have demonstrated that, upon internalization by endocytosis, NSP are found in the lysosomal compartment within a few hours.[172] Here, we show that after internalization, NSP accumulate in the peri-nuclear region for both cells lines. The increased number of NSP internalized did not affect their ability to undergo peri-nuclear localization. Microfilaments of both cell types were well organized and filamentous, with parallel stress fibers distributed throughout the cytoplasm. These cytoskeletal components displayed characteristic endothelial and fibroblastoid phenotypes for HUVEC and ADMSC, with microtubules radiating from the peri-nuclear area throughout the cell body as previously observed.[173, 174] These observations indicated that cytoskeletal elements were largely unaffected by NSP incorporation.
Figure 4.4 Cellular architecture upon internalization of NSP. Cytoskeleton staining of HUVEC (A) and ADMSC (B) at increasing doses (1:1, 1:5, 1:10) of NSP demonstrated conservation of cellular structure. Microfilaments (f-actin) are in red, microtubules (α-tubulin) in green, nuclei are in blue, and NSP are in green (A) or yellow (B), scale bar = 10 μm.
4.4.4 Ultrastructure

The ultrastructure of cells was also investigated using transmission electron microscopy (TEM) to understand the effect of NSP internalization at the organelle scale. HUVEC (Fig 4.5A) and ADMSC (Fig 4.5B) were exposed to NSP at a ratio of 1:10 and inspected for the integrity of several organelles in close proximity to internalized NSP. The analysis of TEM micrographs confirmed that the nucleus, nucleolus, and nuclear envelope of both cells were unaffected. The proximity to the nuclear envelope did not display any visible damage, and that the organization of heterochromatin and euchromatin within the nucleus was comparable with that of untreated cells, even when internalized NSP appeared to modify the curvature of the nuclear membrane (Fig 4.5B). As in untreated cells (supplementary information), rough endoplasmic reticulum studded with more electron dense areas indicative of ribosomes was found adjacent to the nuclear envelope and mitochondria with defined matrix and membranes were observed and appeared intact and healthy upon NSP uptake. Hence, upon NSP internalization, both cell types retained normal organization of cytoskeletal elements and ultrastructure and appeared unaffected by the presence of NSP.
**Figure 4.5 NSP impact on cellular ultrastructure.** TEM images of HUVEC (A) and ADMSC (B), 24 hours after internalization of NSP. Starting from the left, images were selected to demonstrate the effect of NSP internalization on the nucleus/nucleolus, nuclear envelope, rough endoplasmic reticulum, and mitochondria, indicated by black arrows. Scale bars are 500 nm, except for left most images where it is 10 μm (A) and 2 μm (B).
4.4.5 Tube Formation

Understanding the potential effects that NSP may have on the function of endothelial cells is vital for the development of a drug delivery system with no impact *per se* on cell physiology. For this reason, we chose to investigate if HUVEC retained the ability to form tubular networks, a conventional *ex vivo* angiogenesis assay.[175] HUVEC were exposed overnight to 1000 x 200 nm discoidal NSP at a ratio of 1:40. The following day, HUVEC containing NSP were seeded onto matrigel and assayed for tube formation (Fig 4.6). Initially, HUVEC were seen as single cells (Fig 4.6A), however over time cells migrated and elongated towards other cells and reorganized in space to begin the formation of vessel-like structures. Upon NSP internalization, HUVEC assembled and formed tubes in a similar fashion, indicating that NSP did not alter this process. Quantitative assessment of tube formation was performed using Wimasis Image Analysis software and comparing the percentage of area covered by tubes, and the total number of tubes, branching points, and loops in four separate 4x images taken at 24 hours (Fig 4.6B,C). Compared to control HUVEC, cells containing NSP covered equivalent surface and formed a comparable total number of tubes. Furthermore, the final count of branching points and loops between different treatment groups was similar. In the MTT assay, exposed cells retained the ability to actively reduce the dye, demonstrating their viability post tube-formation (Fig 4.6D). In order to confirm NSP retention (Fig 4.6E) and to analyze cytoskeletal organization (Fig 4.6F), tubes were imaged with a laser scanning confocal system. NSP were discovered predominantly distributed within the center of the tubes and mostly co-localized with nuclei, while fewer particles could be found in the tube projections (Fig 4.6E,F). Internalization was confirmed by inspecting a cross-section displaying x and y plane profiles that localized NSP within the cellular boundaries (Fig 4.6E, right). Lastly, the cytoskeletal organization was inspected by staining f-actin. Consistent with results from monolayer HUVEC culture, tubes
formed by both control HUVEC and HUVEC containing NSP exhibited similar parallel microfilaments, indicating minimal actin reorganization.
Figure 4.6 HUVEC network formation upon internalization of NSP. A) Images were taken at increasing times (starting from left, 0.5, 1, 2, 3, 4, and 6 hours) to investigate the effect of NSP on migration of tube network assembly. B) Quantitative assessment of the percentage of area covered and total tubes formed. Left axis (black): displays the quantity of the percent covered area and right axis (red) is for the total tubes formed comparing control and NSP treated HUVEC. C) Quantitative assessment of the total branching points and total loops detected during tube formation. Left axis (black): plots the total number of branching points. Right axis (red) plots the total number of loops. E, F) Confocal imaging of tubes formed at 24 hours demonstrating retention of NSP after tube formation, with HUVEC in green and NSP in red (E). Maintained cytoskeletal structure after tube formation, microfilaments (f-actin) in green, NSP in red, and nucleus in blue (F). Scale bar = 200 µm (E) and 20 µm (F).
4.4.6 Differentiation & Migration of ADMSC

4.4.6.1 Differentiation

Differentiation is integral for efficient tissue repair and maintaining homeostasis, hence conservation of this function is critical and can be assayed as an indicator of toxicity. Stromal cells, initially isolated as plastic-adherent colony-forming fibroblasts,[176] have a marked capacity to differentiate into mesenchymal cell types, such as adipocytes and osteoblasts.[177] Similar properties have been demonstrated for ADMSC,[178] and here we tested whether NSP affect cell multipotency. As shown above, upon internalization (at 1:5 cell:NSP ratio), ADMSC retained the ability to adhere to plastic. Under induction media, ADMSC containing NSP maintained morphology similar to that of control cells, as evident from toluidine blue staining (Fig 4.7A). Oil Red O, a lipophilic dye that stains lipid droplets, was used to identify adipocytes upon adipogenesis induction. ADMSC containing internalized NSP displayed similar staining for Oil Red O as control cells indicating that ADMSC retained the ability to differentiate into adipocytes. Upon osteoblast differentiation, ADMSC were assessed by Von Kossa, alkaline phosphatase (ALP), and alizarin red staining to determine mineralization, osteoblast activity, and calcium deposition, respectfully. ADMSC with internalized NSP demonstrated comparable staining with all three techniques. Furthermore, a quantitative calcium assay was used to compare ADMSC containing NSP with control cells incubated with inductive media or regular media (Fig 4.7B). ADMSC with internalized NSP produced an equivalent amount of calcium as control cells indicating an unchanged capacity of ADMSC to differentiate into osteoblasts upon internalization of NSP.

4.4.6.2 Migration

Finally, a potentially useful property of mesenchymal cells, including ADMSC, is their trafficking toward tumors.[167, 179] Here, we tested the ability of ADMSC to traffic towards breast cancer cells (MDA-MB-231) using modified Boyden chamber (transwell) and wound-
healing (scratch) assays. The transwell assay was established as depicted in Figure 4.7C, where MDA-MB-231 cells were seeded below the insert within the well while ADMSC (Cell:NSP ratio of 1:1, 1:5, and 1:10) were seeded on the top of the inserts. In each group, ADMSC were found to transverse the membrane, as illustrated by representative images (Fig 4.7D). Quantitation was performed using crystal violet staining and comparing absorbance of each sample (Fig 4.7E) and indicated that ADMSC migration was not significantly affected by internalization of NSP. In the wound-healing assay, ADMSC and cancer cells were seeded into inserts that separated the cells by an average of 500 µm (Fig 4.7F). Snapshots shown at 0 and 18 hours of migration demonstrated that the leading edge (red line) within the different groups was equivalent, suggesting that the incorporation of NSP did not affect migration. Furthermore, inspection of time-lapse microscopy movies confirmed that ADMSC containing NSP displayed comparable migration kinetics.
Figure 4.7 ADMSC differentiation and migration upon internalization of NSP. A) Control and NSP-containing ADMSC were induced to differentiate into adipocytes and osteoblasts. Toluidine blue demonstrates similar cellular morphology, while Oil Red O reveal lipid droplets upon adipogenic differentiation. Von Kossa and ALP staining demonstrate osteogenic differentiation. B) Quantitative determination of solubile calcium upon osteogenic differentiation. C) Schematic of Boyden chamber illustrating the set-up used to verify migration of ADMSC toward MDA-MB-231 cells. D) Sample images of migrated ADMSC containing different ratios of internalized NSP (from left: 1:1, 1:5, and 1:10). E) Quantitative
assessment using Boyden chambers, comparing control ADMSC with ADMSC containing different amounts of NSP. F) Scratch assay still frame images from time lapse microscopy at 0 and 18 hours, demonstrating conserved migration at the different ratios of NSP compared to the leading edge (red-line) within each sample.
4.4.7 Impact of degradation byproducts on cellular function

4.4.7.1 Cellular Architecture

The concerns regarding the exposure of nanovectors to healthy cells must be adequately examined in order to gauge its potential use in the clinic. We investigated the reaction of HUVEC cultures upon introduction to partially degraded NSP (i.e. fragments). We initially sought to understand the impact the internalization of fragments have on the cell architecture, specifically inspecting the microfilaments of the cytoskeleton. Hence, we compared the f-actin staining of HUVEC treated with equal concentrations of intact NSP and NSP fragments. As shown in Figure 4.8A, the microtubules (green) and nucleus (blue) of HUVEC displayed similar staining, comparing untreated and NSP. HUVEC exhibited conserved parallel filamentous microfilament arrangements that assembled equally throughout the entire cell. Previous studies demonstrated that NSP were quickly delivered to the perinuclear region.[180] Similarly, NSP fragments were found to preferentially accumulate around the perinuclear area. Furthermore, nuclear staining revealed consistent shapes upon internalization indicating a negligible cytotoxic effect attributed to fragments. Thus, internalization of NSP degradation by-products exhibited a negligible effect on cellular architecture producing conventional endothelial phenotypes.

4.4.7.2 Cellular integrity

Understanding the potential consequences of complete degradation by-products on normal cellular homeostasis is crucial for evaluating drug delivery vectors. The impact of degradation products on the proliferation and cell cycle maintenance of HUVEC was investigated by exposing cells to completely degraded NSP. The proliferation of HUVEC was studied by comparing the incorporation of MTT to extrapolate the level of metabolic activity. Degraded products from oxidized and APTES NSP exhibited similar proliferation rates when compared to PBS controls (Fig. 4.8B). HUVEC displayed a resilient response to
the degradation by-products of NSP of varying porosities (SP, MP, LP, XLP) preserving an active mitochondrial function. In addition, linear regression analysis revealed that the proliferation rates (i.e., slopes) of HUVEC exposed to oxidized and APTES modified NSP were not significantly different than the PBS control. The different porosities demonstrated that HUVEC tolerated a wide concentration of Si approximately ranging from 30 to 80 pg/cell for XLP to SP, respectively.

Although comprehending the metabolic activity and continued proliferation of cells is useful, it fails to provide detailed information on specific events occurring within cells upon exposure. As shown in Figure 4.8C, we quantified the percentage of HUVEC within the different phases of the cell cycle at 48 hours comparing the effect of degradation products with different surface chemistry and porosities. HUVEC revealed equivalent patterns of distribution across the phases at 48 hours with similar quantities among the different groups. A comparison of these results with control and intact NSP (Fig 4.3) revealed that at 48 hours HUVEC exposed to the degradation products of NSP behaved nearly identical. Control HUVEC displayed 17% in the sub G\(_0\) phase, commonly where apoptotic/inactive cells are categorized, comparable with cells containing NSP degradation products whose values ranged from 15-21%. Furthermore an inspection of combined effort of the G\(_2\)/M and S phases of the cycle, representative of mitotic and DNA replication, respectively, can be used to chronicle the ‘active’ fraction of cell division. Using this benchmark, we confirmed that HUVEC preserved these routine functions in a consistent manner displaying 32% for control cells and ranging from 28-34% for NSP degradation. In addition, analogous HUVEC percentages were discovered in the G\(_0\)/G\(_1\) phase, which recognizes cells in the resting/checkpoint stage. Hence, exposure to NSP degradation products yielded a negligible cellular response from primary endothelial cells. In fact, we recently demonstrated that intact NSP would permit HUVEC and stem cells to conserve their intrinsic functions (e.g., tube formation and multipotent differentiation) upon internalization confirming a higher degree of
biocompatibility.[181] Here, HUVEC maintained proliferation and routine cycling through various cellular phases without influencing DNA replication for several days after the introduction of the degradation products.

Figure 4.8 Cellular architecture and biocompatibility of NSP degradation products. A) The microfilaments (green) and nucleus (blue) of HUVEC were stained to interpret the effect of exposing NSP fragments. Control and non-degraded NSP were used to compare cytoskeletal structure. B) An MTT assay to demonstrate the continued proliferation of HUVEC after internalizing oxidized (left) and APTES (right) degradation products of NSP.
with varying porosities (SP-XLP). Proliferation of HUVEC was compared to sterile PBS, the buffer used to degrade the NSP. The results are shown as mean ± SD. C) Cell cycle analysis at 48 hours after introduction to degradation products of NSP. The cell cycle of HUVEC was conserved independent of surface (oxidized and APTES) and porosity (SP and LP). Cell cycle data was collected by Ennio Tasciotti.
4.4.8 *In vivo Cytokine Expression*

In order to provide insight into the organ response and immunoreactivity upon administration of NSP, a profile of anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-1α, IL-1β, 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 NSP or saline (control). As shown in Figure 4.9, the induction patterns of selected cytokines appeared to differ, such that IL-1β and IL-6 reached maximum levels earlier compared to IL-1α and TNF-α. Differences between NSP treated and control groups across different time points stayed comparatively the same for certain cytokines. For example, NSP treated group showed no significant difference in the expression of IL-1β (Fig 4.9C), IL-7 (Fig 4.9E), and IL-10 (Fig 4.9F) compared to the control group. However, NSP 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-1α, 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.
Figure 4.9 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-1α, C) IL-1β, D) IL-6, E) L-7, F) IL-10. ELISA analysis was performed with the assistance of Iman Yazdi.
4.4.9 Biodistribution

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

4.4.9.1 NIR Longitudinal Imaging

NSP labeled with a NIR fluorescent dyes retained similar fluorescence over several days (Fig 4.10A) and displayed increasing fluorescence as the particle concentration increased (Fig 4.10B,C). Mice were intravenously administered with NIR labeled NSP and imaged longitudinally for fluorescent signal at 0, 0.5, 2, 24, 48, and 168 (1 week) hours (Fig 4.10D). Mice were sacrificed at 2, 4 and 168 hours and organs were collected and imaged (Fig 4.10E). 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 (Fig 4.10F). Minimal autofluorescence from the abdomen was observed in mice at 0 hr. Upon administration, NSP 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 NSP. 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.
Figure 4.10 Noninvasive live animal imaging of NSP distribution. A) NSP were labeled with a NIR dye and B) showed increasing fluorescent signal during serial dilution. C) Left axis, green: NSP demonstrated intensified signal as the concentration increased; Right axis, white: the supernatants collected after washing demonstrated decreases in dye concentration per NSP 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. Data collected by Ennio Tasciotti.
4.4.9.2 Quantitative Biodistribution

Tissues were collected, washed with PBS and imaged for fluorescent intensity and quantified by creating ROI around each organ. The quantitation of fluorescent signal is shown in Figure 4.11A. 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 4.11B. This type of analysis demonstrated almost equivalent concentrations of NSP 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 NSP confirming the NIR imaging data. These results established that after an initial accumulation in many vital organs (heart, lung, spleen, liver), the biodistribution of NSP reached a long-term accumulation in the liver and the spleen.
Figure 4.11 Quantification of NSP 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 NSP. Data collected by Ennio Tasciotti.
4.4.10 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 NSP. 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 and were analyzed, organized, and written with the assistance of Christian Boada.

4.4.10.1 Hematoxylin and Eosin

Harvested Tissues were stained with hematoxylin and eosin (H&E) to understand the cytological and structural impact of NSP on tissues. The lungs exhibited alterations of normal tissue structure (Fig 4.12). 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 (Fig 4.12). 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.[182] 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.[183] 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 (Fig 4.12). Heart tissue maintained myofibril organization and intercalary disks were intact while kidney showed typical glomerular structure across all time points (Fig 4.13). 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.

**Figure 4.12 Histological evaluation of filtering organs 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 hepatocyte structure by 5.5 months. Spleen exhibited no pathological findings of interest at all time points collected.
Figure 4.13 Histological evaluation of heart and kidney using H&E. Heart and kidney demonstrated no structural abnormalities through all time points indicative of no relevant pathological organ response to NSP.
4.4.10.2 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.[184] Within the samples evaluated for this study, the TUNEL staining (Fig 4.14) 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.

![Figure 4.14 Apoptotic events in tissues](image)

**Figure 4.14 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.
4.4.10.3 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.[185] 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 NSP further suggesting the lack of NSP induced tissue toxicity (Fig 4.15).
Figure 4.15 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 NSP.
4.5 Conclusion

In conclusion, we demonstrated that NSP elicited minor responses resulting in minimal toxicity that did not compromise key cellular functions. On account of cells maintaining these functions while containing several NSP per cell, our data demonstrate a remarkable tolerance toward this type of nanomaterial. The assessment of NSP 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 it was possible to achieve a more coherent and insightful analysis of the impact of NSP upon systemic administration. In conjunction with providing a methodological platform for analyzing nanoparticle toxicity, this study provides a rationale for using NSP as a nanomaterial whose compatibility and benign nature may streamline its biomedical applications. The low toxicity and multifunctional nature of NSP 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.
Chapter V: VEGFR2 Targeting of NSP

5.1 Abstract

Nanovectors are a viable solution to the formulation of poorly soluble anticancer drugs. Their bioaccumulation in the tumor parenchyma is mainly achieved exploiting the enhanced permeability and retention (EPR) effect of the leaky neovasculature. In this paper we demonstrate that NSP exhibit rapid tumoritropic homing independent of EPR, relying on particle geometry and surface adhesion. By studying endothelial cells overexpressing vascular endothelial growth factor receptor-2 (VEGFR2), we developed NSP able to preferentially target VEGFR2 expressing tumor-associated vessels. Static and dynamic targeting revealed that NSP conjugated with anti-VEGFR2 antibodies displayed greater than a 5-fold increase in targeting efficiency towards VEGFR2 expressing cells while exhibiting minimal adherence to control cells. Additionally, VEGFR2 conjugation bestowed NSP with a significant increase in breast tumor targeting and in the delivery of a model payload while decreasing their accumulation in the liver. Surface functionalization with an anti-VEGFR2 antibody provided enhanced affinity towards the tumor vascular endothelium, which promoted enhanced adhesion and tumoritropic accumulation of a reporter molecule released by the NSP.

5.2 Introduction

Chemotherapy is the most widely used form of therapy in the treatment of cancer. Traditional chemotherapeutic drugs indiscriminately target and destroy both malignant and healthy cells, resulting in severe side effects [186]. Novel therapeutics, such as monoclonal antibodies and small interfering RNA (siRNA), are well suited to target tumor cells or specific genes but are associated with an increased risk of adverse immune reactions [187, 188] and ineffective delivery to cells [189]. New treatments based on the targeted delivery of
therapeutic agents to the tumor microenvironment [15] rely on nanoparticles (NP) able to encapsulate poorly soluble drugs [190], to provide protection from degradation [191], to enhance cellular internalization [192], or to trigger the release of payloads based on environmental cues [193]. NP can persist in blood circulation and exploit the enhanced permeability and retention (EPR) effect exhibited by tumor-associated blood vessels [194, 195].

In the late 1800s, Rudolf Virchow observed that tumors were highly vascularized but it was not until the early 1970s that controlling angiogenesis became a therapeutic option for cancer [196]. Today, the delivery of therapeutic agents to the tumor microvasculature is a clinical reality. Both endothelial and cancerous cells undergoing angiogenesis express high levels of vascular endothelial growth factor receptor 2 (VEGFR2), a primary mediator of cell proliferation and tumor growth [197-200]. The inhibition of VEGF-mediated angiogenesis with monoclonal antibodies against VEGF (the natural ligand and activator of VEGFR2) culminated in the development of bevacizumab (Avastin), a humanized monoclonal antibody that obtained U.S. Food and Drug Administration approval in 2004 [201, 202]. Based on the success of this approach, to selectively deliver a payload to the cancer site, we developed nanovectors capable of recognizing vascular endothelial cells expressing VEGFR2.

To circumvent the biological barriers encountered by NP during systemic administration, we developed NSP [136]. NSP were rationally designed to exhibit superior margination (i.e., tendency to drift towards the vessel wall) due to their disk-like shape and size [41, 44] and demonstrated efficient adhesion towards endothelial cells [171, 203]. During systemic administration NSP demonstrated rapid (< 1 hour) tumoritropic accumulation independent of EPR [43, 204] and enhanced delivery to the bone marrow upon targeting with E-selectin [205]. The pores and the surface of NSP can accommodate a variety of payloads (e.g., chemotherapeutics [66, 206], drugs [69], NP [207], contrast agents
and their release could be tuned by adjusting the pore size [208] or their surface coating [133, 209, 210].

In this paper, we propose to bestow NSP with anti (α)-VEGFR2 antibodies to specifically target NSP to tumor-associated blood vessels. The in vitro targeting efficiency was studied in static conditions and under physiological dynamic flow on endothelial monolayers expressing increasing amounts of human VEGFR2. Breast tumor targeting and biodistribution was assessed in vivo using near infrared fluorescent imaging. We demonstrated the ability of NSP to associate with VEGFR2 expressing endothelia upon decoration with α-VEGFR2 antibodies. Finally we showed the ability of the NSP to favor the local delivery and accumulation of a model payload in the tumor parenchyma.

5.3 Experimental Materials & Methods

5.3.1 NSP Conjugation

NSP were modified with 2% APTES as previously described. APTES-modified NSP were then functionalized with succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Thermo Scientific) in a 1 mg/mL solution and incubated for two hours with mixing. SMCC-modified NSP were washed three times and stored in a desiccator overnight. Rat anti-mouse VEGFR2 antibody (R&D Systems) was separately prepared and dye-labeled in the following manner. VEGFR2 was incubated at 4°C for one hour while mixing in phosphate buffer (PB, 10 mM, 7.5 pH), AlexaFluor 555 (Invitrogen), and Traut’s reagent (2-iminothiolane hydrochloride; 2 mg/mL, Thermo Scientific) solution. The solution was then filtered through a desalting column to remove free dye. SMCC-modified NSP were suspended in PB with dye-labeled VEGFR2 and incubated at 4°C for two hours with mixing. NSP were washed thrice and stored at 4°C. Labeling of VEGFR2-conjugated NSP (α-VEGFR2) or SMCC-modified NSP (untargeted) was achieved by suspending NSP in PB.
solution containing AlexaFluor 647 (Invitrogen) and incubated for one hour with mixing. NSP were washed with PB and stored at 4°C.

5.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was obtained using a Nicolet 6700 FT-IR Spectrometer machine (Thermo Scientific) scanning a spectrum range of 3600 – 400 cm⁻¹ with an average of 200 scans at 0.09 cm⁻¹ resolution. 5 x 10⁶ NSP samples were collected after each step in the conjugation sequence (oxidation, APTES, SMCC, and α-VEGFR2). All samples were mixed with potassium bromide and placed on a Snap-in™ sample compartment baseplate for transmission-based collection. Spectra results were collected and managed using OMNIC software.

5.3.3 Zeta Potential

The zeta potential of NSP was measured at each step of the conjugation process: 1) after oxidation 2) after APTES modification 3) after conjugation with SMCC 4) and after conjugation with α-VEGFR2. Samples for zeta potential analysis were prepared by diluting 100 µL of each sample into 1.5 mL of PB and measured using a Malvern Zetasizer instrument (Malvern Instruments). Each measurement was made in triplicate and the average zeta potential was determined.

5.3.4 Confocal Imaging of NSP

NSP and PAEC were imaged for fluorescence using a laser scanning Nikon A1 confocal imaging system equipped with a 403, 561.5, and 640.8 nm laser sources and a multi-line 488 nm laser source, an instrument made available by The Advanced Tissue & Cellular Imaging Core of HMRI. Dye and anti-VEGFR2 conjugated NSP were imaged to
determine presence and distribution of antibody. PAEC were imaged for YFP fluorescence and interaction with fluorescent VEGFR2 antibodies.

5.3.5 Melittin labeling and loading in NSP

Conjugation of Melittin with fluorescent dye was performed using a DyLight 555 NHS ester (Pierce) following the manufacturer’s microscale labeling kit protocol. Free dye was then removed using a Centricon centrifugal filter device (Millipore) with a 3000 MW cut-off. Next, labeled melittin was rinsed and suspended in a 20 mM Tris buffer (pH 7.3). Loading of NSP with melittin was achieved by incubating $3 \times 10^6$ NSP suspended in 20 mM Tris buffer with melittin. Unloaded melittin was removed following incubation by briefly centrifuging at 3500 $\times$ g. The supernatant was discarded and NSP were re-suspended in fresh Tris buffer. Fluorescence within NSP was imaged using confocal microscopy.

5.3.6 PAEC Clones

Porcine aortic endothelial cells (PAEC) and hVEGFR2 plasmid were a gift from the laboratory of Dr. Mauro Giacca at the International Centre for Genetic Engineering and Biotechnology (ICGEB in Trieste, Italy). PAEC were maintained using F-12K media (Hyclone, Thermo Scientific) and supplemented with 10% fetal bovine serum. The hVEGFR2 protein was fused with yellow fluorescent protein (YFP) and contained a vector encoding for geneticin (G418) resistance. PAEC were transfected with hVEGFR2/YFP using lipofectamine 2000 by Ennio Tasciotti. PAEC “clones” were achieved using cloning by limiting dilution and arranged based on YFP fluorescence. PAEC clones were analyzed using flow cytometry to distinguish different levels of VEGFR2 expression (VEGFR-LOW, -MED, -HIGH). Maintenance of PAEC clones (i.e., PAEC transfected with hVEGFR2) was achieved by supplementing complete media with 3 mg/mL of G418. Flow cytometry analysis of YFP status on PAEC was performed using a BD FACSFortessa housed within HMRI Flow
Cytometry Core and equipped with four laser (405, 488, 561, 630 nm) excitation sources. The 488 nm source with a 525/50 bandpass filter was used to acquire YFP signal from control (i.e., wild-type (WT)) and VEGFR2-clones.

5.3.7 Targeting of NSP:

**Static**: The static (i.e., without flow) targeting potential of targeted (α-VEGFR2) and untargeted NSP was tested using 8-chamber CultureSlides (BDFalcon) placed on a flat rotator shaker (Thermo Scientific) within the incubator at 37 °C and 5% CO₂. PAEC were seeded at a density of 17,500 cells/chamber for WT and 12,250 cells/chamber for HIGH PAEC, 24 hours prior to treatment. PAEC were treated with NSP at a ratio of 1:10 (PAEC:NSP) and analyzed at pre-determined time points. PAEC were then washed, fixed, and stained with Prolong gold and 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Slides were visualized and imaged using a Nikon Eclipse 80i fluorescence microscope, and data was analyzed using Nikon Elements.

**Dynamic**: Dynamic targeting (i.e., with flow) of NSP in flow conditions was tested using ibidi μ-slide I² Luer pre-coated with fibronectin at 75 μg/mL. 24 hours prior to NSP treatment, PAEC were seeded at a concentration of 1.25 × 10⁶ cells/mL. Slides were flowed with 3 × 10⁷ NSP (targeted and untargeted) at 100 μL/minute for 30 minutes, as previously described [133]. Slides were continuously imaged using an inverted Nikon Eclipse Ti fluorescence microscope equipped with a Hamamatsu ORCA-Flash 2.8 digital camera and fitted with an induction chamber maintaining samples at 37°C and 5% CO₂. Data was analyzed using Nikon Elements.

5.3.8 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\textsuperscript{nu}; 4-6 week old) were purchased from Charles Rivers Laboratories and maintained as previously described [211]. Mouse breast cancer tumors were established by implanting 5x10\textsuperscript{5} 4T1 tumor cells suspended in PBS into the mammary fat pad of female nude mice for non-invasive imaging.

5.3.9 Non-invasive imaging and biodistribution

Nude mice containing 4T1 tumors were randomly divided into groups (n \geq 4) to compare VEGFR2 (i.e., targeted) at 2 and 4 hours versus IgG (i.e, non-targeted) at 2 hours. Mice were injected with 5 x 10\textsuperscript{7} NSP labeled with DyLight 800, as previously described [212]. Mice were imaged using an IVIS Lumina equipped with the indocyanine green excitation and emission filters at pre-determined times. Mice were sacrificed and organs were harvested, washed in PBS, and imaged for fluorescence in each organ. Images of whole animal and organs were analyzed and exported using Living Image 4.0 software.

5.3.10 Statistical analysis

Statistics were calculated with Prism GraphPad software. Statistics for static targeting, dynamic targeting, and in vivo targeting experiments was analyzed using a Two-Way ANOVA followed by a Bonferroni post-test to compare replicate means by rows.
5.4 Results

5.4.1 NSP Characterization

Hemispherical NSP were fabricated as previously reported [135] yielding a homogenous and uniform distribution of 3.2 µm NSP with 15 nm pores. The multistep process required to produce α-VEGFR2 NSP is illustrated in Figure 5.1A. FTIR (Fig 5.1B,C) and zeta potential measurements were collected after each modification to verify successful conjugation. Inspection of the FTIR spectrum between 1300 and 1900 cm\(^{-1}\) (Fig. 5.1C), revealed a broad peak at 1565 cm\(^{-1}\) after APTES modification which corresponds to N-H (i.e., amine groups) and confirmed successful conjugation [193]. SMCC conjugation prompted a peak at 1704 cm\(^{-1}\), consistent with terminal maleimide groups present on the cross-linker. Upon conjugation with VEGFR2 antibodies, the peak at 1704 cm\(^{-1}\) diminished suggesting a decrease in maleimide groups due to the reaction between the sulfhydryl groups on the antibody (enriched using Traut’s reagent) and the available maleimide groups on the surface of NSP. Zeta potential measurements corroborated the modifications to the surface resulting in dramatic changes in surface charge (Fig. 5.1D). For example, upon APTES modification the surface charge increased by +29 mV (consistent with previous publications [136, 212, 213]) followed by decreases of -13 and -22 mV for SMCC and α-VEGFR2, respectively. In addition, the successful conjugation of VEGFR2 antibody to NSP was confirmed using fluorescent microscopy and did not hinder further incorporation of fluorescent molecules within the NSP (Fig. 5.1E). NSP modified with α-VEGFR2 antibodies conserved the ability to retain a therapeutic payload, melittin the primary active component in bee venom and has shown promise for cancer therapy [214] (Fig. 5.1F). Thus upon functionalization with antibodies, NSP conserved the ability to retain payloads for imaging and therapy.
Figure 5.1 NSP characterization for successful conjugation with α-VEGFR2. a, Schematic illustrating the procedure and steps performed to conjugate NSP with α-VEGFR2 antibodies. b, Complete FTIR spectra after each conjugation step: oxidation (i), APTES (ii), SMCC (iii), and α-VEGFR2 (iv); and c, inset from b, displaying FTIR spectra of samples between 1300 to 1900 cm$^{-1}$ to emphasize the emerging peaks within this range. d, Zeta potential values of NSP after each conjugation displaying changes in surface chemistry. e, Confocal images of untargeted and α-VEGFR2 NSP showing conjugation of NSP with fluorescent dye (purple) and anti-VEGFR2 antibodies (green). f, Confocal images demonstrating melittin (red) loading into NSP with or without anti-VEGFR2 antibodies (green). (e,f: scale bar, 1 µm). Melittin loading was performed by Rohan Bhavane.
5.4.2 PAEC Characterization

PAEC cells were transfected with the hVEGFR2-YFP gene, selected for successful integration using G418, sorted using FACS, and cloned using limiting dilution to generate cell populations expressing various amounts of VEGFR2 (Fig. 5.2). From these cells, three were selected to illustrate the low, medium, and high (LOW, MED, and HIGH) range of possible VEGFR2 expression within tumor vasculature [215, 216]. Prior to selection, each clone sub-type exhibited several cells absent in YFP expression (Fig. 5.3A). Clones were then sorted and selected using G418 to yield stable populations of clones (Fig 5.3B-D and 5.4A). Confocal imaging (Fig. 5.3B) validated the pattern of expression for VEGFR2/YFP, progressively increasing from the LOW to HIGH VEGFR2 PAEC consistent with flow cytometry analysis (Fig. 5.3C). High magnification of HIGH PAEC displayed uniform YFP fluorescence (i.e., VEGFR2) on the surface surrounding the entire cell (Fig. 5.5). Western blot analysis confirmed the increasing expression of VEGFR2 in the LOW, MED, and HIGH PAEC populations (Fig 5.3D). HIGH PAEC clones exhibited sustained and uniform expression of VEGFR2 for several weeks (Fig 5.4B) and were used for all the in vitro targeting experiments while non-transfected wild-type (WT) PAEC cells were used as controls.
Figure 5.2 Generation of PAEC Clones. A. PAEC were exposed to 4 and 8 µg of plasmid DNA (hVEGFR2-YFP) and YFP fluorescence was measured using flow cytometry comparing with non-transfected PAEC (WT). B. Bright-field (top) and fluorescent images (bottom) were taken of the surviving cells illustrating successful transfection. C. PAEC from each group (4 and 8 µg) were sorted to collect the populations of cells expressing YFP and re-analyzed using flow cytometry where YP4 and YP8 corresponds to cells exposed to 4 µg and 8 µg, respectively. D. Fluorescent images of sorted PAEC cells showing YFP fluorescence (top) and a merged image (bottom) with the nucleus of cells stained with DAPI (blue). E. YP4 cells were cloned by limiting dilution and flow cytometry analysis of four clones (A-D) are shown demonstrating increasing levels of YFP fluorescence from A to D. PAEC clones were established with assistance of Ennio Tasciotti.
Figure 5.3 Characterization of hVEGFR2 transfection in PAEC. a, Fluorescent images demonstrating expression of VEGFR2 (YFP, yellow) of WT (no VEGFR2) and PAEC clones (LOW, MED, and HIGH) before selection and (b) after selection with G418. (scale bar, 50 µm) c, Flow cytometry and (d) western blot with quantification of PAEC WT and clones after selection. Western blot analysis was performed by Amber Jimenez.

Figure 5.4 Characterization of PAEC clones. A. Cloning by limiting dilution resulted in several clones that were analyzed using flow cytometry and number by increasing YFP fluorescence. B. Clones were maintained using G418 and YFP fluorescence remained consistent throughout three weeks in culture demonstrating the stability of VEGFR2 incorporation within PAEC. PAEC clones were established with assistance of Ennio Tasciotti.
Figure 5.5 Confocal imaging of HIGH PAEC. HIGH (i.e., high expression of hVEGFR2-YFP) PAEC exhibited uniform distribution of YFP/VEGFR2 on the surface of the cell. Selective snapshots of a single cell horizontally (top) and vertically (bottom) are shown for perspective. (scale bar, 5 μm).
5.4.3 Static Targeting of NSP with α-VEGFR2

To evaluate the influence of α-VEGFR2 on NSP targeting, we performed an adhesion assay in static conditions. NSP targeted with α-VEGFR2 showed a preferential targeting and significant accumulation on endothelial cells expressing VEGFR2 (HIGH) versus control (WT) cells (Fig. 5.6). Quantitative data was assessed using low magnification images of the entire well (Fig. 5.7) and then the number of NSP that initially settled down within the first 15 minutes were normalized to 1.0 to compensate all conditions. In WT PAEC, gradual increases were observed for both untargeted and α-VEGFR2 NSP exhibiting a 2-fold increase over 60 minutes (Fig. 5.6A). At all time-points collected on WT cells, no significant difference was detected between untargeted versus targeted NSP. Untargeted NSP demonstrated similar adhesion dynamics on both WT cells and HIGH PAEC clones (Fig. 3c). These values served as the baseline for NSP adhesion to PAEC in order to distinguish unspecific adhesion from targeting. On the contrary, α-VEGFR2 NSP showed a superior targeting effect on HIGH PAEC, exhibiting a significant (p<0.001) 3-, 4-, and 5-fold increase at 30, 45, and 60 minutes respectively (Fig. 5.6C). High magnification images of static targeting at 15 and 60 minutes supported the enhanced targeting of α-VEGFR2 NSP to HIGH PAEC and demonstrated a substantial increase in the number of NSP associated with the cells over time (Fig. 5.6B,D).
Figure 5.6 Static targeting of NSP to VEGFR2 expressing cells. a, Quantitative analysis of the targeting index of untargeted and α-VEGFR2 in WT PAEC (i.e., control cells) without flow, normalizing the number of NSP per cell with each field of view (FOV) at 15 minutes. b, Fluorescent images at 15 and 60 minutes for targeting in WT PAEC with nucleus (blue) and NSP (red). c, Quantitative analysis of targeting in HIGH PAEC (i.e., VEGFR2 expressing cells); d, Fluorescent images at 15 and 60 minutes for targeting in HIGH PAEC. Data is plotted as mean with SEM. *** = p < 0.001; (scale bar, 20 µm).
Figure 5.7 Fluorescent images for static targeting. Low magnification images of WT and HIGH PAEC at 15, 30, 45, and 60 minutes after targeting with untargeted and α-VEGFR2 NSP that were used to quantify the targeting index.
5.4.4 Dynamic Targeting of NSP with α-VEGFR2

To simulate the shear forces experienced during systemic administration, the targeting of α-VEGFR2 NSP was tested under dynamic flow conditions (Fig. 5.8). A continuous monolayer of PAEC (HIGH or WT) was seeded into slides pre-coated with fibronectin and stimulated with tumor necrosis factor – alpha to recreate the tumor vasculature for dynamic testing. α-VEGFR2 NSP demonstrated enhanced targeting and firm adherence towards VEGFR2 expressing cells exhibiting a significant increase in NSP adhesion after 20 minutes. At all time-points, untargeted and α-VEGFR2 NSP showed similar accumulation on WT PAEC (Fig. 5.8A), and provided information on the unspecific basal binding of NSP to endothelia under flow conditions. When α-VEGFR2 NSP were flowed on HIGH PAEC (Fig. 5.8C) we reported a significant increase in targeting after 20 minutes (p<0.01 at 20 & 22 min; p<0.001 from 24-30 min). In HIGH PAEC, α-VEGFR2 NSP exhibited greater than a 4-fold increase in targeting efficiency from 20 to 30 minutes over untargeted NSP. The corresponding images at 10, 20, and 30 minutes of PAEC under flow confirmed the progressive increase of NSP accumulation over time (Fig. 5.8B,D).

The in vitro static and dynamic studies were instrumental to optimize NSP for efficient targeting. In the experiments for static conditions we removed the impact of flow and demonstrated the ability of NSP to preferentially dock to endothelial cells expressing increased amounts of VEGFR2 (Figure 5.12C). The experiments in dynamic conditions were instead devoted to investigate the ability of the targeting molecule to increase the firm adhesion of NSP to the endothelial cells in the presence of a physiological flow, as depicted in Figure 5.12D. Combined, the in vitro static and dynamic results demonstrated that a 4-fold increase was observed upon targeting NSP with α-VEGFR2. Furthermore, no significant difference in docking (static) or firm adhesion (dynamic) was observed for NSP directed at control cells or for untargeted NSP directed towards VEGFR2 expressing cells. Selective targeting is critical for drug delivery platforms to avoid unnecessary accumulation in healthy
tissues and to improve the delivery of payloads to the target site (i.e., increased therapeutic index).

Figure 5.8 Dynamic targeting of NSP to VEGFR2 expressing cells. a, Quantitation on time-lapse microscopy images on WT PAEC comparing the number of NSP observed between untargeted and α-VEGFR2 under physiological flow conditions (i.e. dynamic). b, Representative images at 10, 20, and 30 minutes from time-lapse microscopy merging transmitted light and fluorescence (NSP, red). c, Quantitative analysis of dynamic targeting in HIGH PAEC. d, Representative images at 10, 20, 30 minutes for targeting in HIGH PAEC. Data is plotted as mean curve ± SEM. ** = p < 0.01; *** = p < 0.001; (scale bar, 50 µm).
5.4.5 Impact of NSP on PAEC after internalization

Upon docking on the cell surface, NSP were internalized regardless of their surface modification. The cells retained normal cytoskeletal structure displaying parallel actin filaments and microtubules radiating throughout the cell (Fig. 5.9). The static and dynamic images showed that NSP initially adhere to PAEC at 15 minutes concentrating distantly from the nucleus and eventually become internalized as they migrate to the perinuclear region of the cell, as previously demonstrated in other endothelial cell lines [172]. To further validate the biocompatibility of NSP upon internalization by endothelial cells, PAEC treated with up to 20 NSP/cell continued to proliferate and displayed similar metabolic activity as untreated cells (Fig. 5.10).
Figure 5.9 Cytoskeleton of PAEC was conserved upon NSP internalization. A. WT PAEC stained with DAPI (nucleus, blue) and phalloidin (f-actin, red). B. WT PAEC containing internalized NSP (purple) and stained for nucleus and f-actin. (scale bar, 50 µm). C. High magnification of B showing preserved cytoskeletal structure upon NSP internalization. (scale bar, 10 µm). E-G. Confocal images of WT PAEC containing NSP (purple) with labeled nucleus (blue), alpha-tubulin (green) and f-actin (red) separated per channel (D), merged (E), and slice view (F). (scale bar, 10 µm).
Figure 5.10 Continued Proliferation of PAEC upon internalization of NSP. NSP were exposed to NSP at concentrations of 1:10 and 1:20 (PAEC: NSP) and measured for MTT incorporation at 24 and 48 hours. Compared to WT, no significant difference was observed between the two groups.
5.4.6 Tumor targeting and biodistribution of α-VEGFR2 NSP

The biodistribution of untargeted and α-VEGFR2 targeted NSP was followed in breast tumor bearing mice using whole body near infrared (NIR) fluorescent imaging. Untargeted and α-VEGFR2 NSP were conjugated with NIR fluorescent dyes after antibody conjugation as described earlier. The signal from targeted and untargeted NIR-labeled NSP (Fig. 5.11A) was equivalent to APTES modified NSP previously used in other studies [211, 212]. Quantification of the injected dose was measured using the NIR signal within the syringe prior to the injection and subtracting the residual signal after the injection (Fig. 5.11A). Mice were imaged at 0 (i.e., immediately after injection), 2, and 4 hours. All animals displayed a prominent NIR signal from the abdomen with a predominant left side accumulation near the anatomical location of the liver (Fig. 5.11B,D). NIR imaging of harvested organs confirmed a considerable accumulation of NSP in the liver, spleen and kidney (Fig. 5.11C,E). The heart, lungs, and tails (Fig. 5a) of mice treated either with untargeted or targeted NSP showed minimal NIR signal. Higher accumulation in tumors treated with α-VEGFR2 NSP increasing from 2 to 4 hours was observed (Fig. 5e). Fluorescence quantification of the NIR signal of NSP from each organ confirmed the biodistribution of α-VEGFR2 NSP (Fig. 5.11F). At 2 hours, α-VEGFR2 NSP exhibited a significantly (p < 0.05) lower accumulation in the liver along with a highly (p < 0.001) significant increase in tumor accumulation yielding a 3-fold increase in targeting (9.5% versus 30.5%). Other organs (e.g., spleen, kidney, lung, and heart) demonstrated no significant difference at 2 hours. At 4 hours, α-VEGFR2 NSP continued to accumulate in the tumor exhibiting a 4-fold increase in targeting. In addition, α-VEGFR2 NSP exhibited superior tumor-to-liver ratios of 0.982 and 0.997 for 2 and 4 hours, respectively, compared to 0.210 for untargeted equating to a cumulative 5-fold increase in tumor biodistribution.
Figure 5.11 Targeting and biodistribution of α-VEGFR2 NSP to breast tumors. a, NSP (untargeted and α-VEGFR2) were labeled with NIR fluorescent dyes and imaged in the syringe before and after injection, minimal signal was found in tails after tail vein injection. b, Non-invasive NIR optical imaging of mice treated with untargeted NSP at 0 and 2 hours. c,
Harvested organs from mice with untargeted NSP at 2 hours. d, Optimal imaging of mice treated with α-VEGFR2 NSP at 0, 2, and 4 hours. e, Organs from mice with α-VEGFR2 NSP at 2 (top) and 4 (bottom) hours. f, Quantification of the biodistribution of NSP comparing untargeted and α-VEGFR2 in the collected organs based on NIR signal. Data is plotted as mean with SEM. * = p < 0.05; *** = p < 0.001. Data was collected by Ennio Tasciotti.
The data suggest that the fluorescent signal in the tumors could originate from the accumulation of the NIR dye released from the surface of NSP (20% and 60% release at 2 and 4 hours) [212]. These results validate the working mechanism of NSP as illustrated in Figure 6. The shape of NSP was designed to flow and drift towards the vessel wall [41, 203, 204] while the surface allows for the attachment of α-VEGFR2 antibodies (Fig. 5.12A) and fluorescent molecules (Fig. 5.12B). α-VEGFR2 NSP are bestowed with enhanced recognition and docking features (Fig. 5.12C) and displayed firm adhesion on endothelial cells expressing VEGFR2 even in the presence of physiological flow (Fig. 5.12D). After targeting the tumor vasculature, NSP begin to shed the fluorescent molecules from within the pores of the NSP (Fig. 5.12E), which locally accumulate in the tumor. As previously demonstrated, upon release, the NSP undergo degradation and are either cleared by liver macrophages or are internalized by endothelial cells (Fig 5.12F) [217, 218].

Supporting the results of the in vitro static and dynamic analyses, the systemic administration of α-VEGFR2 NSP in breast tumor bearing mice confirmed the ability of NSP to target the tumor vasculature and showed a significant increase in the delivery of reporter molecules released from the pores and surface of NSP. The increased accumulation of a therapeutic payload at the tumor site and the avoidance of healthy tissues is a key feature of effective treatments with high therapeutic index. The liver-to-tumor ratios suggest that α-VEGFR2 successfully redirected NSP to the tumor vasculature while simultaneously reducing liver accumulation. The rapid accumulation of the model payload to tumors bestowed by NSP at 2 and 4 hours was comparatively greater than the previously reported accumulation of bevacizumab in tumors at 24 hours acquired using positron emission tomography imaging [219].
Figure 5.12 Schematic representation of the multistage delivery strategy with α-VEGFR2 NSP. a, NSP are conjugated with α-VEGFR2 antibodies (b) followed by conjugation with fluorescent molecules on the surface and pores of NSP to serve as a model payload. c, NSP furnished with α-VEGFR2 demonstrated preferential docking and (d) exhibited enhanced firm adhesion under physiological flow conditions targeting VEGFR2-expressing endothelia. e, After adhesion, the payload begins to shed away from the NSP and (f) diffuses into the tumor microenvironment. NSP then detach and are either internalized by endothelial/tumor cells or are cleared to the liver.
Targeting the tumor vasculature represents a promising strategy for the delivery of cancer chemotherapeutics. Due to their geometry and increased margination towards the vessel wall, NSP are endowed with the ability to drift during systemic circulation with an increased probability of preferentially docking to the tumor vasculature. The functionalization of the surface of the NSP with a α-VEGFR2 antibody increased the specificity towards VEGFR2 expressing cells in vitro and promoted the preferential targeting of the tumor-associated vessels. The increased affinity, docking, and sustained adhesion to VEGFR2 expressing cells favored the accumulation of the model payload effectively demonstrating the mechanism of action of the multistage delivery strategy.
5.5 Conclusions

The preferential delivery of therapeutic agents to the target location while minimizing the distribution in healthy tissues is the Holy Grail of drug delivery. NSP have been exploited to locally deliver chemotherapeutics, anti-angiogenic agents, biologics, contrast agents, and nanoparticles for therapeutic and diagnostic applications [73, 76, 77, 220, 221]. Here, we demonstrate, for the first time in vivo, that NSP functionalized with α-VEGFR2 could recognize, dock, and firmly adhere to endothelial cells and tumor vessels overexpressing VEGFR2 resulting in a greater than 5-fold enhancement. Targeted NSP accomplished this task independently from the EPR effect, and rapidly homed to the tumor site exhibiting a significant increase in tumor accumulation. The delivery of a model payload to the tumor mass was demonstrated following the release of fluorescent molecules from the NSP pores, thus recapitulating the working mechanism that governs NSP [136]. Due to their ideal physical and chemical properties, their ability to enhance therapeutic agents [222], and their tunable nature that allows the controlled delivery of payloads [213], NSP can enable the spatial and temporal control of drug release at specific cellular targets.
Chapter VI: Stem cell mediated targeting of NSP to inflammatory sites

6.1 Abstract

Cell-based therapies stand to dramatically impact the future of targeted drug delivery. The success of genetically engineering T cells has ushered interest in using cells to provide therapy. Mesenchymal stem cells represent a source of cells capable of providing powerful solutions for regenerative medicine and inflammatory targeting. Herein, we demonstrate that the incorporation of multi-stage nanoporous silicon particles within MSC does not negatively impact the advanced cellular functions of the stem cells, including proliferation, differentiation, migration towards cancer, extravasation at inflamed endothelia, and accumulation at inflammatory sites in vivo. In addition, the loading of multi-stage particles with nanoparticles did not alter their cellular viability and bestowed MSC with ability to be remotely activated or carry a toxic formulation.

6.2 Introduction

The emerging use of cell-based therapies has gained praise over the past few years [223]. The use of stem cells in patients is not new and hematopoietic stem cell transplantation has been successfully used in the clinic for more than 45 years to replace the hematopoietic system of patients with cancer and autoimmune diseases [224]. Mesenchymal stem cells (MSC) represent a cell source with substantial promise and are currently being investigated in ongoing clinical trials for the treatment of cardiovascular disorders, liver and eye diseases, graft versus host and other autoimmune diseases, diabetes, and cancer [88]. The inflammation that is triggered by these conditions is believed to be the driving force governing the homing of MSC to these sites [225].

MSC for cancer therapy has been of major interest over the past decade [87]. These approaches developed genetically engineered MSC in order to express or secrete
interleukins [226], interferons [83, 227], prodrugs [228], viral vectors [229], and pro-apoptotic proteins [230]. Although these approaches hold great promise, a method that will permit a quick adjustment in the given payload and can deliver traditional chemotherapeutics and contrast agents can greatly benefit MSC-mediated cancer therapy.

Nanoparticles have demonstrated the ability to encapsulate multiple payloads and poorly soluble drugs, provide protection of premature degradation, and their surface can serve to conjugate other molecules to enhance internalization [192, 231]. The application of nanoparticles and MSC has been investigated, but predominately concentrate on the ability of metallic nanoparticles for diagnostic imaging and MSC tracking [232, 233] with few studies highlighting the use of polymeric nanoparticles (50-80 nm) for the delivery of anti-cancer agents either by internalization [234] or on the surface [235]. However, the internalization of such small nanoparticles may undergo exocytosis before reaching the target site [236] and surface modification may negatively impact the homing properties and promoting an immune response. Furthermore, both methods may result in the premature release of cytotoxic payloads triggering cellular death and failure of MSC to reach intended target.

Here, we propose on using micron-sized NSP to load and retain nanoparticles within MSC. Previous reports have demonstrated the ability of NSP to enhance the diagnostic [76] and therapeutic [77] effect of the embedded nanoparticles. The versatility of NSP allows for the loading and retention of virtually any nanoparticle and when coupled with the dynamic inflammatory homing of MSC would generate a powerful delivery platform capable of delivering therapeutic and diagnostic payloads. This chapter investigates the impact of NSP on MSC and provides the proof-of-principle information necessary to assess the promise of this “Trojan Horse” approach.
6.3 Experimental Materials & Methods

6.3.1 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, University of Texas Health Science Center, and Houston Methodist Research Institute’s Institutional Animal Care and Use Committee. Depending on the experiment, female athymic nude mice (NCr-Fox1\textsuperscript{nu}; 4-6 week old) or female BALB/c (BALB/cAnNCrl; 4-6 week old) were purchased from Charles Rivers Laboratories and maintained as previously described [211]. Mouse breast cancer tumors were established by implanting $1 \times 10^5$ 4T1 tumor cells suspended in PBS into the mammary fat pad.

6.3.2 Cell Culture

Mesenchymal stem cells (MSC) were isolated from the bone marrow of healthy BALB/c as previously described [237]. Briefly, mice were sacrificed and the femurs and tibia were harvested, stripped of muscle, crushed using a mortar and pestle, and plated into a large petri dish with alpha MEM supplemented with 20% fetal bovine serum (FBS, Thermo Scientific). Upon reaching confluency, cells were collected and sorted with a FACS Aria III with the assistance of Dr. Bruna Corradetti and HMRI Flow Cytometry Core. Mouse pulmonary vein endothelial cells (MPVEC, Cell Biologics) were maintained using complete mouse endothelial cell medium (Cell Biologics) per manufactures recommendation, 4T1 (ATCC) were maintained in RPMI 1640 supplemented with 10% FBS, and MDA-MB-231 (ATCC) and ID8 cells were maintained in DMEM-High glucose supplemented with 10% FBS.

6.3.3 Internalization of NSP in MSC
MSC were seeded at near confluency and exposed to NSP at various ratios allowing for internalization overnight. Unless otherwise stated, 3 µm NSP were used and this was the typical procedure used to ensure efficient NSP internalization.

TEM: Samples were seeded to obtain 80-90% confluency, treated with 0.5, 1, and 3 µm NSP, and then fixed in 2% paraformaldehyde (PFA)(Electron Microscopy Sciences) and 3% glutaraldehyde (Sigma) in PBS and stained for TEM as previously described.

Confocal Microscopy: The cytoskeletons of MSC were stained for alpha tubulin and f-actin using anti-alpha tubulin (DM1A) FITC (Abcam) and phalloidin-555 (Invitrogen), respectively. Nuclei of MSC were stained with DRAQ5 (Biostatus) and NSP were visualized using reflectance acquired using confocal microscopy on a upright Leica DM6000 housed at The University of Texas Health Science Center’s Institute of Molecular Medicine. For various NSP per MSC, NSP were labeled with Alexa Fluor 647 and the nuclei were counterstained with DAPI. Samples were then imaged using a Nikon Eclipse 80i fluorescence microscope and exported using Nikon Elements.

Flow Cytometry: 8x10^4 MSC were plated into 12 well plates and allowed to adhere overnight. Wells were treated with various concentrations of fluorescently labeled NSP (1:0 [WT], 1:20, 1:40, 1:60, 1:80, 1:100) for 24 hours. The following day, MSC were collected and analyzed for fluorescence using flow cytometry on a FACSCalibur, as previously described.

6.3.4 Proliferation of MSC

MTT: Proliferation of MSC containing NSP (at MSC:NSP of 1:10 or LOW; and 1:20 or MED; and 1:40 or HIGH) were incubated with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyletetrazolium bromide) dye at 0.5 mg/mL for 90 minutes followed by incubation with dimethyl sulfoxide for 30 minutes under gentile agitation. Plates were then measured for absorbance at 570 nm using a Synergy H4 BioTek plate reader.
Cytoskeleton: MSC were allowed to internalize 0.5, 1, and 3 µm NS. MSC were followed for 5 days and the cytoskeleton of the cells were stained as previously described and imaged on a Nikon Eclipse 80i fluorescence microscope.

6.3.5 Multipotent Differentiation of MSC

The multi-potent differentiation of MSC was confirmed by running traditional osteogenesis and adipogenesis assays with the assistance of Bruna Corradetti. Briefly, MSC were exposed to NSP at a MED ratios overnight. The following day MSC were collected, counted, and seeded into 24 well plates containing DMEM-low glucose at 20,000 and 30,000 cells per well for osteogenesis and adipogenesis assays, respectively. The following morning, the media for pre-determined wells were changed for induction media. Media in wells (both inductive and non-inductive) was changed every 3-4 days. After three weeks, samples were either collected for quantitative polymerase chain reaction (PCR) or fixed in the well for staining. MSC for PCR analysis were collected by incubating cells with Trizol (Invitrogen) and recovering the solution. MSC were stained with oil red o and von kossa for adipogenesis and osteogenesis, respectfully. PCR was done for the following mRNA markers of adipogenesis: peroxisome proliferator activated receptor (PPAR)-γ, and alkaline phosphatase (ALP) for osteogenesis.

6.3.6 Interaction with MPVEC

Activation: MPVEC and MSC were labeled with DiO and DiD (Invitrogen), respectfully. Cells were stained with these lipophilic dyes at 1x10^6 cells/mL in phosphate buffered saline (PBS) containing the respected dye at 10 µM at room temperature for 20 minutes followed by washes in PBS. For static assessment, 7x10^4 MPVEC were seeded into four-chambered glass slides (LabTek) and allowed to adhere overnight. The following day, MPVEC were either treated with complete media (non-inflamed or control) or complete
media containing 50 ng/mL of murine tumor necrosis factor-alpha (TNFα, eBioscience) (i.e., inflamed or activated) for 18 hours. 2x10^4 MSC (+/- NSP at 1:25 ratio labeled with Alexa Fluor 555) were added to MPVEC and were allowed to adhere for 60 minutes at 37°C and 5% CO_2 and then were carefully washed with PBS, fixed with 4% PFA for 10 minutes, and mounted with Prolong Gold containing DAPI (Invitrogen). For dynamic assessment, MPVEC were seeded into ibidi µ-slide I0.4 Leur pre-coated with human fibronectin at 75 µg/mL at a density of 1.5x10^6 cells/mL and were allowed to adhere overnight before treatment with complete media or 50 ng/mL of TNFα in complete media for 18 hours. DiD labeled MSC (+/- NSP labeled with Alexa Fluor 555) were seeded at 1x10^6 cells/mL and allowed to adhere on inflamed/non-inflamed MPVEC for 20 minutes. Slides were then exposed to 0.5 dyne/cm^2 and imaged continuously for 60 minutes. Images from each chamber or µ-slide were acquired using inverted Nikon Eclipse Ti fluorescence microscope equipped with a Hamamatsu ORCA-Flash 2.8 digital camera and fitted with an induction chamber maintaining samples at 37°C and 5% CO_2. MSC in each image was counted using Nikon Elements.

**Adhesion:** MPVEC were seeded on ibidi µ-slide I0.4 Leur pre-coated with human fibronectin at 75 µg/mL at a density of 1.5x10^6 cells/mL and were allowed to adhere overnight before treatment with 50 ng/mL of TNFα in complete media. For fixed shear, individual µ-slides were treated with WT MSC (i.e., control, without NSP) or MSC+NSP. MSC and were allowed to adhere to MPVEC for 3 minutes seeding at 0.5x10^6 cells/mL. Slides were exposed to 0.1, 0.3, 0.5, or 1.0 dyne/cm^2 and were continuously imaged as described earlier for 5 minutes. Each slide was analyzed for the number of MSC present before and after the addition of flow. For varying shear, WT MSC and MSC+NSP were equally mixed together to create a solution of 1x10^6 cells/mL and seeded onto the same µ-slide containing inflamed MPVEC and allowed to adhere for 5 minutes. The shear stress exposed to cells began at 0.1 dyne/cm2 and was increased after 120 seconds to 0.3
dyne/cm². This increase in shear stress continued for the reminder of the experiment increasing to 0.5, 1.0, 2.0, 5.0, and 10.0 dyne/cm² (10.0 was exposed only for 30 seconds). Cells were monitored continuously for 750 seconds as described earlier.

Transmigration: 9x10⁴ MPVEC were seeded onto 8 µm transwells (Greiner Bio-One) in 24 well plates and allowed to adhere overnight. The following day, MPVEC were activated with 50 ng/mL of murine TNFα for 18 hours. 1.7x10⁴ and 3.5x10⁴ DiO labeled MSC (+/- NSP labeled with Alexa Fluor 555) in serum-free media were incubated for 24 hours at 37°C and 5% CO₂ with MPVEC and MSC in the top chamber with complete MSC media in the bottom chamber of the transwell. The top portion of the transwell was carefully washed with PBS to remove any non-adhered / migrated cells. The transwells were then transferred to a well containing pre-warmed TrypLE EXPRESS (Invitrogen) and incubated at 37°C and 5% CO₂ for 10 minutes. Transwells were then removed and fixed in 4% PFA containing 10 µM DRAQ5 for 30 minutes. Afterwards, the membranes were extracted, placed on a glass slide, and mounted with Prolong Gold. While cells collected from the bottom of the transwell (i.e. transmigrated MSC) in TrypLE solution were spun down at 300 x g for 5 minutes, transferred to four chamber slides, and allowed adhere at 37°C and 5% CO₂ for 2-3 hours. Slides were then fixed in 4% PFA, mounted with Prolong Gold containing DAPI. Transwells were imaged using a Nikon A1 Confocal Imaging System housed in HMRI Advanced Cellular and Tissue Microscopy Core Facility. Slides of transmigrated cells were imaged using Nikon Eclipse 80i fluorescence microscope by taking 10 random fields of view per slide and counting MSC using Nikon Elements software.

6.3.7 Migration of MSC

Crawling & Spreading: Ibidi culture inserts within a ibidi 35 mm high µ-Dish were seeded with DiO labeled 4T1 (2x10⁴) and DiD labeled MSC (5x10³; +/- NSP at LOW, MED, and HIGH ratios labeled with Alexa Fluor 555) in different compartments and allowed to
adhere overnight. The following day, inserts were removed and the dishes were left at 37°C and 5% CO₂. The migration of cells was monitored at 0, 8, and 24 hours using the Nikon inverted scope described above. Analysis of the percent closed and rate of migration was performed using Nikon Elements.

**Invasion:** 2x10⁴ 4T1 (breast cancer) and ID8 (ovarian cancer) murine cancer cells were seeded in different wells of 24 well plates and allowed to adhere overnight. The following day, 8.0 µm pore transwells were placed into wells, the media of the cells was changed, and MSC (2x10⁴; +/- NSP at MED ratio labeled with Alexa Fluor 555) were seeded in the top-chamber of the transwells in serum-free media. Wells containing PBS rather than cancer cells were used as negative controls. Plates were left at 37°C and 5% CO₂ for 48 hours. MSC that successfully invaded the transwell were recovered as described earlier for the transmigration experiment. Recovered cells were collected, centrifuged, and placed in four-chamber glass slides for 2-3 hours at 37°C and 5% CO₂ to allow for attachment. Cells were then fixed in 4% PFA and mounted with Prolong Gold containing DAPI. Slides of MSC were imaged using Nikon Eclipse 80i fluorescence microscope by taking 10 random fields of view per slide and counting MSC using Nikon Elements software.

**Infiltration:** Ibidi culture inserts within a µ-Dish were seeded in different compartments with MSC (1x10⁴ +/- NSP at MED ratio labeled with DyLight 649) and MDA-MB-231 (3x10⁴) that were transduced by Dr. Brian Rabinovich at The University of Texas M.D. Anderson Cancer Center in the Department of Pediatrics with mCherry and green fluorescent protein, respectfully. Inserts were removed and left at 37°C and 5% CO₂ for 120 hours, removing inserts at 18, 24, 48, and 120 hours for pictures taken with a Nikon TS100 equipped with a DS-Fi1. At 24 and 120 hours, inserts were analyzed with a Nikon A1 confocal to image the cellular border and infiltration of MSC into cancer cells.

*6.3.8 Remote Activation with NIR*
Hollow Gold Nanoshells (HAuNS) were synthesized by Dr. Chun Li’s group as previously described [28, 238]. NSP were loaded with HAuNS as previously described [77]. Briefly, 1x10^8 APTES modified 1 µm NSP were incubated with 1.5x10^10 HAuNS in water for 30 minutes. NSP were then washed twice with water to remove HAuNS that were not loaded within the porous core. MSC were seeded into wells of a 96-well plate at 6x10^3 per well and allowed to adhere for 4-8 hours, separating wells assigned for near infrared (NIR) laser treatment or no treatment. Furthermore, the groups were separated onto different plates (max of 2 groups per plate) to minimize the time spent outside of incubator. 1.2 x 10^7 NSP (+/- HAuNS) were added to each well and were allowed to internalize overnight. The following morning, MSC were washed and then were irradiated with near infrared (NIR) laser at an output of 2 W for 3 minutes with the assistance of Tess Melancon. MSC were then incubated with MTT dye as described earlier for 90 minutes, images were captured on a Nikon TS100 equipped with a DS-Fi1, and absorbance was measured at 570 nm on the Synergy H4 BioTek plate reader.

6.3.9 DOX Formulation

DOX micelles (M-DOX) were synthesized and characterized as previously described. M-DOX were loaded into NSP and then were exposed to MSC. The dose-dependent cytotoxicity of DOX was examined in MSC, 4T1, and MDA MB 231 cell lines. Each cell line were placed into 96 well plates and treated with free DOX from 0 to 100 µg/mL for 4 hours. At 48 hours, cells were incubated with MTT and measured for cellular viability as described earlier.

Internalization: NSP (+/- 1 µg/mL MDOX) labeled with DyLight 800 (Thermo Scientific) were exposed to 2x10^4 MSC at ratio of 1:10 in four-chambered glass slides and allowed to internalize for up to 24 hours. At 1, 4, and 24 hours MSC were fixed in 4% PFA
and mounted with Prolong Gold containing DAPI. Slides were then imaged using an inverted TE 2000 Nikon microscope.

**Annexin V:** NSP (+/- 1 µg/mL MDOX) were exposed to 2x10^5 MSC at ratio of 1:10 in six well plates and allowed to internalize. For annexin V (Invitrogen) expression, MSC (and cellular debris within the well) were collected after 1, 4, and 24 hours and stained with annexin V Alexa Fluor 488 as previously described. Briefly, cells were re-suspended in annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2, pH 7.4) containing annexin V in a total volume of 105 µL. Samples were incubated at room temperature for 15 minutes and then mixed with 400 µL of additional annexin binding buffer. Samples were then analyzed on a FACSCalibur (BD) for fluorescence.

**MTT:** To assess the proliferation, MSC were collected after 4 hours of internalization with NSP and seeded into 96 well plates at 1x10^3 MSC per well with complete media. At predetermined times, plates were treated with MTT (as described above) and read for absorbance at 570 nm on a Synergy H4 BioTek plate reader.

**6.3.10 Intravital microscopy (IVM)**

BALB/c mice were administered retro-orbitally with either free NSP or NSP within DiD labeled MSC (at a ratio of 1:25). Initially, one set of mice, were imaged continuously with intravital confocal microscopy (ICM) for NSP and MSC accumulation in the liver for 60 minutes. Organs (lungs, liver, spleen, kidney, heart, brain) were then harvested and imaged at ICM by taking 20 random high-power images. In addition, at other pre-determined times, the organs of mice were collected and imaged for NSP and MSC distribution using ICM following the same procedure. Afterwards, organs were split for quantitative analysis and frozen sectioning. The ICM was equipped with an upright Nikon A1R laser scanning confocal microscope equipped with a resonance scanner, motorized and heated stage, and Nikon long working distance 4x and 20x dry plan-apochromat objectives.
6.3.11 Inflamed Ear Homing

We utilized a model in which we induced inflammation at the ear of mice using *E. coli* lipopolysaccharide (LPS; Sigma Aldrich) as previously described [239]. Briefly, mice were anesthetized followed by an injection of 30 µg of LPS (1 mg/mL) in PBS was injected into the base of one ear while 30 µL of PBS was injected into the other.

MSC were treated with NSP at a ratio of 1:25 and allowed to internalize overnight. The following day control and MSC+NSP were collected, stained with DiD, and passed through a 40 µm cell strainer (BD). For each mouse, $1 \times 10^6$ MSCs were injected retro-orbitally 24 hours post LPS injection. The mice were imaged using ICM at 24, 48, and 72 hours. For delineation of vasculature, 40 µL of FITC-dextran (70 kDa; Sigma Aldrich) were injected retro-orbitally just prior to imaging. Images were acquired by taking a z-stack of 50 µm with a step size of 5 µm and analyzed for the number of MSC present in each section using Nikon Elements.

6.3.12 Noninvasive imaging

Bioluminescence imaging (BLI) was also used to increase the sensitivity of tracking stem cells compared to fluorescence imaging. Erika Spaeth transduced MSC with firefly luciferase. Skin on the lower back of nude mice were carefully lacerated and sutured to create a wound model. MSC were then treated with 0.5, 1, or 3 µm NSP or PBS (WT) and injected in the tail vein, followed by BLI over 5 days. In BALBc mice, a breast lung metastasis model was established using 4T1 cells. MSC were treated with NIR labeled NSP at a MED ratio for 4 hours and injected in the tail vein. Mice were imaged with BLI (MSC) at 36 hours, sacrificed, and then organs were imaged with BLI and NIR. Images were analyzed with Living Image Software 4.0 for both bioluminescent and fluorescent signals.
6.3.13 IHC

H&E sectioning was done by Sara Amra at UTHSC as previously described. Unstained sections were treated with xylene and decreasing concentrations with alcohol to remove the paraffin from sections and rehydrate the slides, followed by heat activated antigen retrieval. Tumor sections were then stained with an anti-luciferase antibody (Promega) and stained with a LSAB peroxidase-based visualization kit with DAB (Dako). Slides were then imaged with Nikon Eclipse 80i equipped with a Nikon DS-Fi1 color camera.

6.3.14 Statistical analysis

Statistics were calculated with Prism GraphPad software. Statistics were analyzed using a Two-Way ANOVA followed by a Bonferroni post-test to compare replicate means by rows. In all cases, * p < 0.05, **, p < 0.01, ***, p < 0.001.
6.4 Results

6.4.1 Internalization of NSP in MSC

NSP of various sizes (Fig 6.1A) can be internalized efficiently into MSC (Fig 6.1B). Inspection of TEM images confirmed that NSP were internalized and entrapped within membranes exhibiting perinuclear trafficking as previously demonstrated in endothelial cells [172]. Due to their size, the remainder of studies (unless otherwise noted) was performed using the 3 µm NSP. As further validation of the internalization, confocal microscopy and flow cytometry were used to confirm internalization (Fig 6.2). In Figure 6.2A, a sliced view of a confocal image of MSC stained for cytoskeletal elements (actin and tubulin) demonstrate that NSP were within the limits of the cell and accumulated in the perinuclear region. Using flow cytometry, Figure 6.2B illustrates that MSC substantially increased in shape and fluorescence upon internalization of fluorescently labeled NSP and could internalize large concentrations of NSP exhibiting increasing fluorescence after exposure to 80 NSP per cell. Furthermore, the incorporation of NSP into MSC at several ratios (Fig 6.3) did not induce any immediate effect on the cytoskeleton of MSC, even after internalizing more than 25 NSP. As observed earlier, the cytoskeleton demonstrated normal f-actin and α-tubulin structure across various concentrations of internalized NSP.
Figure 6.1 Electron microscopy images of NSP and internalization within MSC. A) SEM images of (top to bottom): 3 μm, 1.0 μm, and 0.5 μm NSP. (scale bar: 3 μm, 500 nm; 10.5 and 1 μm, 250 nm). B,C) Low magnification (B) and high magnification (C) TEM micrographs of NSP internalization within MSC. Red box indicates where the image shown in C originates. (scale bar: B, 2 μm; C, 500 nm;).
Figure 6.2 Confocal microscopy and flow cytometry validation of NSP internalization.

A) Maximum intensity projection (top) and slice-view (bottom) confocal images of MSC after internalizing NSP. NSP in yellow, α-tubulin in green, and f-actin in red. B) Flow cytometry analysis of side scatter or MSC shape (top) and fluorescence of NSP (bottom). (scale bar, 25 µm).
Figure 6.3 MSC can manage the internalization of several NSP per cell. Fluorescence images of MSC incorporating and retaining several concentrations of NSP per cell. NSP in yellow, α-tubulin in green, and f-actin in red. (scale bar, 25 µm).
6.4.2 Conserved Proliferation and Cellular Architecture

After MSC successfully internalized NSP they continued to proliferate investigating three different concentrations/ratios of MSC to NSP: LOW (1:10), MED (1:20), and HIGH (1:40). As observed in Figure 6.4A, MSC at all three ratios continued to proliferate and demonstrated equivalent mitochondrial function as control cells exhibiting active reduction potential of the MTT day. In addition, the impact of internalization on the cytoskeleton of MSC over time was investigated (Fig 6.4B). As expected, MSC conserved their cellular architecture over time and retained cytoskeletal structure over the course of five days.

Figure 6.4 Proliferation of MSC containing NSP. A) MTT comparing the proliferation of WT (1:0), LOW (1:10), MED (1:20), and HIGH (1:40) for six days after internalization, ratios given as MSC:NSP. B) Fluorescence images comparing the cytoskeleton of MSC and MSC+NSP as they proliferate over five days. NSP in yellow and f-actin in red. (scale bar, 25 µm).
6.4.3 Differentiation of MSC

As demonstrated earlier, MSC conserved the ability to undergo osteogenesis and adipogenesis after treatment with a few NSP per cell (i.e. 1:5). Here, we examine the MED ratio and examine the mRNA expression of early markers for both osteogenesis and adipogenesis (Fig 6.5). Oil Red O staining of lipids in both WT and NSP treated MSC displayed similar results with large red vesicles observed in both samples (Fig 6.5A). Quantification of the mRNA expression of PPAR-γ (an early indicator of adipogenesis) showed that NSP had higher expression of this gene but was not statistically significant (Fig 6.5B). For osteogenesis, MSC exhibited similar Von Kossa staining as control cells indicative of continued similar levels of mineralization (Fig 6.5C). In addition, the expression of ALP illustrated similar levels of expression for both WT and NSP treated MSC (Fig 6.5D). Thus, the MED ratio (~1:20) did not impact or alter the ability of MSC to differentiate into osteoblasts or adipocytes. Furthermore, these genes (PPAR & ALP) have important roles in providing inflammatory relief and thus the incorporation of NSP should conserve the immunosuppressive properties of MSC.
Figure 6.5 Conserved multi-potent differentiation of MSC. A) Oil red O images at both low magnification (left) and high magnification (right), comparing control (WT) MSC and those containing NSP after incubation for three weeks in inductive media. B) PCR results of mRNA collected from cells to evaluate the expression of PPAR-γ in control (not induced MSC), WT (inductive media without NSP), and MSC+NSP. C) Von Kossa images at both low magnification (left) and high magnification (right), comparing control (WT) MSC and those containing NSP after incubation for three weeks in inductive media. D) PCR results of mRNA collected from cells to evaluate the expression of ALP in samples.
6.4.4 MSC interaction with endothelial cells

A critical process for MSC to home to inflammatory sites is their ability to interact with the inflamed endothelia and extravasate to the inflammatory site. To this extent, we chose to study how mouse MSC interact with MPVEC (mouse endothelial cells), paying attention to activation, firm adhesion, and transmigration.

The ability of MSC to recognize and become activated in response to inflammation was tested in both static and dynamic (i.e., flow) conditions. In both conditions (Fig 6.6 & 6.7), resting MPVEC displayed minimal adherence of MSC for both WT and NSP conditions. However, when activated with TNF-α, MSC displayed a significant attraction (Fig 6.6A) or adherence (Fig 6.7) to activated endothelium. This ability was conserved and the number of MSC activated was not significantly different from WT after incorporation of NSP (Fig 6.6B,C). Furthermore, this activation was conserved in dynamic conditions or with the application of flow simulating the shear stress experienced by MSC during systemic administration (Fig 6.7A,B). Thus, NSP did not interfere with the ability of MSC to recognize and become activated upon interaction with inflamed endothelia, one of the early critical processes for homing to inflammatory sites.

Next the firm adhesion of MSC was investigated (Fig 6.8). Here, only activated endothelium was tested based on the results of activation. Investigations were performed to examine the response of MSC at fixed shear stress (0.1, 0.3, 0.5, 1.0 dyne/cm²) and in varying shear stresses where MSC were exposed to gradual increases from 0.1 to 10 dyne/cm². The shear stresses were chosen to represent the physiological range of stresses experienced in venules and large arteries. In both fixed (Fig 6.8A,B) and varying shear stresses (Fig 6.8C), MSC containing NSP displayed a similar fraction of bound cells as WT cells. Surprisingly, MSC demonstrated a robust response and nearly 80% remain bound at 10.0 dyne/cm², nearly approaching the shear stress experienced in arteriolar flow of 15 dyne/cm².
Figure 6.6 Static activation of MSC to inflamed endothelia. A) Fluorescence images of MSC (control, left; MSC+NSP, right) seeded after one hour onto resting (i.e., not inflamed with TNFα) and activated (i.e., inflamed with TNFα) on top and bottom, respectively. Nucleus in blue, MPVEC in red, MSC in green, and NSP in orange. (Scale bar bar bar bar, 100 µm). B) Quantification comparing the activation response of WT and MSC+NSP to TNFα, resting and activated on top and bottom respectively. C) Quantification comparing how WT (top) and MSC+NSP (bottom) individually responded comparing resting and activated endothelia. ** = p < 0.01; *** = p < 0.001.
Figure 6.7 Retention of MSC activation in dynamic conditions. A) Representative fluorescent images displaying MSC+NSP at 0, 10, and 60 minutes of flow taken from time lapse microscopy, comparing resting (left) and activated (right) conditions. MSC in green and NSP in orange. B) Quantification of the area fraction bound comparing resting and activated conditions for MSC+NSP at 0, 10, and 60 minutes. ** = p < 0.01; *** = p < 0.001.
Figure 6.8 Firm adhesion of MSC. A,B) Quantification of the percentage of WT (A, blue) and MSC+NSP (B, red) that remained adhered at fixed shear stresses. Pre-flow (black bars) were used to normalize for the number of MSC initially adhere prior to starting flow. C)
Quantification of the number of MSC that remain adhered during varying shear forces. WT and NSP MSC were allowed to initially adhere on same slide and then shear was started at 0.1 dyne/cm² for 120 seconds and then increased every 120 seconds until reaching 720 seconds where cells were left at 10.0 dyne/cm² for 30 seconds. Data shown as mean values plus and minus one standard deviation, blue for WT and red for MSC+NSP.
The final step that must be achieved by MSC in order to extravasate is the ability to transmigrate past the endothelial barrier into the target site, a process also known as diapedesis. Figure 6.9 validated that the integration of NSP into MSC preserved their ability to undergo transmigration. At the two concentrations (5x10^5 and 1x10^6) tested, there was no significant difference between the two groups. In addition, confocal microscopy revealed that MSC containing NSP could perform paracellular diapedesis (Fig 6.9B,C) as previously reported with MSC [240].

In conclusion, these results indicate that NSP did not negatively influence the ability of MSC to interact with inflamed endothelia and could undergo the necessary steps to achieve extravasation upon homing to the inflamed target during systemic circulation.
Figure 6.9 Transmigration of MSC. A) Quantification of the number of MSC that successfully transmigrated through the transwell comparing WT and MSC+NSP after 24 hours. Both low (5x10^5 cells/cm^2) and high (10x10^5 cells/cm^2) concentrations of cells seeded were tested. B) Low magnification of a slice view confocal microscopy image of MSC+NSP that remained on the transwell. C) High magnification slice view confocal image of MSC on transwell showing diapedesis. Nucleus in purple, MSC in green, and NSP in red. (scale bar, 25 µm).
6.4.5 Migration of MSC

The retained migration of MSC is vital to ensure that they can sense and home to their target. Here, we chose to use cancer as the model inflammatory site and investigated the ability of MSC to crawl or spread, invade, and infiltrate upon carrying NSP.

Using a modified wound-healing assay, the crawling and spreading of MSC was investigated using fluorescence microscopy to track MSC, NSP, and cancer cells at 0, 8, and 24 hours (Fig 6.10A). MSC carrying NSP at LOW, MED, and HIGH exhibit similar crawling and spreading dynamics towards the cancer cell barrier and by 24 hours are the leading edge of MSC (yellow lines) meet the cancer cells in all tested conditions, which crawled and spread minimally compared to MSC. The percent of the gap between MSC and cancer cells closed (Fig 6.10B) and the rate of migration (Fig 6.10C) between the groups was determined by measuring the distance the leading edge of the MSC groups traveled. The percent closed at 8 and 24 hours showed no significant change between the groups, while the rate revealed that at 24 hours the LOW and HIGH groups exhibited a significant increase in the rate of migration compared to WT.

The invasion properties of MSC were investigated using transwells, an assay based on the Boyden chamber principal. These studies evaluated the ability of MSC to invade into a porous membrane in response to a given stimuli. Here, we tested if MSC would migrate towards cancer cells but not PBS. Figure 6.11A & B demonstrated that MSC with NSP could invade in response to both breast and ovarian cancer cells to the same extent as WT cells, but not in response to PBS. The MSC that successfully migrated and were recovered (Fig 6.11C) demonstrated the dramatic flexibility and versatility of MSC that were capable of reorganizing their structure and membrane to accommodate large payloads of NSP while undergoing the deformation necessary to invade the cancer cells below.
Figure 6.10 Crawling and spreading of MSC towards cancer cells. A) Fluorescence images of WT, LOW, MED, and HIGH (left to right) MSC at 0, 8, and 24 hours (top to bottom). Yellow line marks the leading edge of MSC in each image. 4T1 breast cancer cells in red, MSC in green, and NSP in orange. B) Quantification of the percent gap closed between cancer cells and MSC at 8 and 24 hours. C) Quantification of the rate of MSC at 8 and 24 hours. * = p < 0.05; *** = p < 0.001.
Figure 6.11 Invasion of MSC toward cancer cells. A,B) Quantification of MSC migration towards breast (A) and ovarian (B) cancer cells, comparing migration towards PBS (negative control), WT, and MSC+NSP. Values are normalized to WT and are shown as mean plus standard error of mean. C) Representative images of MSC+NSP that successfully invaded cancer cells. (scale bar, 5 µm).
Infiltration was tested by allowing cells to spread past the cellular border and infiltrate into the cancer mass and imaging the distribution of MSC/NSP after 120 hours of migration (Fig 6.12). As observed earlier, MSC migrated to the cellular border of cancer cells by 24 hours followed by the successful infiltration by 48 and 120 hours. Confocal microscopy confirmed that at 24 hours MSC retain NSP and imaging within the cancer mass showed both MSC and NSP within this region surrounded by cancer cells (Fig 6.12B).

**Figure 6.12 Infiltration of MSC into cancer cells.** A) Low magnification images of WT (top) and MSC+NSP (bottom) at 0, 18, 24, 48, and 120 hours. B) Confocal images of MSC at 24 hours imaged at the border of the cancer cell and MSC interface and at 120 hours imaged past the interface to locate infiltrating MSC. MSC in red, MDA-MB-231 in green, and NSP in yellow. (scale bar, 50 µm).
6.4.6 Remote Activation of MSC

The versatility of NSP allow for the incorporation of countless nanoparticle payloads. The ability to temporally regulate the death and release of payloads is of great interest. To this extent, we explored the use of HAuNS to respond towards NIR laser excitation upon loading into NSP. Thus, NSP were loaded with HAuNS and were exposed to MSC for internalization. Figure 6.13 shows MSC stained with MTT treated with NIR (+NIR) or untreated (-NIR) and quantification of the data. The MTT staining showed that loaded MSC exhibited death specifically where the NIR laser was exposed (red line) permitting cells in the border of the well alive where the laser did not irradiate (Fig 6.13A). WT and empty NSP treated MSC displayed minimal change in viability and retained active mitochondria for the reduction of MTT dye. On the other hand, MSC containing NSP loaded with HAuNS exhibited a significant reduction (p < 0.01) in viability upon irradiation with NIR laser (Fig 6.13B).
Figure 6.13 Remote activation of MSC with HAuNS. A) Images of MSC stained with MTT dye and treated with NIR irradiation (right) are shown. Starting from the top: WT, MSC+NSP, and MSC+NSP loaded with HAuNS were compared. The right dashed line represents the outer diameter of the NIR laser. B) Quantification of MSC viability comparing the impact of NIR laser irradiation. ** = p < 0.01.
6.4.7 Toxic Formulation

The retention of toxic payloads within MSC without impacting viability of the carrier is imperative for the optimal performance for this drug delivery system. Here, we examined the ability of MSC to retain DOX as a model toxic formulation. DOX exhibited a dose-dependent response towards MSC, 4T1, and MDA-MB-231 (Fig 6.14) with more than a 50% reduction for MSC at low concentrations compared to ~80% for cancer cells indicating MSC were more sensitive towards DOX treatment.

![Graphs showing the toxicity of DOX on MSC, 4T1, and MDA-MB-231 cells.](image.png)

**Figure 6.14 Toxicity of DOX on cell lines.** The cell growth of MSC, 4T1, and MDA-MB-231 cells after treatment with DOX for 4 hours was investigated. Cell growth was measured at 48 hours and was normalized to the viability of cells without DOX.

M-DOX (i.e. micelle encapsulated DOX) was loaded into NSP and then exposed to MSC. Fluorescence microscopy (Fig 6.15A) and Annexin V analysis at flow cytometry (Fig 6.15B) were used to explore the impact of MDOX+NSP on MSC for the first 24 hours. Fluorescence microscopy revealed that M-DOX treated cells showed the DOX fluorescence accumulating in the nucleus, while that incorporated within NSP remained perinuclear co-localizing with the fluorescence of NSP and with minimal (to none) DOX fluorescence within the nucleus of these samples.

Flow cytometry on the expression of Annexin V (Fig 6.15B) provides information regarding the apoptotic induction of MSC. MSC treated with M-DOX exhibited a gradual
increase of Annexin expression with a substantial shift towards increased expression over 24 hours. On the other hand, MSC treated with NSP+M-DOX exhibited minimal Annexin V expression and thus avoided apoptotic induction demonstrating similar levels of expression as WT cells. An MTT assay confirmed that MSC containing NSP+M-DOX continued to proliferate over one week showing minimal difference between WT and NSP MSC (Fig 6.15C). As expected, MSC treated with M-DOX did not proliferate and were stagnant after two days.

Figure 6.15 Retention of a toxic formulation within MSC. A) Fluorescent images at 1, 4, and 24 hours of MSC treated with media (WT), M-DOX, and NSP loaded with M-DOX (top to bottom). Nucleus in blue, DOX in red, and NSP in yellow. B) Flow cytometry measuring the expression of Annexin V on the surface of MSC at 1, 4, and 24 hours of the different groups. C) MTT proliferation assay comparing the viability of MSC treated with M-DOX, NSP, NSP+MDOX, or complete media over the span of six days.
6.4.8 MPS Avoidance

The systemic administration of MSC carrying NSP was compared to free NSP using IVM to understand the biodistribution within the first hour. The liver, spleen, and lung were imaged and NSP in each image were counted. Figure 6.16 shows that upon incorporation within MSC, NSP significantly decreased their accumulation in both the spleen and liver. Furthermore, minimal discernable signal from MSC/NSP were visible within the spleen or liver. The lung was revealed to be the major accumulation site for MSC containing NSP but did not exhibit a significant difference in accumulation compared to free NSP (Fig 6.17A,B). However, when examining the ratio between lung to liver a significant difference was observed confirming the ability of MSC to modulate the biodistribution of NSP (Fig 6.17C). In addition NSP were retained within MSC after several days (Fig 6.17D) thus validating the ability of MSC to carry NSP in vivo and alter the distribution of NSP to avoid sequestration by filtering organs.
Figure 6.16 Accumulation of NSP in liver and spleen. Harvested liver (top) and spleen (bottom) were imaged with IVM and the numbers of NSP were counted in images (left). Representative images in liver and spleen showing free NSP (green), MSC (blue), and NSP within MSC (red). *** = p < 0.001.
LUNGS

Cell 1
Cell 2

Day 1
Day 6

D

Lungs

Free NSP
MSC+NSP

Lung to Liver

Ratio

Free NSP
MSC+NSP

Day 1

Day 6

Fluorescence Intensity

Distance (µm)

Fluorescence Intensity

Distance (µm)
**Figure 6.17 Lung accumulation and retention of NSP within MSC.** A) IVM images of lungs comparing free NSP and MSC+NSP. B) Quantification of NSP within the lungs. C) Quantification of NSP comparing the ratios of lung to liver for free NSP and NSP within MSC. D) IVM images of MSC after one hour (top, Day 1) and six days (bottom) post systemic administration. MSC were outlined in dashed yellow line and were analyzed using an intensity plot (yellow arrow). Intensity plot generated graphs (right) that demonstrate the boundaries of MSC (blue) and NSP (red) based on fluorescent signal over the distance covered by the yellow arrow. Plots show graphs at Day 1 and Day 6 showing overlapping signals. For images, free NSP were in green, MSC in blue, and NSP within MSC in red. *** = p < 0.001.
6.4.9 Conserved inflammatory homing in vivo

To investigate the ability of MSC to target inflammatory sites, an inflammatory model using LPS was established in the ears of BALBc mice to maintain the syngeneic design. The ears of mice allow for longitudinal imaging of each group with ease using intravital imaging and can serve as their own control, such that one ear is left untreated. Figure 6.18A shows representative images at 24, 48, and 72 hours for both saline and inflamed (i.e., LPS treated) ears for both WT and NSP groups. The green in the images represent the vasculature within the ear, while the red represents MSC. Several MSC in both groups were found in the inflamed ear with apparent increases over time. Furthermore, both groups demonstrated substantial extravasation by MSC over time such that more MSC were observed further away from the vessels. On the other hand, saline ears exhibited minimal targeting and very few MSC were observed over 72 hours. Quantification of each condition revealed a significant increase in targeting comparing saline versus inflamed ears (Fig 6.18B). Furthermore, at each time measured there was no significant difference between WT and NSP MSC thus confirming that the homing of MSC towards inflammatory sites was conserved upon incorporation with NSP.
Figure 6.18 Homing of MSC to inflamed ears. A) IVM images of saline treated or control ears (top) and LPS treated or inflamed ears (bottom) at 24, 48, and 72 hours. Vessels imaged with dextran in green and MSC in red. (scale bar, 100 µm). B) Quantification of the number of MSC that homed to saline and inflamed ears for WT and MSC+NSP.
An additional inflammatory model was investigated that established small wounds on the lower back of mice that were immediately sutured. In this case, firefly luciferase modified MSC containing 0.5, 1, or 3 µm NSP were imaged over 72 hours (Fig 6.19). Longitudinal imaging (Fig 6.19A) showed that MSC initially accumulated in the lungs after tail vein injection and efficiently escaped the lung by 18 hours. Quantification of the BLI signals (Fig 6.19B) revealed substantial decreases in the retention of MSC within the lung in the first 24 hours for all groups. Simultaneously, the amount of MSC accumulating in the wound gradually increased reaching a plateau after 36 hours for all groups. Thus, as well in this model, MSC retained the ability to home to sites of inflammation after the incorporation of NSP at several sizes.

**Figure 6.19 MSC escape lung vasculature and accumulate at wounds.** A) BLI of mice treated with (from left to right): WT, MSC + 3µm NSP, MSC + 1µm NSP, and MSC + 0.5µm NSP comparing 0 (i.e., immediately after systemic administration) and 18 hours. B) Quantification of bioluminescent signals in lungs (dashed lines) and wounds (solid lines) for WT (black), MSC + 3µm NSP (red), 1 µm NSP (blue), and 0.5 µm NSP (green). The 0 hour time point for lungs was omitted as it was much higher than remaining values.
6.4.10 Cancer Targeting

Preliminary cancer targeting of a syngeneic cancer model of 4T1 was performed, examining targeting of both primary and metastatic cancer (Fig 6.20). In primary breast targeting, MSC were located in harvested tumors by staining for luciferase (Fig 6.20A) or using fluorescence (Fig 6.20B). In both cases, MSC were found in the periphery of the tumor, consistent with previous reports [241]. Targeting of breast lung metastasis, displayed a high retention of MSC in the lung at 36 hours (Fig 6.20C). In previous experiments in mice without lung metastasis (Fig 6.19), MSC were observed to escape the lung within the first 24 hours thus indicating the presence of cancer nodules in the lung permitted the retention of MSC within the lung. BLI (Fig 6.20D) and NIR (Fig 6.20E) imaging of harvested lungs revealed both MSC and NSP (respectively) at high concentrations illustrating the retention of NSP upon targeting by MSC (Fig 6.20E). In addition, histology images of lungs showed abnormal tissue architecture and the presence of large accumulation of NSP typical of MSC mediated delivery rather than free NSP that would result in single particle accumulation and not aggregation (Fig 6.20F. red square). Although, the work is preliminary this data represents promising results that indicate MSC can be used to specifically deliver NSP to cancer while avoiding MPS sequestration. In the future, further validation will need to be performed to confirm these results.
Figure 6.20 Preliminary primary and metastatic breast cancer targeting of MSC.

Primary cancer (A,B) and metastatic (C-F) breast targeting data is shown. A) Breast tumor stained with an antibody for luciferase (brown). B) IVM of a breast tumor showing MSC (blue) carrying NSP (red) along the tumor border (dashed yellow line). C) BLI imaging of mouse lungs containing breast cancer lung metastasis at 36 hours treated with MSC+NSP. D) BLI imaging and quantification of lungs for MSC distribution comparing to WT lungs previously imaged in 6.19. E) NIR imaging and quantification of lungs for NSP distribution. F) H&E images of normal lung (top) and metastatic lung containing several NSP accumulated together (red box).
6.5 Conclusion

In summary, the incorporation of NSP within MSC did not induce any significant alteration in biological activity of the stem cell. The NSP payload bestowed MSC with the ability for remote activation and to carry a toxic formulation. Thus when coupled with the specific targeting of MSC and retention of payloads of NSP can enable the spatiotemporal delivery of payloads to be released at any intended site thereby potentially reducing the delivery of therapeutics to healthy tissues and increasing the therapeutic index. Furthermore, the delivery of NSP via MSC dramatically altered the first hour of distribution within animals effectively imparting NSP with the ability to avoid MPS sequestration. Hence, the combination of MSC plus NSP has the potential to provide a powerful solution for the delivery issues that have plagued nanoparticles.
Chapter VII: Discussion

The work demonstrated above shows the promise of NSP as a drug delivery vector. The loading and release of NP, impact of fabrication and environment factors on the degradation, and the impact of internalization and systemic administration on primary cells and tissues of NSP was investigated to assess its bioactivity. In addition, the functionalization of NSP with VEGFR2 antibodies and incorporation within MSC was explored to evaluate the impact on altering the distribution of NSP.

The loading of various NP (QD, micelles, liposomes) substantiates the versatility of NSP to load virtually any NP given favorable electrostatics. Using QD as a model payload, the loading revealed that larger pored NSP retained more QD and release data exhibited delayed release. We hypothesized that this effect was attributed to the depth of penetration achieved by QD within larger pored NSP. Adapting a continuum diffusion model, confirmed this hypothesis assuming XLP were 100% loaded, release data could be matched for the other NSP assuming a varying degree of penetration. This work provides vital information on how the pore size can be used to modulate the release kinetics of the embedded therapeutic thus enabling the pharmacokinetics of the drug.

The degradation of NSP in physiological solutions was investigated using several experimental methods: SEM, ICP-AES, flow cytometry, Z2 coulter counter, and zeta potential. Increasing the porosity induced faster degradation kinetics measured by examining the amount of silicon deposited into solution. Flow cytometry and Z2 volumetric analysis demonstrated that NSP substantially decreased in overall shape and size with time and exhibited dramatic shifts between the various porosity tested, again confirming the higher porous NSP degrade quicker. SEM provided images of NSP at each time-point permitting us a view at the shape/size of NSP as well as the pores. In addition, the impact of
the nucleation layer was investigated. The nucleation layer is a thin layer of small pores (< 2 nm) that formed at the backside of NSP and can serve to prevent payloads from freely passing through. Removal of this nucleation layer triggered a premature collapse of the porous structure of NSP without significantly affecting the overall shape and size or the amount of silicon deposited into solution. Hence, the nucleation layer also has an instrumental role in ensuring pore stability.

The effect due to environmental influences was investigated examining the effect due to changes in buffers (cell culture media vs saline) and pH. NSP in cell culture media experienced accelerated degradation dynamics possibly due to the added proteins and amino acids commonly found in these buffers and absent from saline. Hence, environments rich in proteins and amino acids should favor the quicker degradation of NSP such as those within tissues. Higher pH also induced quicker degradation due to increased nucleophilic attack of hydroxide ions on the NSP surface.

Inspection of SEM images coupled with the other data collected, we observed a distinct degradation mechanism of NSP. The first major feature to degrade was the porous ring surrounding the core resulting in progressively smaller NSP ultimately leaving the central porous core. Simultaneously, the pores in the core initially start to gradually increase in size but are accelerated once the ring has degraded away. After several hours, the pores lack the structural stability and eventually collapse and then complete degrades into mono-orthosilic acid. These degradation byproducts did not induce any significant change in the cell cycle or proliferation of HUVEC nor impact their cellular architecture.

The biocompatibility of NSP was investigated examining both cellular and tissue responses. Traditional viability and toxicity assays showed minimal impact on proliferation or release of LDH from primary human and mouse cells. Furthermore, the conserved cellular integrity was further investigated demonstrating that treatment with NSP did not cause any
adjustment in the distribution of cell cycle phases nor induction or increased expression of PS, an early indicator for apoptosis. Fluorescence and electron microscopy showed that NSP incorporation into primary cells did not negatively impact their cytoskeletal architecture or their ultrastructure showing preserved organelle and sub-cellular structure. In addition, internalization of NSP failed to interfere with advanced cellular functions, such as the ability of HUVEC to form tubes or the ability of ADMSC to undergo osteogenesis or adipogenesis or in their ability to sense and home to cancer cells.

The tissue response and biodistribution upon systemic administration of NSP was evaluated. Biodistribution of NSP showed substantial accumulation in the spleen and liver even after one week. Evaluation of cytokine analysis revealed a mild transient inflammatory response exhibiting a minor increase in TNFα expression at early time points that then returned to basal levels for all pro-inflammatory cytokines. Analysis of the H&E images of liver and lung sections were also consistent with this mild transient inflammatory response exhibiting sub-chronic and reversible alterations of tissues. TUNEL and Ki-67 displayed minimal positive staining indicating negligible induction of apoptosis and liver regeneration in response to NSP administration.

In summary, cells and tissues exhibited a healthy response to NSP administration. In addition we contended that future investigations regarding the biocompatibility of bio/nano-materials should aim to investigate advanced cellular functions (e.g., tube formation, differentiation) rather than relying on traditional assay (e.g., MTT, LDH) and coordinate methods to examine data using in vitro and in vivo correlates to gain a better understanding of the potential immune response.

The successful conjugation and characterization of α-VEGFR2 antibodies onto NSP was described. After conjugation with α-VEGFR2, NSP conserved the ability to retain both therapeutic (melittin) and diagnostic (NIR fluorescent molecules) either passively or tethered
within the pores. In order to investigate the targeting specificity of α-VEGFR2 NSP, we developed and characterized an endothelial cell line expressing various levels of hVEGFR2. 

*In vitro* testing showed that α-VEGFR2 bestowed NSP with enhanced docking and firm adhesion features, resulting in a 4-fold increase in targeting compared to untargeted NSP. Systemic administration of α-VEGFR2 NSP in mice implanted with breast tumors displayed a 5-fold increase in rapid tumor targeting (< 2 hours) compared to untargeted NSP. Furthermore, due to the surface degradation and subsequent release of attached molecules, that data also illustrates the working mechanism that governs the multi-stage delivery strategy. This was illustrated in Figure 5.12. Thus α-VEGFR2 NSP permitted a 5-fold increase in local delivery of fluorescent molecules attributed to their superior ability to marginate within the blood flow, recognize the tumor endothelia, and then firmly adhere. While adhered to the tumor associated endothelia, NSP begin to locally release their model payload (fluorescent molecules within the pores) and are eventually cleared by the immune system transported to the liver or are internalized by the endothelial cells.

The potential use of MSC as cell carriers for NSP was demonstrated. This “Trojan Horse” approach is illustrated in Figure 7.1. The proposed work aims to couple the unique features of NSP, NP, and MSC into a single potent cancer therapeutic. Within this strategy, NSP will initially be pre-loaded with therapeutic or diagnostic NP and then exposed to MSC for internalization. NP will serve as the diagnostic or therapeutic component and thus can be loaded with chemotherapeutics or contrast agents; NSP will carry, concentrate, and protect cells from NP and prevent their exocytosis; MSC will direct the cytotoxic payload specifically to tumor sites.

The work above demonstrates the feasibility of this approach providing a proof-of-principle to validate our hypothesis: MSC will preserve their primary functions and retain homing potential to inflamed endothelia upon internalization with loaded NSP. Here we
demonstrated that MSC could tolerate high ratios of NSP (up to 40 3µm NSP per cell) without any significant impact on their ability to proliferate, undergo osteogenesis or adipogenesis, interact with inflamed endothelia, \textit{in vitro} migration towards cancer cells, \textit{in vivo} sensing and homing to inflammatory sites, and targeting cancer sites. Furthermore, upon loading NSP with NP unique attributes were bestowed on these Trojan Horse vehicles. For example using HAuNS and NIR laser irradiation, we demonstrated that NSP loaded with HAuNS could be efficiently internalized by MSC and showed a significant reaction towards NIR excitation resulting in MSC death. In addition, NSP allowed MSC to carry a toxic formulation. We demonstrated that upon loading NSP with M-DOX, MSC remained viable and lack any significant increase in the expression of apoptotic markers within the first 24 hours and permitted MSC to continue proliferating over six days. When treated with free M-DOX, MSC showed a substantial increase in apoptotic induction and did not proliferate. Lastly, MSC also works synergistically to modulate the distribution of NSP upon systemic administration. Upon incorporation within MSC, NSP successfully avoided the rapid sequestration by the MPS exhibiting significant reduction in both the liver and spleen.

Hence, the combination of MSC and NSP to develop Trojan Horse vehicles has the potential to provide dramatic benefits for both components effectively generating a drug delivery platform capable of providing site specific delivery of therapeutics to cancer lesions and for other inflammatory disorders. The specificity and dynamic targeting of MSC will allow for decreased detrimental effects on healthy normal tissues and potentially increasing the therapeutic index of the embedded payload.

If successful, findings from this work will have the potential to dramatically impact current cancer treatment providing a site-specific, individualized therapy for multitudes of cancers and diseases. This approach has the potential to be used in both healthy patients for early diagnosis and in patients currently undergoing treatment. Therefore, populations with both early-stage and advanced disease will benefit from the clinical translation of this
technology through the creation of potential nanotechnology solutions to improve the delivery of drugs for the diagnosis and therapy of cancer. Furthermore, due to the versatility of this platform unnecessary costs can be avoided by offering the ability to assess the efficacy early during the course of treatment, allowing for prompt interventions and a switch to an alternative therapeutic strategy.
Figure 7.1 Schematic of Trojan Horse delivery platform. (A) NP are loaded into NSP, (B-C) which are then internalized and retained by MSC. (D) MSC carrying NP loaded NSP are administered into mice and (E) home towards tumor site. (F) Upon reaching the tumor lesion, (G) NSP+NP will be released from MSC where (H) they can provide local therapy to the cancer lesion.
7.1 Future Directions

The ability of NSP to provide spatiotemporal tumor delivery of embedded payloads in vivo has not been studied. Based on our previous reports, we can modulate the release rate by adjusting the pore size of NSP. We can use IVM to follow this delivery in real time and use NSP of various pore sizes loaded with QD. In addition, we can conjugate NSP with α-VEGFR2 and load them with QD to further validate our claims regarding the working mechanism of the multistage drug delivery system.

After demonstrating successful homing of Trojan Horse vehicles to inflammatory sites, the delivery of anti-inflammatory therapeutics is the next logical step. To this extent, we will investigate the ability of NSP to carry dexamethasone or non-steroidal anti-inflammatory drugs and release them locally upon reaching the tumor site. The inflamed ear model will be used and MSC will be followed using IVM and the thickness of ears will be measured at 24, 48, 72, and 120 hours. Ears will be collected and analyzed using H&E to understand effect on tissue structure. This type of work will significantly assist in validating the versatility of this drug delivery system and future work can also be done to deliver other beneficial therapeutics for other inflammatory disorders, such as atherosclerosis and tissue regeneration.

Further work for targeting cancer will be performed. We will concentrate on targeting 4T1 breast cancer pulmonary metastasis in a syngeneic setting and comparing the ability of free NSP and NSP within MSC to specifically target metastatic lesions within the lung. In order to facilitate the tracking of MSC and 4T1 one cells, we will use IVM and 4T1 cells transfected with GFP. We will examine the targeting over 120 hours to examine the clearance from lung of both MSC and free NSP.

Coupled with targeting data mentioned above, we can validate the potential of this system to provide spatiotemporal delivery of payloads by examining the ability to induce
remote activation. In addition to using NIR, we will explore the use of ultrasound and radiofrequency to induce the release of chemotherapeutics co-loaded within NSP. To examine the effect in vivo, we will use intra-tumor injections and image tumors for the release of DOX (as a model chemotherapeutic) using IVM. The combination of this data plus the targeting of MSC will provide rationale for engineering MSC to achieve the spatiotemporal induced release of chemotherapeutics as dictated by the user. This therapeutic strategy will enable the activation and release of the treatment only at the desired locations and at the specified times.

Also, NIR-based therapy combined with radiotherapy resulted in enhanced cancer stem cell destruction and triggered increased targeting of circulating MSC when exposed to low-dosed radiotherapy [38, 242]. We will study if our therapeutic approach would work cooperatively with current clinical treatments of radiotherapy for pulmonary metastasis. The synergy between basic science and cutting edge clinical applications relies on the further understanding of these mechanisms. Thus, an effective interaction between the proposed platform with current clinical treatments will potentially provide accelerated translational potential for the treatment of metastasis.

Lastly, we can investigate the diagnostic potential of Trojan Horse vehicles by examining the effect of loading cobalt or iron oxide NP into NSP. We will characterize the magnetic properties within NSP, including hysteresis loop measurement, relaxivity measurement, and MRI images in agarose. The tracking of stem cells using MRI can also be used for other applications and projects, such as ones that require monitoring for tissue regeneration projects currently being studied in Dr. Tasciotti’s laboratory.
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Vita

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