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Metallic Cation-Mediated Entrapment of Nucleic Acids on Mesoporous Silica Surface: Application in Castration Resistant Prostate Cancer

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Abstract

The use of exogenous nucleic acid technologies to modulate aberrant protein expression resulting from genetic mutations is a promising therapeutic approach for treatment of diseases such as advanced prostate cancer (PC). The promise of nucleic-based therapeutics is dependent on the development of platforms that effectively protect nucleic acids from nuclease degradation and deliver the nucleic acids to the cytosol of target cells. In this work, we present the development of a divalent metal-mediated nucleic acid entrapment strategy withing porous silica matrix. This simple strategy results in efficient loading percentages of both siRNA (>60%) and mRNA (>80%) as well their release within relevant biological environments (80%). Additionally, our data supports that the current method reduces endosomal entrapment. and supports the lipid coating of mesoporous silica nanoparticles (LC-MSNs). The metal-enhanced nanosystem is assessed for biocompatibility, stability, and circulation within *in vitro*, *ex ovo* and *in vivo* models of PC.

1. Intro/Background

Treatment of advanced prostate cancer (PC) has moved to FDA approved androgen signaling inhibitors (enzalutamide and abiraterone) with some increase in overall survival. Unfortunately, advanced PC diseases become castration resistant after relapse and metastasis to sites such as bone, liver, lymph nodes and brain are observed.¹ To reduce the burden of these diseases and improve patient outcomes, it is vital to develop new therapeutic approaches that address hard to treat cancers such as advanced prostate cancer. The limitations and issues associated with traditional prostate cancer therapeutics as well as enhanced understanding of the molecular mechanisms leading to disease progression have driven the emergence of nucleic acid-based gene therapies.²⁻⁷ More specifically, the utilization of synthetic siRNAs or mRNAs for modulation of gene expression are attractive for use in therapeutics due to their high specificity and the ability to tailor their design for a broad range of genetic targets.^{8,9}

In the last ten years, numerous scientific studies have been reported in the literature focused on gene therapy for cancers, however very few gene therapies have achieved FDA approval for use in clinical settings. The reason for this is related to issues associated with the delivery of naked DNA/RNA molecules in biological systems such as degradation by nucleases, rapid clearance by the kidneys, poor cellular uptake and non-specific biodistribution.¹⁰ These physiological barriers for nucleic acid delivery necessitate the development of carriers that provide safe and efficient delivery of nucleic acids to target tissues. The application of nanotechnology for nucleic acid delivery has enabled the development of nonviral systems that provide effective gene modulation. Material properties of nanoparticle systems must enable stable blood circulation after intravenous injection, evasion of the mononuclear phagocytic and reticuloendothelial systems, facilitate uptake by target cells and subsequent endosomal escape for cytosol delivery upon cellular internalization. Lipid-based nanocarriers systems (namely liposomes and solid lipid nanoparticles) have emerged as promising delivery agents for nucleic acids due to their biocompatibility, ability to protect naked DNA/RNA from degradation and clinical translatability.¹¹⁻¹⁴ While promising, liposomal based nanoparticles have some limitations in their stability, loading capacity and multi-cargo accommodation and reliance on specific lipid components for effective nucleic acid encapsulation.^{15,16} Mesoporous silica nanoparticles (MSNs) resulting from surfactant templated sol gel processes have garnered significant attention within biomedical research due to their colloidally stability, controllable size and morphology, chemical versatility, excellent biocompatibility and tunability to a variety of nucleic acid cargos.¹⁷⁻²¹ Additionally, MSNs have been shown to improve liposome stability, enhance blood circulation and stability as well as offer controlled release characteristics.^{17,22,23} Native bare silica matrix exhibit a net negative charge in aqueous environments due to the presence of silanolate groups at their surface which presents an unfavorable electrostatic repulsive interaction between silica mesopores and negative charged nucleic acid molecules. To overcome these interactions, various so called “core-shell” constructs have been developed in which silica surface modifications^{24,25} and cationic polymers²⁶⁻³⁰ or cationic lipid coatings^{13,31} are employed. While these cationic modifications of the silica surface have had success in loading of nucleic acids, they face shortcomings in 1) the blood circulation as positively charged molecules tend to stick to negatively charged membrane of vasculature epithelial cells 2) the ability to release cargos in biological conditions and 3) issues with cationic mediated toxicity.

In this study, we present the development of a new nucleic acid entrapment strategy that reduces the need of positively charged organic moieties, polymers, or complicated silica surface functionalization steps to a single metallic cation that efficiently intermediates the interaction between the silica surface and nucleotide's phosphate locking-in the nucleic acid. This method results in efficient loading via a straightforward incubation, but also quick release within biologically relevant environments. This simple strategy also allows and supports the addition of a lipid coating on the silica surface hence combining physiologically relevant liposomes with colloidally stable silica nanoparticles for delivery of nucleic acids in the advanced PC landscape. The addition of this metal cation entrapment technique also serves to provide a mechanism of endosomal escape for cytosolic delivery of siRNA or mRNA. Finally, the LC-MSN's nucleic acid delivery ability, circulation and biocompatibility were evaluated within relevant *in vitro*, *ex ovo* and *in vivo* models of PC.

2. Experimental Section

Chemicals. Tetraethylorthosilicate (TEOS), cetyltrimethylammonium chloride (CTAC), cetyltrimethyl ammonium bromide (CTAB), triethanolamine (TEA), calcium chloride (CaCl_2), sodium chloride (NaCl), magnesium sulfate (MgSO_4), iron III chloride (FeCl_3), aluminum III chloride (AlCl_3), ammonium nitrate (NH_4NO_3), hydrochloric acid (HCl), cyclohexane, (3-Aminopropyl)triethoxsilane (APTES) and dimethylformamide (DMF) were purchased from Sigma Aldrich. Cyanine 3-NHS (Cy3) was purchased from Luminoprobe and DyLight 633-NHS was purchased from ThermoFisher. Potassium chloride (KCl) and ammonium hydroxide (NH_4OH) were purchased from Macron Chemicals. Anhydrous ethanol was purchased from Thomas Scientific. Magnesium chloride (MgCl_2) was purchased from Mallinckrodt Chemicals. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG₂₀₀₀), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000, NHS ester] (sodium salt) (DSPE-PEG₂₀₀₀-NHS) were purchased from Avanti Polar Lipids.

Synthesis of monodisperse 2nm pore size hexagonal silica nanoparticles. In a 150 mL beaker, 0.29g of CTAB was combined with 150g of a 0.32M NH_4OH in water solution. The beaker was then covered with parafilm before being placed in an oil bath at 40 °C. The reaction mixture was stirred at 600 rpm for one hour. The parafilm was then removed and a solution of 0.6 mL TEOS in 2.4 mL ethanol was added to the beaker and reacted for 30 minutes before stirring was stopped. The reaction was allowed to age at 40 °C overnight before being placed in an oven at 70 °C for a 24-hour hydrothermal treatment. Finally, the resulting suspension of MSNs was centrifuged at 50,000 g. Surfactant removal was achieved via successive washing steps with NH_4NO_3 (6 g/L) and HCl (1% in ethanol, twice). All centrifugation steps were conducted at 50,000 g for 15 minutes. Finally, the resulting MSNs were stored in pure ethanol.

Synthesis of monodisperse 8 and 5nm pore size spherical silica nanoparticles. In a 100 mL round bottom flask, 0.181g of TEA, 24 mL of CTAC and 36 mL of DI H_2O were mixed in an oil bath at 55°C for one hour with a stirring speed of 400 rpm. The stirring speed was then reduced to 200 rpm before adding dropwise a solution of TEOS in cyclohexane (2 mL TEOS, 18 mL cyclohexane). The mixture was then allowed to react for 18 hours before being removed from heat and stirring. The upper organic phase was removed from the flask and the resulting aqueous phase was centrifuged at 50,000 g. Surfactant removal was conducted in the same manner as described for hexagonal MSNs. The resulting MSNs are stored in pure ethanol.

For *in vitro* and *in vivo* studies, MSNs were dyed with either Cy3 or Dylight 633. For Cy3 labeled MSNs, a solution of Cy3 and APTES in ethanol (2 mg Cy3, 2 μL APTES, 1 mL ethanol) was added to the aqueous phase 10 minutes before TEOS addition. For Dylight 633 labeled MSNs, APTES (110 μL APTES in 200 μL ethanol) was added to the aqueous phase during the MSN synthesis (18 hours after TEOS addition) and allowed to react for three hours before surfactant removal. The resulting amine functionalized MSNs were readily reacted with AF 633 NHS for 2 hours. The MSN suspension was spun down and the supernatant was replaced by a solution of

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3 succinic anhydride (100 mg in 4 mL DMF) and the mixture was sonicated for 2 min and kept at
4 RT for at least 24 h. The labeled MSNs were then spun down and washed once by DMF and twice
5 by ethanol.
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7 **Nanoparticle Characterization.** TEM/STEM and SEM images were obtained on a JEOL
8 NEOARM 200 kV aberration corrected instrument equipped with a Gatan Orius digital camera
9 system. Energy dispersive spectroscopy (EDS) mapping was also conducted on the JEOL
10 NEOARM STEM instrument equipped with JEOL SDD 100 mm² EDS detectors. MSN
11 hydrodynamic size, zeta potential and polydispersity were assessed using dynamic light scattering
12 (DLS) on a Malvern Zetasizer Nano-ZS equipped with a He–Ne laser (633 nm) and non-invasive
13 backscatter optics. All samples for DLS measurements were suspended in distilled water or ethanol
14 at a 1 mg mL⁻¹ concentration. Samples were washed three times through centrifugation prior to
15 measurements. All measurements were acquired at 25 °C. DLS measurements for each sample
16 were obtained in triplicate and the Z-average diameter was used for all reported hydrodynamic
17 size values. The zeta potential for all the samples was measured in distilled water in triplicate
18 according to the Smoluchowski method. All reported values correspond to the average of three
19 independent measurements. Nitrogen adsorption–desorption isotherms of MSNs were obtained on
20 a Micromeritics ASAP 2020 at 77 K. Samples were degassed at 60 °C for 12 h before
21 measurements. The surface area was calculated following the Brunauer–Emmet–Teller (BET)
22 equation and the pore size was obtained using the standard Barrett–Joyner–Halenda (BJH) method
23 applied to both the adsorption and desorption branches. For visualization of LC-CaMSNs, samples
24 (1 mg/mL in 1x PBS) were plunge frozen into liquid ethane using a Thermo Fisher Scientific
25 Vitrobot, then Cryo-TEM imaging was performed on a Talos L120C operating at 120 kV using a
26 Gatan 626 holder.
27

28 **Metal Based Cation siRNA Loading.** The metal-based cation loading procedure described
29 here was adapted and developed from a previously described method for porous silicon.³² Stock
30 solutions of cation solutions were prepared by adding corresponding mass of cation salt to 10 mL
31 of DI H₂O to achieve final concentrations of 0.4M, 1M or 4M and used immediately for loading
32 experiments. 1 mg of MSNs were washed twice in DI H₂O (1 mg/100 μL) before being incubated
33 with a 5% weight ratio of Cy3 siRNA (ThermoFisher AM4621) or Cy5 firefly luciferase mRNA
34 (APExBio R1010) for 10 minutes. 750 μL of the cation solution was then added to the MSN-
35 nucleic acid suspension and allowed to incubate at room temperature for 1 hour. The resulting
36 nucleic acid loaded MSNs were then spun down at 20,000 g and washed twice with DI H₂O (Sigma
37 Aldrich W4502 pH=8.8). Loading efficiencies were quantified using a Biotek microplate reader.
38 Loading efficiencies were calculated as the ratio of fluorescence signal in supernatant over
39 fluorescence signal of the overall suspension.
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41 **Liposome Preparation and Fusion to MSN.** Lipid formulations were prepared from pure
42 lipid stock solutions at 25 mg/mL in chloroform kept in an argon glove box. Lipid components in
43 chloroform were mixed and chloroform was evaporated using a rotary evaporator. The obtained
44 lipid films were then rehydrated to a concentration of 5 mg/mL in 1x PBS. The resulting liposomes
45 were then characterized by DLS to determine hydrodynamic size and polydispersity index. Fusion
46 of lipids to the MSN surface was done at a weight ratio of 1:5 MSN to lipids. MSN were suspended
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3 in DI H₂O at a concentration of 1 mg/mL. Liposomes were added to MSNs under sonication while
4 pipetting up and down ~25 times. The resulting LC-MSNs were then spun down and washed once
5 with 1x PBS.
6

7 **GRP78 Ligand Conjugation Strategies.** For NHS-NH₂ conjugation of GRP78, the NHS lipid
8 film was rehydrated with a 1 mg/mL stock solution of GRP78 in 1x PBS (CPC Scientific 937657)
9 to a final concentration of 5 mg/mL liposomes and sonicated for 30 minutes. The resulting
10 liposomes were then fused to the surface of MSNs pre-suspended in DI H₂O at a 1:5 MSN to lipid
11 weight ratio and incubated at room temperature for 30 minutes. The resulting LC-MSNs were then
12 spun down at 20,000 g (10 min) and resuspended in 1x PBS to a final concentration of 1 mg/ mL.
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15 **LNCaP Cell Culture and Cytotoxicity Analysis.** The androgen sensitive human prostate
16 adenocarcinoma metastatic cell line (LNCaP) was cultured in RPMI 1640 media with L-glutamine
17 (Corning 10-040-CV) supplemented with penicillin/streptomycin (ThermoFisher 15140122) and
18 10% fetal bovine serum (EMD Millipore 12306C). Cell cultures were maintained at 37°C and 5%
19 CO₂. For cytotoxicity studies, LNCaP cells were seeded in 96 well plates at 10,000 cells per well
20 and incubated with LC-MSN system components for 24 or 48 hours. Analysis of LC-MSN
21 cytotoxicity with the LNCaP cell line was assessed on a Biotek microplate reader using the
22 commercially available Cell Titer Glo assay (Promega G7571) according to the manufacturer's
23 protocol.
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26 **Confocal Microscopy Imaging.** For visualization of LC-MSN cellular interactions, LNCaP
27 cells were grown on No. 1.5 coverslips in 6 well plates at a density of 250,000 cells per well
28 overnight. Lysotracker (Invitrogen L7528) was incubated with live cells at 50 nM according to the
29 manufacturer's protocol. The next day, Cy3 labeled LC-MSNs were added for the indicated times
30 to their respective wells and incubated at 37 °C. Cells were then washed twice with pre-warmed
31 1x PBS, fixed with 4% paraformaldehyde for 15 minutes at room temperature. Following fixation,
32 cells were permeabilized using 0.1% Triton-X in PBS for 15 minutes. Cells were then incubated
33 in a 1% BSA blocking buffer for 20 minutes. All cell staining solutions were prepared by diluting
34 fluorescently conjugated antibodies in 1% BSA in PBS. Rab5a-Alexa Fluor 488 (Santa Cruz
35 Biotechnology sc-46692) was diluted at 1:100 and incubated with cells at 4 °C overnight. Clathrin
36 heavy chain-Alexa Fluor 475 (Invitrogen MA1-065) was diluted to 5 µg/mL and incubated with
37 cells at 4 °C overnight. Cell samples were then rinsed twice with 1x PBS before being mounted to
38 microscopy slides in Prolong Gold with DAPI (Thermo Fisher P36935). All microscopy images
39 were obtained using a Leica DMi8 TCS SP8 confocal microscope. Confocal images were
40 deconvoluted using Huygens Deconvolution software package.
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43 **Flow Cytometry Analysis of LC-MSN Uptake Kinetics.** LNCaP cells were seeded in 6 well
44 plates at 250,000 cells per well and incubated overnight at 37 °C. The next day, cells were
45 incubated at the indicated times with 50 µg/mL Cy3 LC-MSNs. Cell samples were then washed
46 twice with 1x PBS and collected using 0.05% Trypsin-EDTA (Thermo Fisher 25300054) before
47 being fixed with 4% PFA in 1x PBS. Cell samples were analyzed on a Thermo Fisher Attune NxT
48 flow cytometer.
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Human Blood Hemolysis Analysis. Whole human blood was obtained from (Innovative Research, IWB1K2E10ML). Red blood cells (RBCs) were then isolated using Ficoll-Paque PLUS (Cytiva #17144002) and density gradient centrifugation at 800 xg for 8 minutes. RBCs were then purified with successive 1x PBS washing and centrifugation steps (800 xg, 8 minutes) before being suspended in 1x PBS (~4 x 10⁶ cells/mL). Isolated RBCs were then incubated with relevant MSNs, CaMSNs or LC-CaMSNs at 37 °C for three hours. Samples were then centrifuged and resulting supernatant hemoglobin amounts were assessed via plate reader endpoint absorbance measurements (570 nm). The percentage of hemolysis for each sample was calculated using Equation 1 below. Negative control samples corresponded to RBCs suspended in 1x PBS at physiological pH while positive control samples corresponded to RBCs suspended in DI H₂O.

$$\% \text{ Hemolysis} = \frac{\text{Sample abs} - \text{Negative Control abs}}{\text{Positive Control abs} - \text{Negative Control abs}} * 100 \quad [1]$$

LNCaP mRNA Proof of Concept Experiments. LNCaP cells were seeded in 96 well plates at 10,000 cells per well and incubated overnight at 37 °C. The next day media was removed and replaced with media containing Lipofectamine/mRNA complexes or mRNA loaded LC-MSNs and incubated at 37 °C for 24 hours. Lipofectamine MessengerMax (Invitrogen LMRNA003) complexes were prepared and added to cells according to manufacturer protocol based on recommended high and low amounts of reagents.

Real-time qPCR Analysis. LNCaP cells were seeded in 6 well plates at 250,000 cells per well and incubated overnight at 37 °C. Relevant LC-CaMSNs loaded with luc-mRNA were incubated with cells (50 µg/mL) for 24 or 48 hours. RNA was then extracted using an IBI reagent Kit (IB47602, IBI Scientific, Dubuque, IA). qPCR was performed on a LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). The mRNA level was measured by qPCR. The gene levels were normalized by 18S. Expression level changes were calculated by comparing to a non-treated control.

Luciferase F: TGCACATATCGAGGTGGACATC

Luciferase R: TGCCAACCGAACGGACAT

Ex ovo chick chorioallantoic membrane model (CAM) Maintenance and Analysis. Fertilized white leghorn chicken eggs were purchased from a local farmer on embryonic developmental day zero (EDD). Eggs were placed in a GOF 1500 cabinet incubator with 60% humidity at 38 °C and allowed to rock until EDD 3. On EDD day 3, a Dremel tool equipped with a No. 5 blade was used to score the eggs along the shortest circumference. The eggs are then cracked into a sterile weigh boat. Viability of chick embryos out of the shell in the weigh boats was confirmed by observing an intact yolk and a visible heartbeat. Embryos were then covered with a square plastic petri dish cover and placed in a VWR Symphony incubator at 37 °C and 0% CO₂ until EDD 16. On EDD 16, embryonic vasculature was labelled by microinjecting LCA fluorescein (1:100, 250 µL) and allowed to circulate for 15 minutes before microinjecting Cy3 labeled LC-MSNs. Embryos were then imaged using a Zeiss Axio Examiner Z1 microscope. All experiments were conducted in accordance with the regulations and guidelines of the Institutional Animal Care and Use Committee at the University of New Mexico (IACUC Protocol # 21-201149-T-MC).

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3 **Mouse Models and *in vivo* Experimentation.** Adult male NOD scid gamma (NSG) mice at
4 the University of New Mexico were maintained on a 12-hour light-dark cycle with *ad libitum*
5 access to food and water. Animals were group housed and all experiments were conducted in
6 accordance with the regulations and guidelines of the Institutional Animal Care and Use
7 Committee at the University of New Mexico (IACUC Protocol # 22-201155). LC-MSNs were
8 administered to adult male NOD scid gamma (NSG) mice via retro-orbital injections under
9 isoflurane anesthesia (1 mg/mL, 100 μ L). For xenograft establishment in NSG mice, LNCaP cells
10 were suspended to a final concentration of 5x10⁷ cells/mL in serum free, phenol red free RPMI
11 media. Cell suspensions were then mixed at 50% volume with phenol red free Matrigel (Corning
12 356237) before subcutaneous injection in the right flank (200 μ L final injection volume). Tumor
13 growth was monitored serially over time. Fluorescent LC-MSN and bioluminescence signal was
14 tracked in mice using a Perkin Elmer IVIS Spectrum *in vivo* imaging system.
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18 3. Results and Discussion

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20 **3.1 MSN Morphology and Pore Size Modulation.** MSNs in this study were synthesized via a
21 sol gel surfactant template approach in which a silica precursor (TEOS) is condensed over an
22 aqueous surfactant self-assembly (Figure 1a). This approach is attractive for engineering
23 nanocarriers due to its ease of synthesis as well as the high level of control over reaction products.³³
24 The interchanging of surfactants, conditions of the reaction scheme and varying additions of the
25 silica precursor enabled the creation of a library of MSNs having varying morphologies and pore
26 sizes as for subsequent tailoring to nucleic acid cargos. First, hexagonal MSNs having a 2nm pore
27 size structure were synthesized via CTAB templating base catalyzed by NH₄OH according to
28 previous work.³⁴ Secondly, spherical MSNs having either dendritic or worm-like pores were
29 synthesized using a biphasic reaction setup adapted from our previous work.³⁵ All synthesis
30 approaches resulted in highly monodisperse nanoparticle populations based on the low
31 polydispersity indices (PDI \leq 0.2) obtained from DLS measurements of resulting reaction batches
32 (Figure 1b). The DLS zeta potential measurements of the synthesized nanoparticles (-28.9 \pm 2.4
33 mV) were indicative of a pure silica matrix in water (Figure 1c). TEM indicated highly ordered
34 resulting mesoporous structures for the hexagonal (Figure 1d, left), 5nm wormlike (Figure 1d,
35 middle) and 8nm dendritic nanoparticles (Figure 1d, right).
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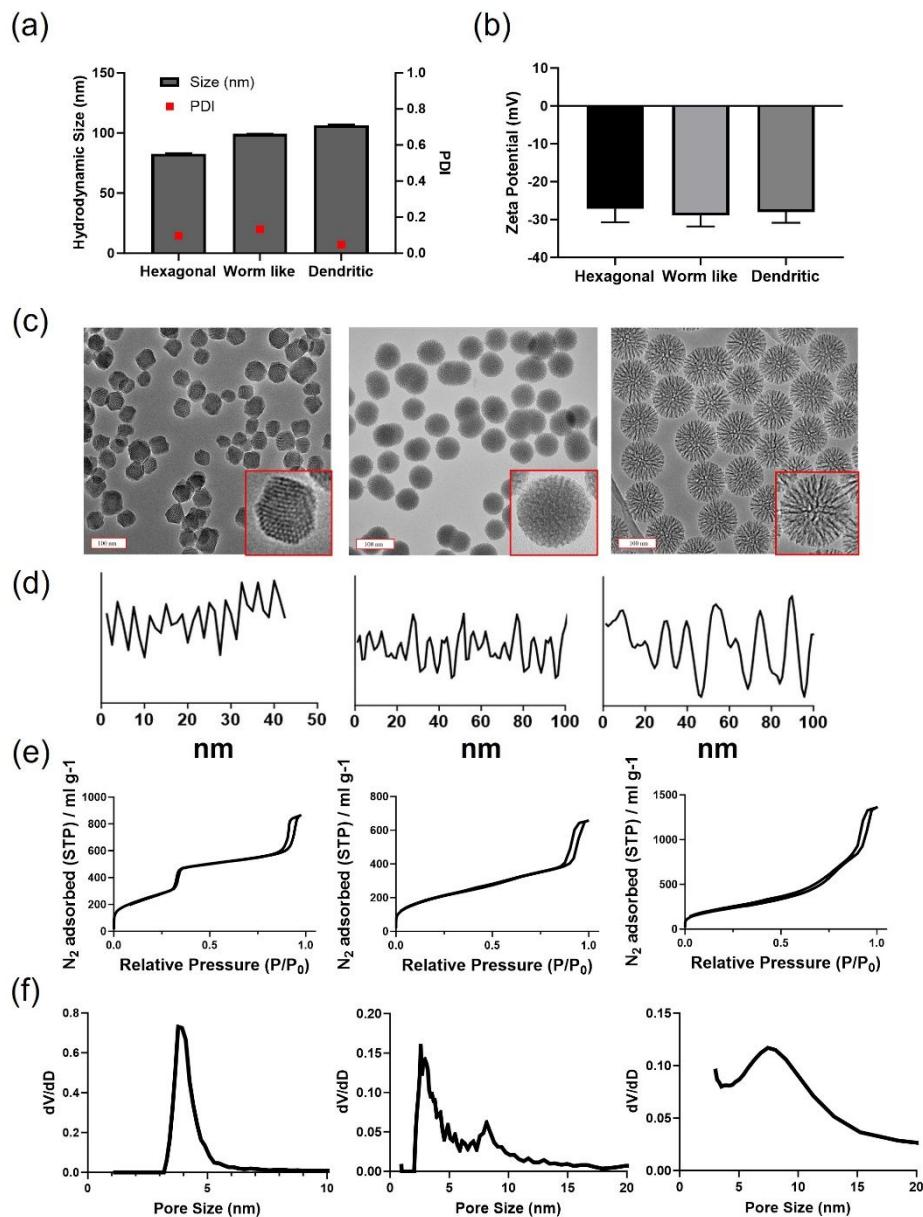


Figure 1. MSN pore size and morphology modulation. (a) DLS measurement results for as synthesized MSNs, (b) DLS measurement results for zeta potentials of as synthesized MSNs (c) Representative TEM images of as synthesized MSNs left: 2 nm hexagonal middle: 5nm worm like right: 8 nm dendritic MSNs, insets are zoomed in images to demonstrate morphology, (d) Profile topography of hexagonal (left), worm like (middle) or dendritic (right) MSNs (e) Nitrogen adsorption isotherms for hexagonal (left), worm like (middle) or dendritic (right) (f) Corresponding calculate pore size distributions for hexagonal (left), worm like (middle) or dendritic (right).

3.2. Metallic Entrapment for MSN Loading of Nucleic Acids.

2.1. siRNA Loading and Pore Entrapment. Engineering of chemical and physical characteristics for improved siRNA or mRNA loading into silica mesopores must also consider the need for efficient release kinetics and biocompatibility within biological systems. Additionally, reducing the complexity of such systems boosts their reproducibility as well as streamlines future use in clinical settings. To avoid the reliance on cationic polymers, cationic lipids or additional surface functionalization, we have shown that the utilization of metal cations can link silanolate ($\text{Si}-\text{O}^-$) groups and negatively charged nucleotides by overcoming electrostatic repulsive forces. We hypothesized that complexation between nucleic acid molecules, metal cations and silanolate groups on the surface of the silica nanoparticles and within the pores would enhance siRNA or mRNA loading into negatively charged MSN mesopores and that the resulting complexation would serve to trap nucleic acids within the pores (Figure 2a).

Impact of MSN morphology/pore size. In order to evaluate surface morphology impact on nucleic acid loading using metallic cations, we have adapted a previously reported procedure that uses high concentration of calcium chloride to load siRNA into porous silicon by forming insoluble calcium silicate species.³² As expected, bare MSNs loaded negligible siRNA amount, due to natural electrostatic repulsion while the use of the 4M CaCl_2 solution drastically increased the loading efficiency with a ratio 1/0.75/0.5 as a function of pore size.(Figure 2b). These results suggest a dependence on MSN pore size and morphology for calcium mediated loading of siRNA.

Evaluation of siRNA loading in function of cation radius and solution pH. We then hypothesized that the loading extent of siRNA could be further modulated as a function of ionic radii of the cations. To test this hypothesis, we used Mg^{2+} (ionic radius=72 pm), Ca^{2+} (Ca^{2+} = 100 pm), Na^+ (ionic radius=102 pm), and K^+ (ionic radius=138 pm) in 4 M cation chloride solutions to load fluorescent siRNA. Loading efficiency (<10%) results along with zeta potential measurements (~ -20 mV, with no difference compared to bare MSN) indicated ineffective loading with monovalent cations (Na^+ , K^+) in both acidic and basic pH conditions, in contrast to an effective loading (>80%, pH 9.5) with divalent cations (Mg^{2+} , Ca^{2+}) ruling out the effect of the ionic radius effect in favor of the cation valency. Additionally, it is noteworthy that acidic pH reduced to 30% the loading efficiency using divalent cations (Figure 2d, 2f) implying that pH and hence, the degree of ionization of both silanols and phosphate (nucleotide), plays a key role in the metallic entrapment process. Considering the Hendersson Hasselbach relationship ($\text{pH} = \text{pKa} + \log[\text{A}^-]/[\text{AH}]$), the ratio of both base/acid couples SiO^-/SiOH and $\text{PO}_4^-/\text{PO}_4\text{H}$ groups will drastically evolve from ~ 20 at pH=3.5 to ~10⁷ at pH=9.5 suggesting that an electrostatic link plays a key role in the metallic entrapment process. Our results from STEM imaging do not show any morphological or contrast difference of MSN incubated with and without concentrated CaCl_2 (Figure 2e), as previously shown with the silicon surface in a reported study.³² These results suggest a nucleic acid entrapment strategy via an electrostatic metallic link without formation of observable silicate precipitate.

We then extended the loading procedure to other cations having higher valences. Prepared 4 M solutions of FeCl_3 and AlCl_3 resulted in pH <1. Adjusting the pH to >3 resulted in important precipitation on a wide range of concentrations (0.04 M-0.4 M) (Figure S1). The obtained loading

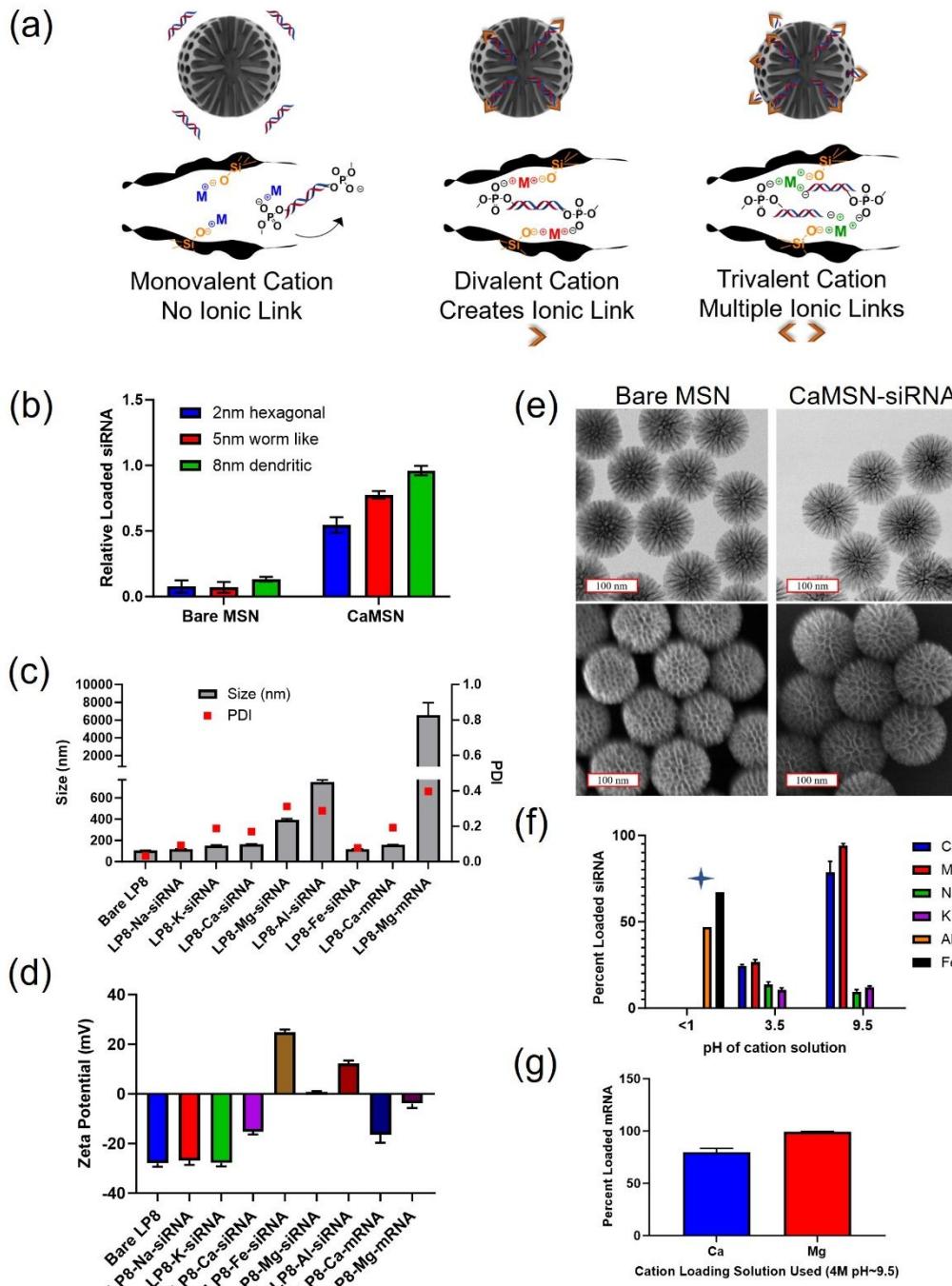


Figure 2. Metal based cation loading of siRNA or mRNA. (a) Schematic showing cation loading and entrapment of RNA (b) Initial siRNA loading as a function of pore size and morphology. pH=4.5 (c) DLS characterization of resulting cation loaded MSNs (d) DLS characterization of zeta potential for resulting cation loaded MSNs (e) STEM images of siRNA loaded MSNs (f) Cation siRNA loading efficiencies, blue star indicates cation loading solutions not applicable to pH adjustments (g) Cation mRNA loading efficiencies.

efficiency results (at unadjusted pH<1) for trivalent cations (Fe^{3+} , Al^{3+}) also indicated relatively good loading (~80%), however these conditions would create technical challenges and would not

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3 be applicable to therapeutically relevant nucleic acids.
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5 We then sought to understand the interaction between the silica surface and siRNA/cation
6 complexes with a hypothesis that the state of silanol groups would play a key role in the ability of
7 calcium loading to occur. To test this hypothesis, we conducted fluorescent tracking of siRNA
8 loading utilizing CaCl_2 solutions at a higher pH (pH~9.5). In respect to our hypothesis, at higher
9 pH the siRNA loading efficiency was increased to approximately 80% (Figure 2f). This is likely
10 due to the fact that at lower pHs, the silanol groups on the surface are protonated (SiOH), inhibiting
11 optimal interaction with cation/siRNA complexes. At pHs well above the pK_a of silanol groups
12 we expect the surface to be significantly deprotonated, facilitating effective interactions between
13 Si-O^- groups and cation/siRNA complexes.
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16 **3.2.2 Metallic entrapment efficiently loads messenger RNA.** After confirming the capability of
17 divalent metallic cations to entrap siRNA (~50 nucleotides, double stranded), we wanted to
18 evaluate the efficacy of this strategy with mRNA, a significantly larger molecule (1921
19 nucleotides, single stranded) with comparable chemistry. The loading data shows 85-100% loading
20 efficiency, suggesting that divalent cations can exert the same effect on the mRNA nucleotides
21 and assist by folding mRNA within the pores. The ability of divalent cation loading was also
22 applicable to mRNAs as indicated by results from tracking of a fluorescent Firefly Luciferase
23 mRNA (Figure 2g). DLS measurement results for cation loaded MSNs indicated optimal
24 hydrodynamic size (<200 nm) and PDI (<0.2) of the CaMSN and was therefore chosen for
25 subsequent experimentation (Figure 2c). STEM imaging results after Ca^{2+} loading indicated that
26 nucleic acids are loaded into pores and that MSN morphology is retained (Figure 2e). Finally, EDS
27 elemental analysis of nucleic acid loaded CaMSNs confirmed the presence of calcium and
28 phosphorous attributed to the phosphate backbone of the siRNA (Figure S2). Considering these
29 results, we decided to move forward with the calcium rich MSN (CaMSN) as divalent cations
30 showed optimal loading conditions compared to mono and trivalent metallic ions, and Ca-MSN
31 exhibited optimal stability as compared to Mg-MSN.
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34 **3.3 Fusion of Liposomes to MSN for LC-MSN Formation.** The addition of liposomes to coat
35 the CaMSN was hypothesized to enhance nanoparticle biocompatibility, improve nanoparticle
36 stability within biologically relevant medias as well as provide a modifiable surface for subsequent
37 functionalization for targeting within the aggressive LNCaP cell model of PC (Figure 3a). Table 1
38 in the Supplemental Information lists the liposomal formulations developed in this study. The
39 colloidal stability of neutral and GRP78 encapsulated CaMSNs was confirmed by DLS
40 measurements where the full LC-CaMSN construct and its individual components remained in the
41 150-200 nm range with a low polydispersity indices (PDI<0.2) indicating homogeneous
42 nanoparticles (Figure 3b). DLS zeta potential measurement results further indicated successful
43 encapsulation of CaMSNs (Figure 3c). The addition of liposomes to coat the metallic enhanced
44 MSN was also hypothesized to enhance the resistance of $\text{SiO}_2\text{-M-PO}_4$ (nucleotide) to other ionic
45 species that can overlap with the nucleotide's phosphate. For instance, incubating siRNA-CaMSN
46 in PBS resulted in a brutal release of the nucleic acid (100% within 30 min) due to the replacement
47 of siRNA's phosphate groups by the free and labile phosphate from PBS or sulfates in the case of
48 the endosomal mimicking solution, MES (Figure 3d). The lipid coating does not affect the loading
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of the nucleic acid meaning that lipid protection of the metallic entraptions occurs faster than their destabilization resulting from PBS. To confirm this, we tracked the release of fluorescently labeled siRNA or mRNA in PBS and an endosomal mimicking solution (MES) for 4 days. Significant release of siRNA was observed (up to 80%) within MES while release within PBS having a physiological pH was minimal (< 15%) (Figure 3e). DLS hydrodynamic size measurement results indicated that lipid encapsulation conferred superior stability properties of neutral LC-CaMSNs over bare CaMSNs for up to 4 days in PBS and complete cell media (Figure S3). LC-CaMSNs incubated in pure serum degraded over time agreeing with our previous work.¹⁷ LC-CaMSNs incubated in MES exhibited instability, further supporting a pH triggered disruption and subsequent release of nucleic acids (Figure S3). Further confirmation of successful lipid coating of RNA loaded CaMSNs was enabled via cryogenic electron microscopy. The lipid coating appears as a distinct halo ring surrounding the dendritic MSN (Figure S4).

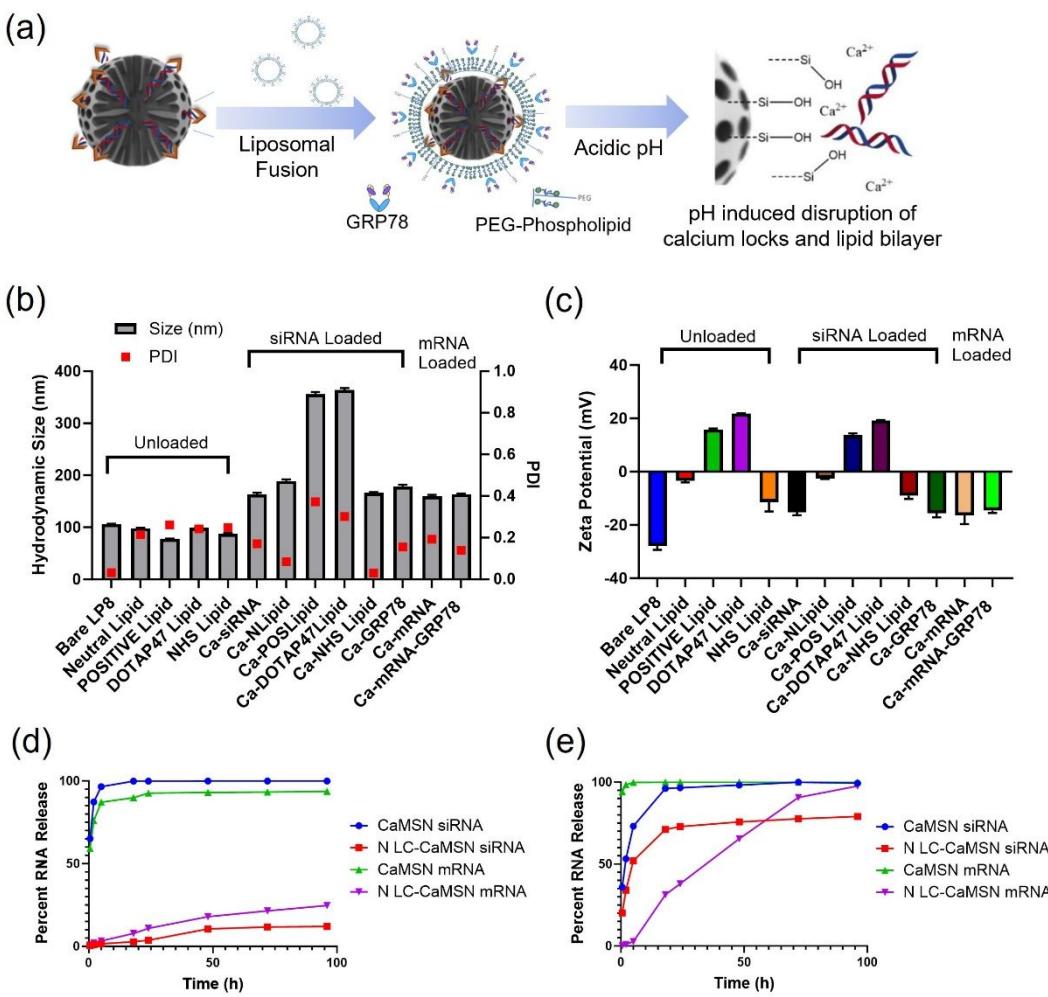


Figure 3. Lipid encapsulation of CaMSN loaded with RNA. (a) Schematic of lipid coating of calcium MSN and RNA release mechanism. (b) DLS characterization LC-CaMSN construct and components. (c) DLS characterization of zeta potential of LC-CaMSN construct and components. (d) Release kinetics for RNA loaded CaMSN or LC-CaMSN incubated in 1x PBS. (e) Release kinetics for RNA loaded CaMSN or LC-CaMSN incubated in MES.

3.4 LC-CaMSN Interactions with LNCaP PC cell line and Human Red Blood Cells. The LC-CaMSNs were then introduced to the LNCaP cell line for *in vitro* analysis. The biocompatibility of the LC-MSN system was analyzed using the Cell Titer Glo® assay in which cell viability is characterized based on the presence of ATP, signaling metabolically active cells. After 48 hours of incubation with a LC-CaMSN having a neutral lipid coating, no significant reduction in cell viability was observed, indicating encapsulation with neutrally charged lipids may reduce cytotoxic effects of the bare MSNs (Figure 4c). To further assess the biocompatibility of the LC-CaMSN system within the bloodstream, we then tested the hemolytic activity of particles incubated with human red blood cells for 3 hours at 37 °C. Bare MSNs caused significant hemolysis regardless of the concentration compared to bare CaMSNs which exhibited slightly less hemolysis likely due to incomplete covering of silanol groups with calcium (Figure 4a). CaMSNs coated with either neutral lipids or GRP78 lipids exhibited significantly less hemolytic activity (Figure 4a), further supporting the use of lipid coatings for enhancement of biocompatibility. Cellular uptake kinetics for non-targeted (Figure S6 a) or targeted (Figure 4b) were evaluated by flow cytometry. Representative gating parameters used to determine the percentage uptake for flow cytometry experiments are given in Figure S6. In the case of non-targeted LC-CaMSNs, rapid uptake was observed for positively charged particles as compared to neutrally charged particles, most likely due to nonspecific electrostatic interactions with cellular membranes. GRP78 minibody targeted particles exhibited superior uptake kinetics, indicating the need for active targeting strategies in the LNCaP cell line to achieve rapid uptake in cancerous cells.

The majority of nanoparticle systems are internalized within cells via endocytotic pathways in which nanoparticles are trapped in vesicles (endosomes) which are trafficked to lysosomes for hydrolytic enzyme degradation.^{36,37} We first investigated the neutral lipid LC-CaMSN uptake by flow cytometry and confocal microscopy. Flow cytometry (Figure 4b) and confocal microscopy (Figure 4f) results showed full association and internalization within cells at 48h. In line with studies that confirmed the capacity of cells to readily uptake positively charged nanoparticles, we then evaluated our LC-CaMSN having positive liposomal encapsulations, namely POSITIVE and DOTAP47 (inspired by our previous work) with which internalization via endocytosis was previously demonstrated in HeLa and A549 cells.¹⁷ Confocal microscopy data showed a significant amount of nanoparticles associated with LNCaP cells at 2 hours (Figure S7 and Figure S8). Deconvoluted 3D rendered images obtained from z-stack confocal imaging further demonstrated internalization of the POSITIVE or DOTAP47 LC-MSNs with or without calcium by 6 hours (Figure S9 and Figure S10). Additionally, POSITIVE or DOTAP47 LC-MSNs colocalized (yellow areas, Pearson correlation coefficient PCC = 0.5236) with the early endosomal marker Rab5 at 6 hours, supporting an internalization mechanism via endocytosis (Figure 4f). We hypothesized that the addition of calcium to LC-MSNs would not only load RNAs but could also provide an endosomal escape release mechanism. Endosomal escape was assessed by tracking colocalization between Rab5 (early endosomes) and LC-MSNs or LC-CaMSNs via confocal microscopy. At 24 hours, deconvoluted confocal microscopy images revealed that LC-CaMSNs colocalized less with Rab5 as compared to LC-MSNs lacking calcium, supporting calcium's role in endosomal escape (Figure 4f). To further support qualitative confocal imaging, colocalization was quantified by tracking Pearson correlation coefficients (PCC) between Rab5 and LC-MSNs or LC-CaMSNs as a function of incubation time with LNCaP cells. Results demonstrated LC-CaMSNs internalized

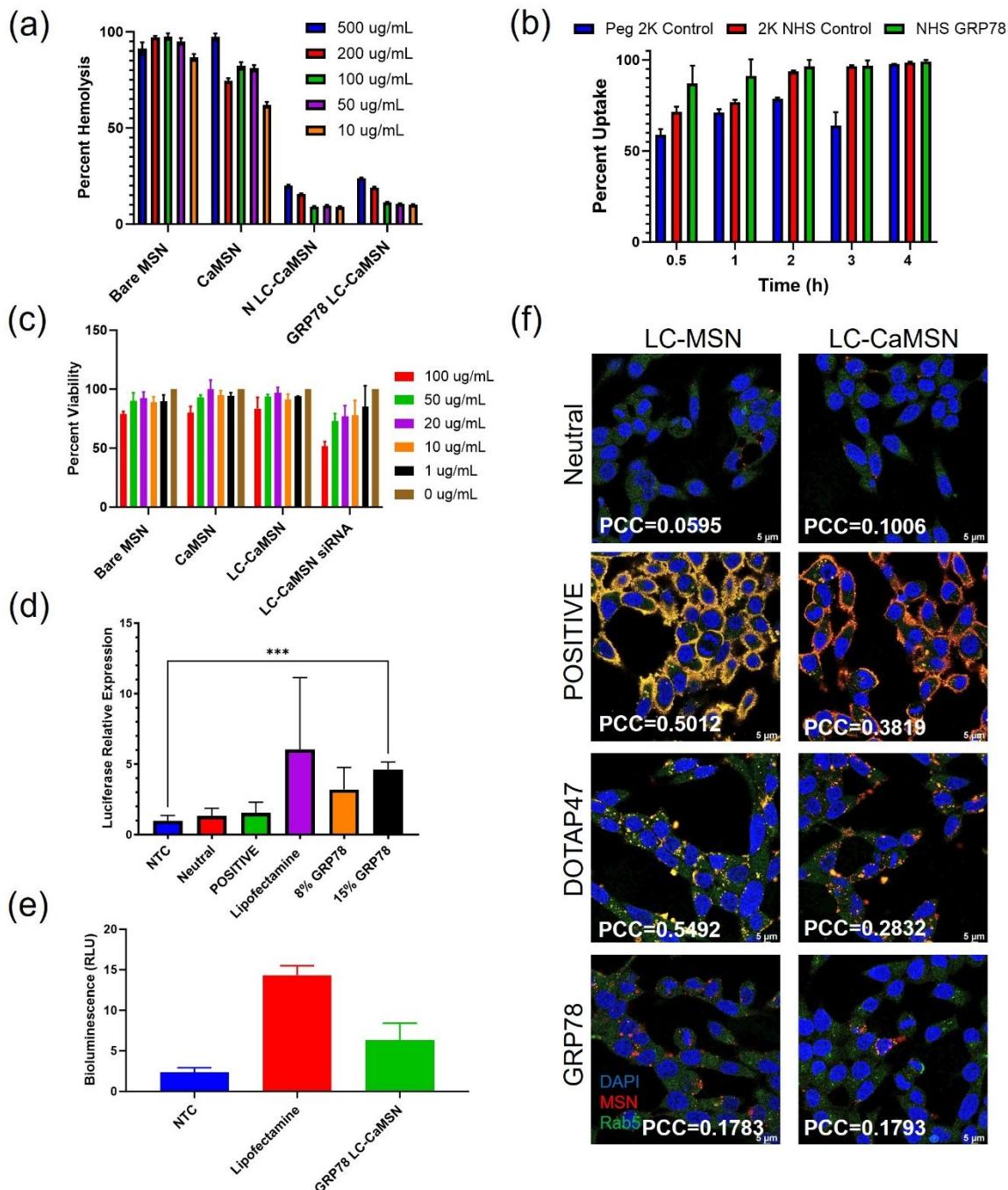


Figure 4. Analysis results LC-CaMSN and LNCaP or human RBC interactions. (a) Calculated percent hemolysis as a function of MSN or LC-CaMSN concentration, (b) Flow cytometry analysis of cells following incubation with targeted LC-CaMSN (c) Cytotoxicity analysis of 72-hour incubation with MSN and LC-MSN (d) Quantitative RT-PCR analysis results for luciferase mRNA delivery via LC-CaMSN. (e) Plate reader results of cell bioluminescence following 24h LC-CaMSN incubation. (f) Deconvoluted confocal images of LNCaP cells following 24h incubation with LC-CaMSNs having various lipid coating with or without calcium using a 63x oil immersion objective. Scale bars indicate 5 μ m.

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3 via endocytosis escaped endosomes more quickly or completely as compared to LC-MSNs lacking
4 calcium (Figure S11). Glucose Receptor Protein 78 KDa (GRP78) has been previously
5 established as an overexpressed receptor on LNCaP cells, and has been previously validated as a
6 functional molecular moiety for targeted delivery systems in PC therapeutic applications.^{38,39} We
7 hypothesized that LC-MSNs with or without calcium that are functionalized with a GRP78
8 minibody would enable quick association with LNCaP cells. Flow cytometry results confirmed
9 almost complete uptake of GRP78 LC-CaMSNs within 30 minutes of incubation at 37 °C (Figure
10 4b). Additionally, GRP78 functionalized LC-MSN or LC-CaMSN systems lacked colocalization
11 with Rab5 (Figure 4f, Figure S7 and Figure S8) suggesting an alternative internalization pathway.
12 3D rendered confocal images of LC-MSN or LC-CaMSNs following incubation with LNCaP cells
13 confirmed internalization by 2 hours (Figure S9 and Figure S10). To confirm that GRP78
14 functionalization results in LC-MSN or LC-CaMSN binding to surface expressed GRP78
15 exclusively, we pre-incubated LNCaP cell with free GRP78 (insert concentration) for 4 hours
16 before incubating cells with LC-CaMSNs. Deconvoluted confocal microscopy results
17 demonstrated a lack of LC-CaMSN association or internalization supporting that GRP78
18 functionalized LC-CaMSNs bind exclusively to surface expressed GRP78 (Figure S12).
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Successful delivery of mRNA within the LNCaP cell line was first assessed via quantitative RT-PCR after cells were incubated with various LC-CaMSNs. The commercially available *in vitro* transfection agent Lipofectamine MessengerMax® was used as a positive control. Results indicated that as compared to the non-treated control, GRP78 targeted LC-CaMSNs significantly delivered more mRNA to cells and that the relative gene expression could be increased with increasing amount of NHS within the lipid formulation (Figure 4d). We then assessed subsequent protein expression after mRNA delivery in LNCaP cells via expression of luciferase and subsequent bioluminescence as measured by plate reader. Similarly to RT-PCR results, better bioluminescence expression was observed in cells incubated with targeted LC-CaMSN systems (Figure 4d). While Lipofectamine® outperformed the LC-CaMSNs as far as bioluminescence signal, cytotoxicity analysis in which volumes of LC-CaMSN and Lipofectamine® were matched revealed that Lipofectamine® was significantly more toxic than the LC-CaMSN carrier. (Figure S13). This indicates that Lipofectamine® would not be appropriate as a therapeutic delivery agent and should only serve as a control for experiments.

3.6 LC-MSN system vascular margination, circulation and stability within *ex ovo* chick chorioallantoic membrane model (CAM). The CAM can be classified as a bridge between *in vitro* and *in vivo* systems making it an ideal biological barrier platform for evaluating vascular margination, binding, circulation time and stability of the LC-MSN system.⁴⁰ Bare CaMSNs and LC-CaMSNs having varying liposomal coatings were injected into CAM vasculature and imaged after allowing 20 minutes of circulation (Figure S7). Bare MSNs were observed to aggregate and bind to vasculature walls (Figure 5) and remaining non-aggregated MSNs were observed to quickly be removed from circulation most likely via innate immune system opsonization.⁴¹ LC-CaMSNs having a positive charge (POSITIVE) exhibited relatively longer circulation within CAM vasculature but were quickly bound to vasculature walls and red blood cells most likely due to

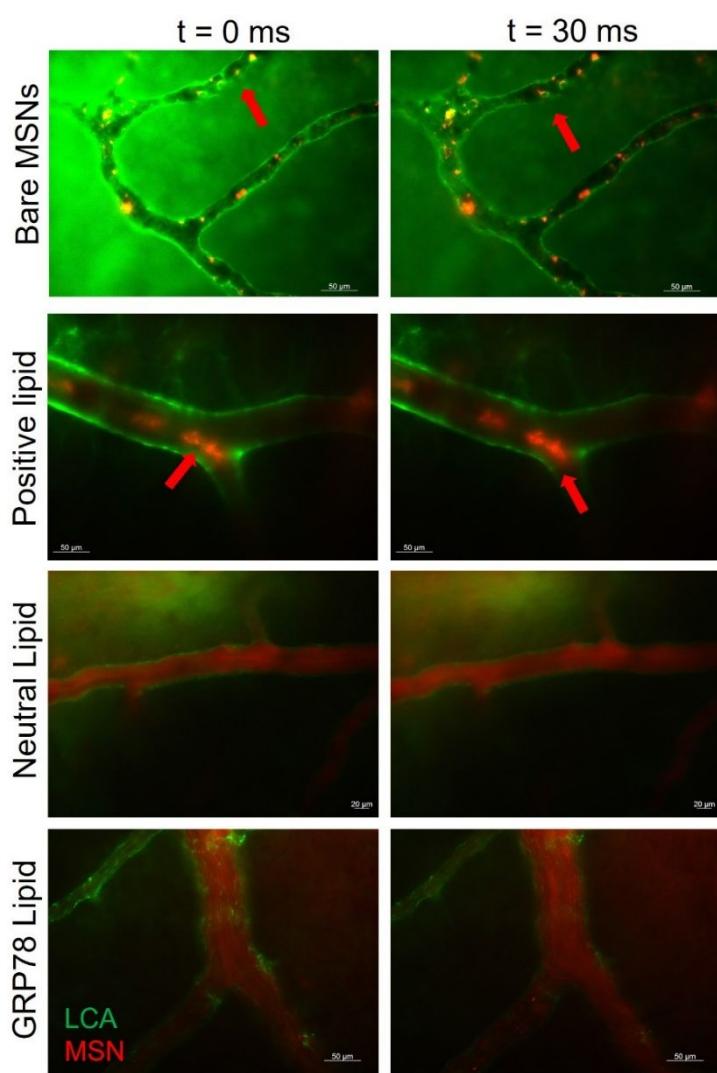


Figure 5. Timelapse images of LC-MSN circulation within CAM vasculature taken at 40x. Red arrows indicate MSN aggregation.

electrostatic interactions between negatively charged membranes and the positively charged LC-CaMSN (Figure 5). Additionally, positively charged LC-CaMSNs ultimately exhibited aggregation. Finally, neutrally charged LC-CaMSNs with or without the GRP78 targeting ligand exhibited stable circulation, no vasculature binding, and no apparent reduction in circulation due to opsonization (Figure 5). Videos of LC-CaMSN live circulation within the CAMs are provided in supplemental information of online version.

3.7 LC-CaMSN *in vivo* Tumor Retention. We tested the retention of DyLight 633 labeled LC-CaMSNs after subcutaneous (SC) injections within NSG mice engrafted with LNCaP tumors for up to 48 hours. Whole live animal IVIS imaging results for SC injected LC-CaMSNs indicated superior retention at the tumor site in the case of GRP78 LC-CaMSNs as compared to non-targeted N LC-CaMSNs (Figure 6a). After live animal imaging, mice were euthanized and necropsied to assess LC-CaMSN presence in organs and tumors. Enhanced tumor retention in the case of GRP78 targeted LC-CaMSNs as compared to non-targeted neutral LC-CaMSNs was confirmed via quantification of Dylight 633 LC-CaMSN signal in dissected organs and tumors (Figure 6b). IVIS

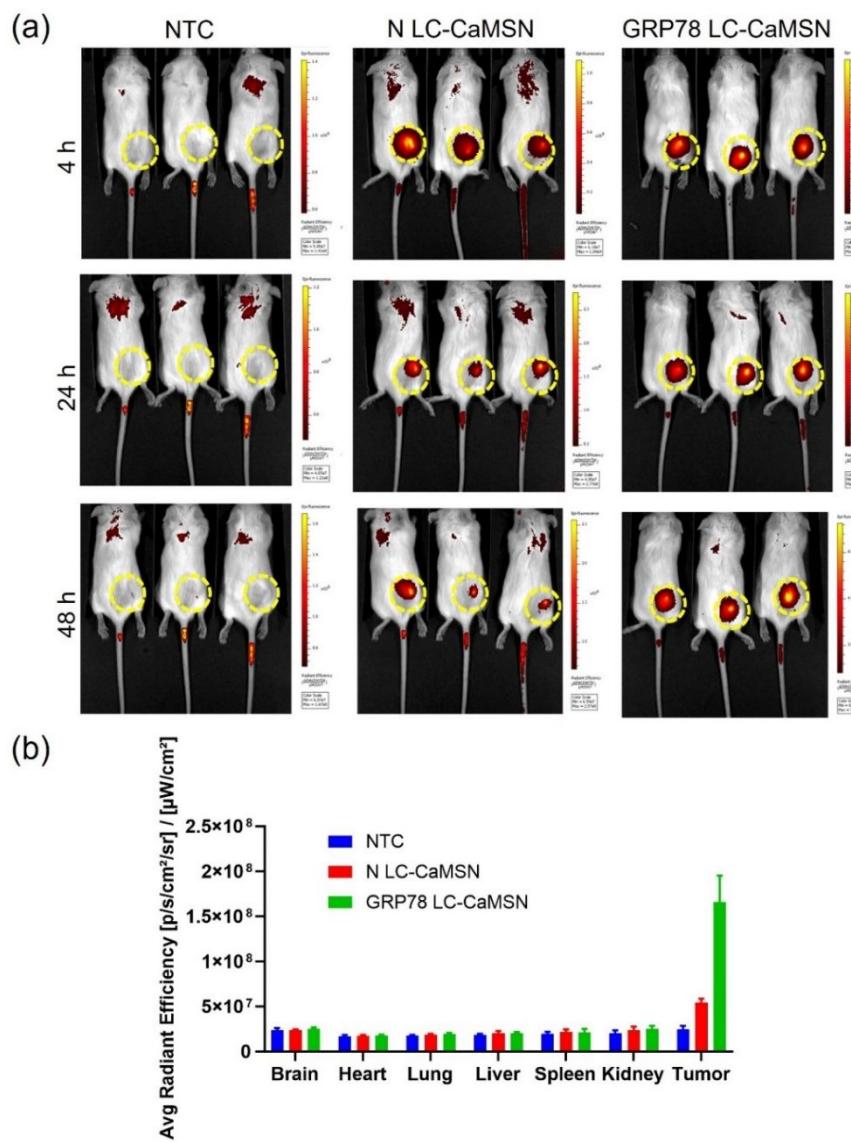


Figure 6. Imaging of NSG mouse LNCaP xenografts obtained from IVIS imaging system after right flank subcutaneous Dylight 633 mRNA loaded LC-CaMSN injections. (a) Whole body live images of dorsal side of mice. Dashed yellow lines indicate tumor site. (b) Quantification of fluorescent signal in mouse organs dissected 48 hours after injections.

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2 images of necropsied organs upon which this quantification were carried out over are given in
3 Figure S15.
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5 **4. Conclusions.** In summary we have presented the development and characterization of a lipid
6 coated mesoporous silica nanoparticle that is capable of loading either siRNA or mRNA via metal
7 cation complexation. To the best of our knowledge this is the first report of successful mRNA
8 loading using metallic cation mediated entrapment. We demonstrated colloidally stable constructs
9 that exhibit pH triggered release for endosomal escape within LNCaP cells. *Ex ovo* results
10 supported the biocompatibility of the system along with promising stability and circulation
11 characteristics. The inclusion of calcium cations to the LC-MSN system provided an enhanced
12 endosomal escape mechanism as seen by confocal microscopy. The tailorabile and biocompatible
13 nature of the LC-CaMSN along with high loading efficiencies for siRNA and mRNA allowed for
14 the addition of tumor retention ligands as shown in the intratumoral retention of the GRP78 LC-
15 CaMSN. Future work will be focused on the systematic investigation of fine-tuning liposomal
16 formulations and GRP78 conjugation strategies for CaMSN encapsulation for optimized tumor
17 accumulation upon IV administration and subsequent reduction of tumor burden.
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19 **Supporting Material Available:** Table of liposomal formulations, picture of FeCl_3 solutions and
20 corresponding Fe loaded MSNs, EDS elemental analysis of siRNA loaded MSNs, DLS
21 hydrodynamic size characterization of bare or lipid encapsulated CaMSN incubated in various
22 physiological solutions, Cryo-EM images of LC-CaMSNs, Analysis of LC-CaMSN hemolysis,
23 Representative flow cytometry gating parameters, 2D and 3D surface-rendered deconvoluted
24 confocal images of LNCaP cells, Graph of PCC for LC-CaMSN colocalization with Rab5,
25 Deconvoluted confocal images of GRP78 pre-incubated LNCaP cells, Cytotoxicity of
26 Lipofectamine® results, CAM injection of LC-MSNS imaging setup, Necropsied IVIS images of
27 mouse organs and tumors.
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37 **Notes**

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