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Enhanced siRNA delivery using a combination of an arginine-grafted bioreducible polymer, ultrasound, and microbubbles in cancer cells

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Abstract

RNAi-based gene therapy for cancer treatment has not shown significant clinical impact due to poor siRNA delivery to the target site. Here, we design a non-viral siRNA gene carrier using a combination of an arginine-grafted bioreducible polymer (ABP), microbubbles (MB), and ultrasound (US), for targeting vascular endothelial growth factor (VEGF) in a human ovarian cancer cell line. Newly designed MBs with a perfluorocrownether gas core show higher stability compared to controls. Further, MBs in combination with polyplexes show a significant higher loading capacity compared to naked siRNA. Lastly, only siRNA-ABP-MB (SAM) complexes in combination with US show significant VEGF knock down in A2780 human ovarian cancer cell line compared to naked siRNA when incubated for a short time after sonication treatment.

Keywords

non-viral siRNA delivery; Microbubbles; Ultrasound; Cancer

1. INTRODUCTION

The primary goal of gene therapy is to cure diseases caused by genetic defects and to provide the necessary therapeutic modality through genetic engineering. This approach to prevent and treat various diseases includes the replacing, removing or introduction of genes. For example, adding a gene to a cell to produce a specific missing protein or using antisense molecules to stop producing harmful proteins in cancer disease like the vascular endothelial growth factor (VEGF). In order to deliver genes efficiently to the appropriate tissues including the target cells it is essential to use a gene carrier.[1] RNA interference (RNAi)-based gene therapy using siRNA for protein knockdown has proven to be a feasible strategy for the treatment of genetic diseases.[2] VEGF is known to be one of the key factors for deleterious angiogenesis in cancer and has proved to be an attractive gene therapy target to treat angiogenic tumors.[3] At this time, (RNAi)-based gene therapy has not shown significant clinical impact due to poor siRNA delivery to the target site. When administered systemically, uncoated (naked) siRNA fails to take effect because of the following reasons: first, uncoated siRNA has a poor half-life in the blood stream and is degraded quickly by

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SUPPORTING INFORMATION **AVAILABLE**
Supplement 1 describes the chemical structure of ABP. Supplement 2 shows the loading capacity of MBs depending on incubation volume, incubation time and MB concentration. Supplement 3 represents MTT assay of figure (7A) and (7B). This information is available free of charge via the Internet at http://pubs.acs.org/.

serum nucleases; second, uncoated siRNA has poor therapeutic efficacy due to low accumulation at the target site; lastly, uncoated siRNA has poor cellular-uptake efficiency due to its large size and negatively charged backbone.[4] Therefore, a gene vector that is able to carry and protect genes in the bloodstream from serum nucleases is necessary. Further, a successful gene carrier should be able to facilitate delivery and accumulation of genes at the target site, to attain adequate cell transfection, to produce relevant therapeutic response, and to show an optimal safety profile.

Perfluorocarbon-exposed-sonicated-dextrose-albumin-microbubbles (MB) have been used for the last two decades as ultrasound contrast agents (UCA) for diagnostic imaging purposes. Optison™ is one example of a contrast MB which has been on the market and FDA-approved for the use as a UCA since 1998.[5] Optison™ consists of an albumin shell and a perfluoropropane (PFPr) gas core. The microbubbles used in this study consist of an albumin shell and a perfluoropentane (PFP) or perfluorocrownether (PCE) gas core. The advantage of using PFP/PCE over PFPr is that the boiling points of PFPr, PFP and PCE are −36.7 °C, 29 °C and 146 °C respectively, which makes PFP and PCE much easier to handle.

Ultrasound (US) as a transfer system for gene therapy represents a safe, non-invasive, cost-effective system which can be used for site-specific targeting.[6] MBs in combination with US can be used to deliver DNA systemically while releasing DNA through focused ultrasound treatment (sonication) at specific target sites. Focused sonication at the target site in the presence of MBs enhances delivery of therapeutic agents due to cavitation and microjets/jetstreams. MB implosion perforates the cell membrane, facilitating gene uptake.[7] However, DNA delivery with MB shows only moderate DNA uptake and expression due to degradation of the released DNA at the target site by extracellular nucleases and due to poor cellular uptake efficiency. Therefore, a combination of MBs with non-viral polycationic polymer carriers can overcome the disadvantages of MBs used as gene delivery system alone.

Gene carriers used for nucleic acid delivery can be categorized into two major groups; viral and non-viral. Viral gene carriers show limitations in terms of their safety, particularly immunogenicity and loading capacity of genetic material. Therefore, non-viral polymeric gene carriers offer a safer alternative. non-viral polymeric vectors are non-immunogenic, can be easily modified to achieve multifunctional properties and applications, have lower production costs compared to their viral counterparts, and have a high loading capacity.[1, 8] Novel arginine grafted bioreducible polymer (ABP) (Supplement 1) as non-viral gene carrier has been used to deliver siRNA.[9] ABP has been engineered to electrostatically interact with negatively charged siRNA and form stable complexes smaller than 200 nm called polyplexes. Further, ABP has been shown *in vitro* to protect siRNA from serum protein degradation, to facilitate endocytosis through interaction of the positively charged ABP polyplex with negatively charged cell membranes, to escape the endosome, and to release siRNA into the cytosol through intracellular cleavage of the bioreducible backbone.[10] ABP is a polymeric polycation with a disulfide amine backbone and an arginine residue. The main advantage of ABP is the bioreducibility of disulfide groups in the cytosol through the intracellular reductive agent glutathione that enhances gene release and decreases cytotoxicity.[9] Further, arginine is a recurring motif in cell penetrating peptides and increases transfection efficiency when conjugated to polymers compared to unconjugated counterparts.[11, 12] Recent work in our lab showed that ABP has high transfection efficiency while decreasing cytotoxicity compared to 25k-branched-polyethyleneimine (bPEI), which represents the “gold standard” for non-viral gene therapy.[9, 10] However, polycations show disadvantages if systemically administered because the positive surface charges interact with serum proteins forming aggregates and attract the RES.[13] Lastly, targeting is believed to occur passively based on the Enhanced Permeability and Retention (EPR) effect. Passive

targeting by the EPR effect requires a particle size less than 200 nm for extravasation into tumor tissue caused by leaky tumor vasculature and poor lymphatic drainage. If systemically administered, ABP are exposed to serum proteins that increase particle size to greater than 200 nm through protein interactions, therefore decreasing passive targeting.[14] Active targeting of tumors using ligands attached to gene vector has shown modest *in vivo* success due to immunogenicity and heterogeneity of cancer cells and tumor physiology.[13, 15-19] Overall, attached targeting ligands are dependent on the expression of specific receptors on tumors that may differ in patients and which may only be expressed in certain tumor subpopulations.[14] Therefore, combining advantages of MBs and ABP should produce a gene carrier that is able to deliver genes to the target site and improve therapeutic efficacy.

2. EXPERIMENTAL SECTION

2.1.Materials

Dextrose, HEPES, human serum albumin (HSA), Dithiothreitol (DTT), DMSO, Fluorescein isothiocyanate (FITC), branched polyethylenimine (bPEI, Mw 25,000), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St.Louis, MO). arginine-grafted bioreducible polymer (ABP) was synthesized and characterized as described previously[9, 20]. Human VEGF siRNA (sense, 5′-GGAGUACCCUGAUGAGAUCdTdT-3′; antisense, 5′-GAUCUCAUCAGGGUACUCCdTdT-3′) and AlexaFluor555-labeled siRNA BLOCK-it™ were purchased from Ambion (Foster City, CA). Cy3-labeled siRNA was synthesized by the Health Sciences Center core research facilities at the University of Utah (Salt Lake City, UT). All cell culture products including Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), and fetal bovine serum (FBS) were obtained from Invitrogen (GibcoBRL; Carlsbad, CA). Perfluoropentane (PFP) was purchased from SynQuest Labs Inc. (Alachua, FL) and perfluorocrownether (PCE) from Oakwood Products (West Columbia, SC).

2.2.siRNA-ABP-Microbubble (SAM) complexes formation

A) Microbubble (MB) synthesis—MBs were prepared following the procedure developed by Porter *et al*[21]. Briefly, three parts of a 5% dextrose solution in HEPES buffer were mixed with one part of a 5% HAS solution in HEPES buffer (20 mM, 5% dextrose, pH 7.4), combined with perfluorocarbon gas (10% PFP or 1% PCE), and sonicated with 60% amplitude of a 20 kHz ultrasonic processor with cuphorn (51mm probe diameter, Sonics Vibracell VCX-500 ultrasonic processor, Sonics, Newtown, CT) for 10 seconds. MBs were washed by centrifuging at 118g for 1 min, removing the supernatant and re-suspending in HEPES buffer after each wash step. This step was repeated three times.

B) ABP-siRNA Polyplex formation—Polyplexes were prepared by adding a HEPES buffer solution containing ABP to a HEPES buffer solution of siRNA at a weight ratio 10:1 (w/w; ABP to siRNA), followed by gentle shaking and incubation at room temperature for 30 min.

C) SAM formation—SAM complexes were built by mixing polyplexes (ABP-siRNA) and MB. To form SAM complexes, MB and polyplexes were combined in HEPES buffer solution and incubated for 10 minutes.

2.3.MB, polyplex, and SAM characterization

The concentration (number of MB/mL) of MB was determined immediately after preparation by eye counting with a light microscope using a hemocytometer. The zeta-

potential and particle size of the MB, polyplexes, and SAM complexes were measured using a Zetasizer-Nano ZS (Malvern Instruments, Worcestershire, UK).

2.4.MB stability at storage and body temperature

Stability of PCE and PFP MBs at storage temperature (4° C) and body temperature (37° C) were determined by analyzing both MB concentration and MB particle size over time. Therefore, PCE and PFP MB were stored for 3 weeks at 4 °C. At 0, 3, 7, 10, 14, 17, and 21 time points (days), the concentration of MBs (number of MB/ml) and particle size were determined using a hemocytometer and Zetasizer-Nano ZS, respectively. Concentration and size of MB prepared with PCE and PFP gas were further observed at 0.17, 0.5, 1, 2, 4, 24, and 48 hours for determining MB stability at 37° C .

2.5.siRNA, polyplex, and SAM stability after ultrasound (US) exposure

Degradation/stability of siRNA, polyplexes, and SAM complexes in the presence of US was determined by gel retardation assay. Samples were exposed to different US conditions of 1 MHz, 3.0, 2.0, 1.0 W/cm^2, 10%, 50% and 100% duty cycle for 10 minutes. ABP polymer dissolved in HEPES buffer was mixed with 1 Pg siRNA at a w/w ratio 10:1 (ABP:siRNA). The mixtures were incubated for 30 minutes at room temperature for complex formation and then exposed to US. In cases where release of siRNA from polyplex and SAM complex is desired the samples were mixed with 5 mM dithiothreitol (DTT) (Sigma Aldrich, St Louis, MO) for 30 minutes at 37°C after US exposure to ensure ABP dissociation from intact siRNA followed by gel-electrophoresis analysis on a 2% agarose gel. Electrophoresis was carried out at 120V for 20 min in TAE buffer solution (40mM Tris/HCl, 1% (v/v) acetic acid, 1mM EDTA). The migration of the complexes was visualized by using an image analyzer equipped with UV transilluminator (GelDoc, BioRad, Hercules, CA).

2.6.Fluorescence microscopy

SAM formation was confirmed and visualized by a confocal laser scanning microscope (Olympus Fluoview FV300, Melville, NY) with a 60x water objective lens. siRNA was labeled with Cy3 dye at the 3′-terminal end of the sense strand. FITC-labeled ABP was prepared by a drop wise adding of FITC solution (10 mg/mL) dissolved in dimethylsulfoxide (Sigma Aldrich, St Louis, MO) to ABP in 0.2 M sodium bicarbonate buffer (Sigma Aldrich, St Louis, MO) (pH 9.0). After 5 hours of reaction at 4 °C, FITC-labeled ABP was purified using a Sephadex G-25 column (GE Healthcare, Piscataway, NJ) in the same sodium bicarbonate buffer. The sample solution was dialyzed against ultra pure water using a dialysis membrane (MWCO 1000, Spectrum Laboratories, Inc., Rancho Dominguez, CA) for 4 h, followed by lyophilization. All steps were performed in the absence of light. HSA was labeled with FITC according to the procedure mentioned above.

2.7.siRNA loading capacity of MBs

Loading capacity of MBs was analyzed after SAM formation at different incubation volumes (250, 1000, and 1500 μl), incubation times (1, 5, 10, 20 minutes), and MB concentrations (0.5, 1, 2, 3 × 10^8 MB). First, 0.5 × 10^8 MBs mixed with 500 ng ABP/siRNA-Cy3 polyplexes prepared at a w/w ratio 10:1 (ABP:siRNA) were incubated in 250, 1000, or 1500 μl. After 10 minutes incubation, SAM complexes were centrifuged at 118g for 1 minute and supernatant was collected. The supernatant was analyzed for excess unbound polyplexes by measuring the fluorescence of the Cy3-labeled siRNA using a TECAN infinite® 200 Pro series fluorometer (TECAN group Ltd, Männedorf, Switzerland). Amount of siRNA bound on microbubble was determined by subtracting the unbound siRNA concentration from the starting concentration (500 ng). Further, loading capacity was determined using different incubation time for SAM formation of 1, 5, 10, and 20 minutes

and different MB concentrations of 0.5, 1, 2, and 3×10^8 MB. Final loading capacity was calculated as mentioned above. Loading capacity of MB without ABP was further determined as described above. siRNA loading capacity of MB complexed with polyplexes was compared to that of MB complexed with naked siRNA (without ABP).

2.8.Cell culture

DMEM containing 10% FBS and 1% penicillin-streptomycin was used to grow A2780 cells (human ovarian carcinoma). Cells were seeded on 6-well plates at a density of 16.7×10^4 cells/well. Cells were cultured at 37° C in a humidified incubator and an atmosphere of 5% (v/v) CO_2.

2.9.FACS analysis

The cellular uptake of siRNA was examined using FACS. Cells were transfected with SAM, naked siRNA, or ABP/siRNA polyplexes after 24 hours of seeding, which showed a 30–40% cell density. The cell media was replaced with plain media without serum prior to the transfection. AlexaFluor555-labeled siRNA was mixed with ABP polymer at a w/w ratio 10:1 (ABP:siRNA) in HEPES buffer solution for a final concentration of 10 nM siRNA/well in 2 ml cell media. Polyplexes were incubated with different concentration of MB (MB:cell ratio 25, 50, 100, and 200) for 10 minutes to form SAM complexes. SAM complexes were added to the wells and incubated for 5 minutes prior to US. An Omnisound^R 3000 Pro ultrasound device (ACP, Reno, NV) with a 3 cm diameter US probe was used. The cells were exposed to US conditions of 1MHz or 3MHz, 0.5 W/cm^2, 50% duty. The US probe was rotated constantly in the well for 60 seconds of US treatment. After 4 hours incubation, the cell media was replaced with serum-containing media. The cells were collected for FACS analysis24 hours after transfection. Cells were analyzed by a flow cytometer (FACSCanto™ II, Becton-Dickinson, Mountain View, CA). Only transfected singlet cell population was gated for analysis of siRNA uptake.

2.10.VEGF ELISA assay

Knock down of VEGF protein was quantified by using a human VEGF ELISA kit (Thermo Scientific, Rockford, IL). Cells were prepared as described above. siRNA targeting human VEGF, a model siRNA, was mixed with ABP polymer at a w/w ratio 10:1 (ABP:siRNA) in HEPES buffer solution for a final concentration of 50 nM siRNA/well in 2 ml cell media. ABP/siRNA polyplexes were incubated with MBs at a concentration of 50 (MB:cell) for 10 minutes to form SAM complexes. A GFP-targeting siRNA was used as a negative control. PEI complexed with VEGF siRNA at a w/w ratio (1:1) was used as positive control. Further controls include MB-siRNA and naked siRNA. Complexes were added to the well and incubated for 5 minutes prior to US treatment. Same US conditions as described above were used except for the incubation time post US treatment. After US treatment, cells were incubated for 10 minutes or 60 minutes. Cell media was removed and cells were washed twice with HEPES buffer and replaced with 2 ml of serum-containing culture media. After 48 hours incubation, cell media was collected to determine VEGF concentration in each well.

2.11.Cell viability assay

Cell viability was measured by MTT assay. Cells were seeded, transfected, and exposed to US as described above. After transfection, the cells were incubated for 24 or 48 hours. MTT (200 μL/well with 5 mg/ml) was added to the cells. After 4 hours, media was removed and 2 ml DMSO was added in each well to dissolve the formazan crystals. The absorbance of MTT was determined using a UV-microplate reader at 570 nm. The MTT value for untreated cells (cells not exposed to transfection systems) was taken as 100% cell viability.

3. RESULTS AND DISCUSSION

3.1.Microbubble synthesis and stability

MBs were synthesized using a slightly changed protocol as described by Porter *et al.*[21] Thus, HSA has been labeled with FITC following above-mentioned procedure. Particle size of PCE-MB 1–5 μm was confirmed by fluorescence microscopy (Figure 1A, B) and particle size measurement was performed using a Malvern Zetasizer Nano-series (Figure 1C). MB particle size was 3001 nm (diameter) with a PDI of 0.155, width of 794 nm and a zeta potential of −34.6 mV ± 5.4. MB concentration was determined using a hemocytometer and a light microscope and was observed to be $2.67 \pm 0.14 \times 10^9$ MB/ml. Further, two different perfluorocarbon gases were investigated for the production of MBs. The boiling points of PFP and PCE are 29 °C and 146 °C respectively which influences the stability of the MBs.[22] Therefore, stability at storage and body temperature was investigated to determine which MBs are more stable and should be used for future experiments. First, storage stability of both MBs was investigated by analyzing MB concentration and particle size for 3 weeks at 4 °C. PCE-MBs concentration was constant for over 3 weeks while PFP-MBs concentration decreased by 50% after 3 days and almost completely disappeared after 3 weeks. The difference in stability of PFP- and PCE-microbubbles at storage temperature can be explained by the different Laplace pressure. Laplace pressure describes the pressure difference that occurs between inside and outside the microbubble. Thus, PCE-MBs have a lower inside pressure (vapor pressure) compared to PFP-MB because the PCE boiling point is higher (PFP 29 °C, PCE 146°C), therefore, PCE-MB show a better stability under storage temperature (Figure 2A). The particle size of PCE-MBs was constant for over 3 weeks at 4 °C and PFP-MBs particle size increased after 10 days from 2000 nm to 6000 nm (Figure 2B). Next, stability of both MBs was investigated at body temperature (37 °C). PCE3, PFP-MBs concentration and particle size behave very similar in the first four hours of incubation. However, PCE-MBs are more stable after 24 hours incubation (Figure 2 C, D). Thus, the higher boiling point of PCE gas compared to PFP gas explains the better stability at storage and body temperature, therefore, PCE-MBs were be used for future experiments.

3.2. siRNA and polyplex ultrasound stability

The impact of US on the stability of siRNA and ABP is crucial, therefore, the stability of siRNA and polyplexes were investigated using different US conditions. Polyplexes and siRNA were exposed to various US conditions and analyzed by gel retardation assay, for particle size and for surface charge. As seen in Figure 3A, naked siRNA withstood different US conditions. Only the harsh US treatment conditions of 10 minutes at 1MHz, 3 Watt/cm^2, and 100% duty cycle showed degradation of siRNA. Further, polyplexes (ABP:siRNA) with a w/w ratio of 10:1 were analyzed at low (10%) and high (50%) duty cycles at 1 Watt/cm^2 intensity. First, ABP protected and did not release siRNA after US treatment (Figure 3B **lanes 5–6**). Second, ABP's bioreducibility upon treatment with the reducing agent DTT is not influenced after US treatment (Figure 3B **lanes 8–9**). Lastly, particle size and zeta potential of polyplexes after sonication did not show any significant difference from baseline (Figure 3C). Thus, siRNA and polyplexes showed no drawback after sonication and were used in combination with US for future experiments. However, only siRNA degradation can be shown here. Evaluation of any differences of siRNA activity after sonication were evaluated with the VEGF ELISA kit.

3.3. siRNA-ABP-Microbubble (SAM) formation

The basic principle of SAM complex formation is manifested by the electrostatic interaction between positively charged polyplexes and negatively charged MBs. In general, human serum albumin has an isoelectric point of 4.7, therefore, is negatively charged in the bloodstream pH 7.4.[23] ABP polymer is a poly(disulfide amine) and is positive charged at pH

7.4 due to protonation of the amino groups on the ABP polymer backbone (Supplement 1).[10] Thus, positive polyplexes and negative microbubbles undergo complexation at pH 7.4 through electrostatic interactions to form SAM complexes (Scheme 1A). Depending on the MB stability and US exposure, MBs undergo noninertial or inertial cavitation that leads to polyplex release from the MB shell (Scheme 1B). Inertial cavitation describes the process of a bursting MB after US exposure and noninertial cavitation describes the consistent shrinking and growing motion of MBs without bursting. Both mechanisms lead to polyplex release from the MB shell due to high shear forces, shock waves, jet streams and microstreaming that disrupts the electrostatic forces between the MBs and polyplexes.[24]

SAM formation was evaluated by fluorescence microscopy. The goal of this experiment was to prove the existence of siRNA and ABP on the MB shell and to compare it to a MB-siRNA (no polymer) control, thus, underlying the importance of the use of ABP. Therefore, FITC labeled MBs were combined with polyplexes (ABP-siRNA-Cy3) and formation of SAM complexes was verified by fluorescence microscopy. Figure 4A indicates the formation of SAM complexes, where green fluorescence is FITC-labeled MB albumin-shell, red fluorescence is Cy3 from polyplex of ABP with siRNA-Cy3 and yellow is the merged pictures of green and red, verifying the existence of polyplexes on the surface of MBs, however, it is not clear if ABP polymer is involved in the formation of SAM complexes. Therefore, SAM formation was investigated using unlabeled MBs and combined with polyplexes (FITC-ABP-siRNA-Cy3). As seen in Figure 4B ABP polymer and siRNA-Cy3 show a yellow merged picture and verify the existence of polymer on the surface of the MBs. Next, the ability of naked siRNA-Cy-3 to bind on the surface of MB was tested. Figure 4C indicates that naked siRNA is able to bind on the MB surface but with a much lower efficacy compared to polyplexes. Higher localization of polyplexes on the MB shell compared to naked siRNA is due to electrostatic interaction between positively charged polyplexes and negatively charged MBs. Naked siRNA is negatively charged and explains the lower localization on the MBs shell due to repulsion interaction with the negatively charged MB shell. Thus, polyplexes combined with MBs showed a higher localization of siRNA on the MB shell compared to naked siRNA and underlines the importance of the use of ABP.

3.4. SAM characterization

As described above, the positively charged polymer ABP can interact with the negatively charged MB shell. Therefore, to show the advantage of polyplexes (ABP-siRNA) over naked siRNA we quantified the loading capacity of MBs when combined with polyplexes or naked siRNA. We combined 0.5×10^8 MBs with 500 ng naked siRNA-Cy3 or 500 ng siRNA-Cy3 complexed with ABP at a 10:1 (ABP:siRNA) w/w ratio. Polyplexes were able to load around 300 ng of siRNA on 0.5×10^8 MBs and showed a significant difference ($P < 0.001$) compared to naked siRNA (30 ng of siRNA on 0.5×10^8 MBs). Thus, polyplexes showed around ten times higher loading capacity compared to naked siRNA (Figure 5A). Further, loading capacity was analyzed at different incubation volumes, incubation times and MB concentrations. Hence, incubation of polyplexes with MBs in the lower incubation volume of 250 μl compared to 1500 μl for 10 minutes incubation time showed higher loading capacity due to the higher possibility of interaction of polyplexes with MBs in a smaller volume (Supplement 2A). A longer incubation time 20 minutes showed an increase in loading capacity compared to a shorter incubation time of 1 minute (four fold increase) (Supplement 2B). Therefore, an incubation volume of 250 μl and an incubation time of 10 minutes were used for SAM formation in future experiments.

Zeta potential of SAM complexes was measured using a fixed MBs concentration and an increasing concentration of siRNA (0.5, 1, 5, 10 μg) complexed with ABP. MBs and polyplexes displayed zeta potentials of −34.6 mV ± 5.4 and +20.1 mV ± 4.7, respectively.

After incubation at different polyplex concentrations, SAM complexes were washed by centrifugation at 118g for 1 minute, removing the supernatant and resuspending in HEPES buffer after each wash step. This step was repeated three times to completely remove unattached polyplexes. As seen in Figure 5B, combination of MBs with 0.5 μg of siRNA complexed with ABP showed a zeta potential around 0 mV and combination with 5 μg of siRNA complexed with ABP a zeta potential identical to polyplexes around 20 mV indicating the saturation of the MBs shell with polyplexes. Thus, it is possible to tune the zeta potential on the MBs depending on the polyplex concentration used.

Lastly, release of siRNA from SAM complexes was investigated using sonication and DTT treatment. US- and DTT-treated SAM complexes were able to release siRNA and showed the same intensity in the siRNA band compared to naked siRNA control (Figure 5C).

3.5. FACS assay

MB:cell ratio and US intensity have an effect on siRNA uptake.[25] Therefore, FACS assay was used to determine the best MB:cell ratio and US conditions for siRNA uptake in A2780 human ovarian cancer cell line. Thus, cellular uptake efficiency with 10 nM (final concentration in 2 ml cell media) siRNA in SAM complexes using different MB:cell ratios (25, 50, 100, 200) and 1 MHz US condition at 0.5 Watt/cm^2 and a 50% duty cycle were investigated. Transfection occurred in serum free media for 4 hours. The use of a MB:cell ratio of 50 or higher showed a significantly higher siRNA uptake compared to ratio 25. However, there was no difference between ratios 50, 100, 200 (Figure 6A). Therefore, the MB:cell ratio of 50:1 was used for future experiments.

Next, siRNA uptake efficiency was compared using polyplexes or SAM complexes with and without sonication. Therefore, 10 nM (final concentration in 2 ml cell media) siRNA complexed in polyplexes or SAM complexes and US conditions of 1 and 3 MHz with 0.5 Watt/cm^2 and 50% duty cycle were used. Cells were transfected in serum free media for 4 hours. Cells treated with polyplex and US 1 Mhz condition showed a significant difference compared to unsonicated cells. Further, cells treated with SAM complexes and sonication showed a significant siRNA uptake difference compared to polyplexes without US (Figure 6B). Thus, US in combination with polyplex and US combined with SAM complexes increased the siRNA uptake in A2780 cells. The observed increase in siRNA uptake can be explained by the sonoporation mechanism. Sonoporation is an increased and reversible porosity of the cell membrane caused by US due to mechanical stress. Further, US in combination with MBs enhances the porosity due to cavitating MBs that increase mechanical stress due to jet streams, shock waves and microstreaming.[24, 26, 27] The increased porosity of the cell membrane allows large molecules to enter the cell, therefore, suggesting a mechanism for the increased siRNA uptake in A2780 cells after treatment with US and SAM complexes.

3.6. VEGF ELISA assay

The previous paragraph described the increased uptake of siRNA into A2780 cells after sonication and SAM treatment, however, we need to identify if the siRNA is still functional after uptake by quantifying protein knockdown. Therefore, US enhanced gene knockdown using SAM complexes and compare them to appropriate controls was investigated using a VEGF ELISA kit. First, SAM complexes were compared to polyplexes with and without sonication (1 MHz, 0.5 Watt/cm^2 and 50% duty, 1 minute) in A2780 cell line. Cells were incubated with complexes for 4 hours in serum free medium after sonication. There was no significant difference between SAM with and without US. SAM treated with US should release the polyplexes from the MBs shell due to jet streams, shock waves, microstreaming and show higher gene knockdown when compared to the control group SAM without US.

However, the cells are incubated with SAM complexes for 4 hours with and without US treatment and the same gene knockdown level can be explained by the slow release of polyplexes from the MBs shell into the cell media due to instability of SAM complexes at 37 °C over 4 hours incubation time. Further, there was a significant protein knockdown between SAM and polyplex groups after sonication that can be described as followed; Polyplexes alone increase siRNA uptake and gene knockdown due to interaction of positively charged ABP with negatively charged cell membranes, which facilitates cellular uptake. Additionally, in the cell, ABP interacts with intracellular glutathione that reduces disulfide bonds in ABP backbone and releases siRNA into the cytosol where siRNA targets the mRNA of the desired gene. The combination of US, MB and polyplexes (SAM) increases the sonoporation mechanism as described above and explains the enhanced VEGF knockdown compared to the polyplex with US group (Figure 7A, Supplement 3A).

Next, short time incubation (10 minutes) after sonication was compared to a longer time incubation (60 minutes) using SAM, polyplexes and SAM with a non-VEGF targeting siRNA (GFP-siRNA) as control with and without sonication. The rationale of this experiment and the use of a short incubation time (10 minutes) was to show the impact of sonication on the SAM complex and to verify polyplex release from the MB shell compared to non-US treated and extended (60 minutes) incubation groups. A short duration transfection of 10 minutes with SAM complexes +US showed a significant difference ($P < 0.05$) in VEGF knockdown compared to SAM 10 minutes –US and a significant difference ($P < 0.001$) compared to negative control SAM (GFP) (Figure 7B, Supplement 2B). Thus, a short time transfection of 10 minutes with SAM complexes after sonication proved the release of polyplexes compared to non-US treated SAM group. Further, SAM 60-minute transfection with and without US showed the same protein knockdown and proved SAM stability at body temperature for at least <10 minutes. More, SAM complexes incubated for 10 minutes post sonication showed higher VEGF knock down when compared to polyplex 60-minute treatment. Thus, SAM plus US is able to decrease necessary transfection time by 6-fold.

Lastly, a short time transfection (10 minutes) using same US conditions as described above was performed using appropriate controls to show the importance and underline the impact of the combination of siRNA, ABP, MBs and US for protein knockdown. SAM complexes were compared to controls MB-siRNA (no ABP), polyplex (ABP-siRNA no MBs), bPEI (25k, gold standard), naked siRNA and all groups were exposed to sonication. As seen in Figure 7C, only SAM complexes were able to show a significant difference ($P < 0.05$) in protein knockdown compared to the naked siRNA control. Further, there was no difference in cell viability between each group (Figure 7D). Increased VEGF knockdown by SAM complexes might be explained by enhanced sonoporation mechanism due to combination of MBs and US. In addition, positive charged polyplexes facilitate the cellular uptake by interaction with negative charged cell membrane. Therefore, only combination of ABP, MBs and US leads to the best siRNA delivery and protein knockdown. Furthermore, the synergistic effects of ABP, MBs and US for siRNA delivery with a short time transfection (<10 minutes) will become useful *in vivo* for both local and systemic injections. Intratumoral (i.t.) injections of naked siRNA show only modest transfection efficacy due to siRNA degradation by nucleases and poor cellular uptake. ABP is able to overcome the above-mentioned obstacles and increase transfection in cancer cells. Further, including MBs and US for i.t. injections might facilitate and increase siRNA delivery due to the sonoporation mechanism as described above. Furthermore, systemically delivered naked siRNA is easily and rapidly degraded by nucleases in the bloodstream, has poor tumor targeting and cellular uptake efficacy. siRNA by systemic delivery using SAM complexes could increase accumulation at the tumor site for the following reasons. MBs combined with focused US at the target site after systemic injection leads to rupture of microvessel around

the target site, therefore, increases extravasation of therapeutic agent at the target site.[28-30] So far, tumor targeting depends on indirect targeting by the EPR effect or direct targeting using specific ligands.[14] Therefore, the combination of ABP, MBs and focused US at the tumor site might increase the extravasation of polyplexes at the tumor site due to microvessel rupture around the tumor and facilitate siRNA delivery into the cell due to the sonoporation mechanism. In addition, ABP will protect siRNA from nuclease degradation and facilitate cellular uptake.

To conclude, we designed a non-viral siRNA gene delivery system using a combination of a bioreducible polymer, MBs with HSA shell, and a PCE gas core for targeting VEGF in a human ovarian cell cancer line. Newly designed MBs with PCE gas core showed higher stability compared to controls. Further, MBs in combination with polyplexes showed a significantly higher loading capacity compared to naked siRNA. Lastly, only SAM complexes in combination with US showed significant VEGF knock down in A2780 human ovarian cancer cell line compared to naked siRNA if incubated for a short time (10 min) after sonication treatment.

Faster transfection and siRNA uptake could be key for an effective cancer treatment *in vivo*. Future studies will involve *in vivo* experiments using local and systemic delivery of SAM. Finally, our gene delivery system showed promising *in vitro* results and may open up new perspectives to overcome obstacles in current gene delivery *in vivo* experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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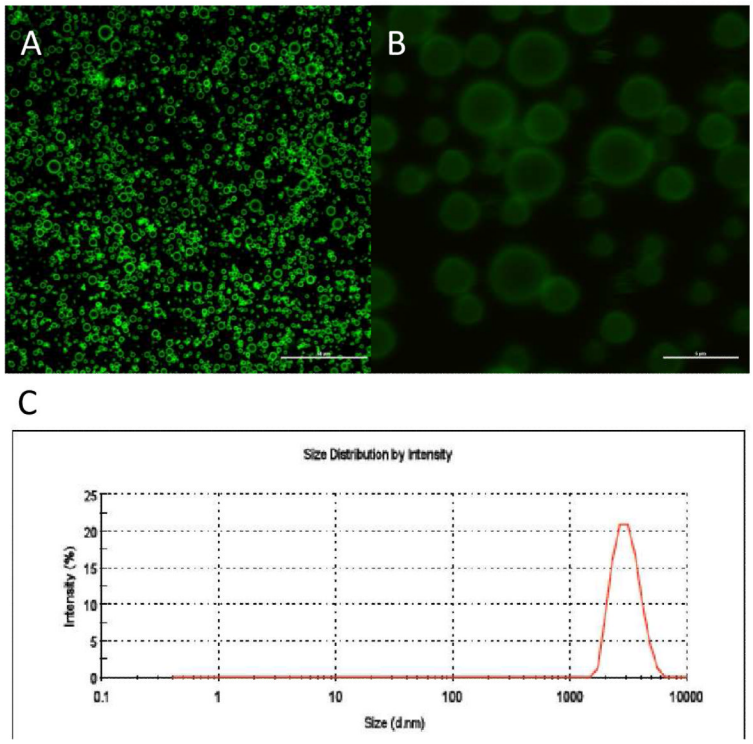


Figure 1.
Fluorescence microscopy of microbubbles and particle size measurement. (A) Overview of microbubbles with FITC-HSA shell (green) scale bar 50 μm. (B) Zoomed area of (A) with scale bar 5 μm. (C) Particle size measurement of microbubbles by dynamic light scattering.

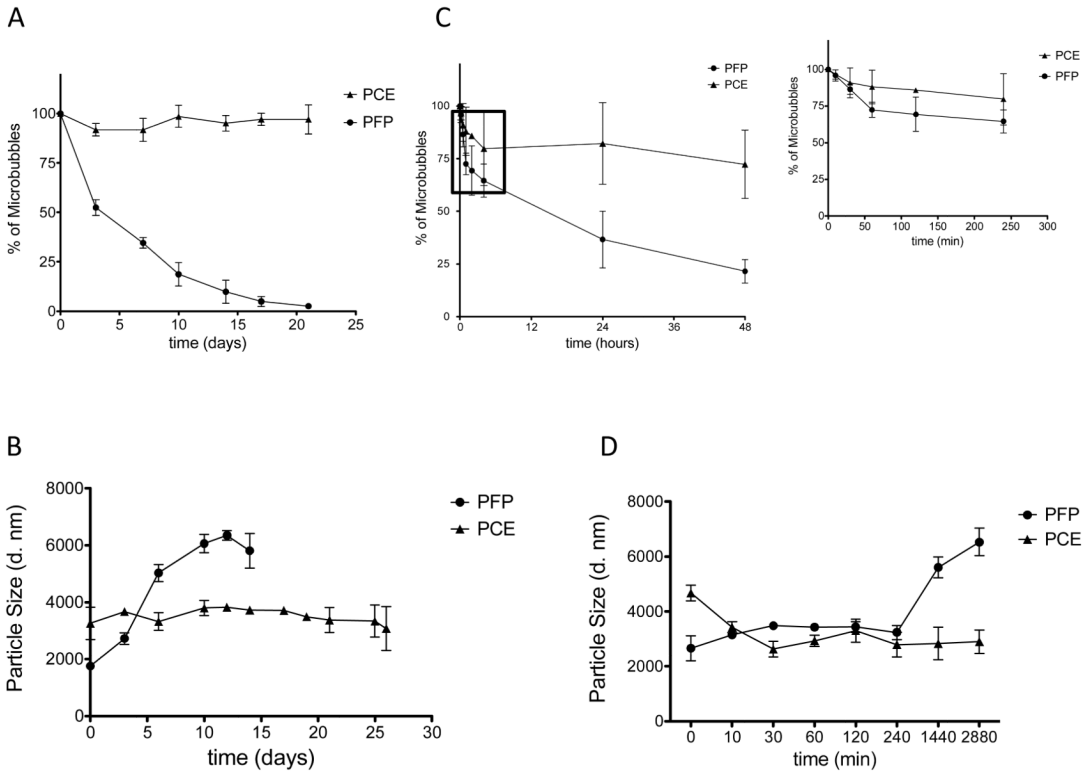


Figure 2.

Concentration and particle size measurements of PFP and PCE MBs at storage temperature (4° C) and body temperature (37° C). (A) Concentration of PFP and PCE after storage in 4° C. (B) Particle size measurement of PFP and PCE after storage in 4° C. (C) Concentration of PFP and PCE after incubation in 37° C (Boxed area represents 0-240 min). (D) Particle size measurement of PFP and PCE after incubation in 37° C. Data represent mean ± SD.

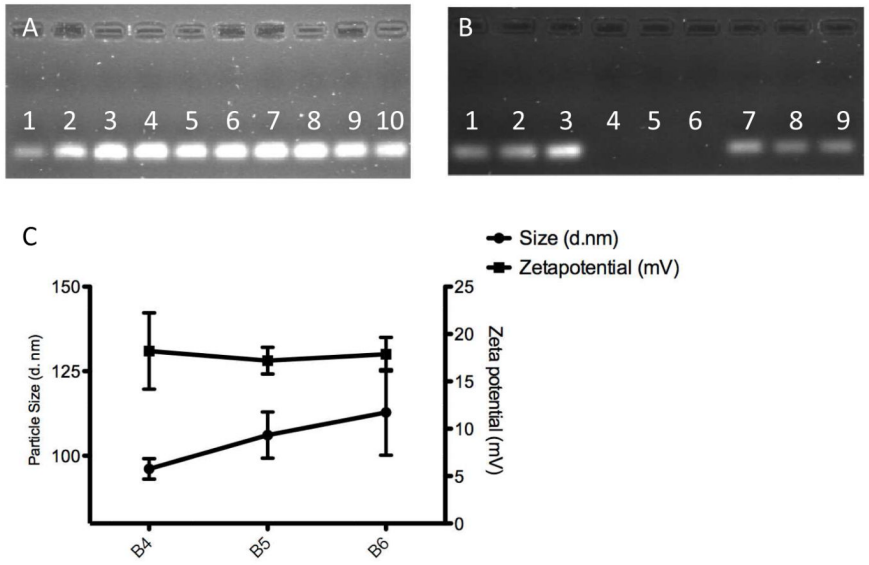


Figure 3.

Gel retardation assay, particle size and zeta potential measurements of siRNA and polyplexes (siRNA-ABP) after US exposure 1 MHz for 10 min. (A) Gel retardation assay of siRNA after US exposure. (1-3) siRNA + 3 Watt/cm^2 100% (1), 50% (2) and 10% (3) duty. (4-6) siRNA + 2 Watt/cm^2 and duty like (1-3). (7-9) siRNA + 1 Watt/cm^2 and duty like (1-3). (10) untreated siRNA. (B) Gel retardation assay of siRNA, polyplexes (siRNA-ABP) after US exposure 1 MHz for 10 min and DTT treatment. (1) untreated siRNA. (2) siRNA + 1 Watt/cm^2, 50% duty and (3) 10% duty. (4) polyplex (siRNA-ABP, 1:10 w/w ratio) untreated. (5) polyplex + 1 Watt/cm^2, 50% duty and (6) 10% duty. (7) polyplex + DTT treatment 2.5 mM for 60 min in 37° C. (8) polyplex + 1 Watt/cm^2, 50% duty, (9) 10% duty and DTT treatment 2.5 mM for 60 min in 37° C. (C) Particle size and zeta potential measurements of (B4) polyplex (siRNA-ABP, 1:10 w/w ratio) untreated, (B5) polyplex + 1 Watt/cm^2, 50% duty and (B6) 10% duty. Data represent mean ± SD.

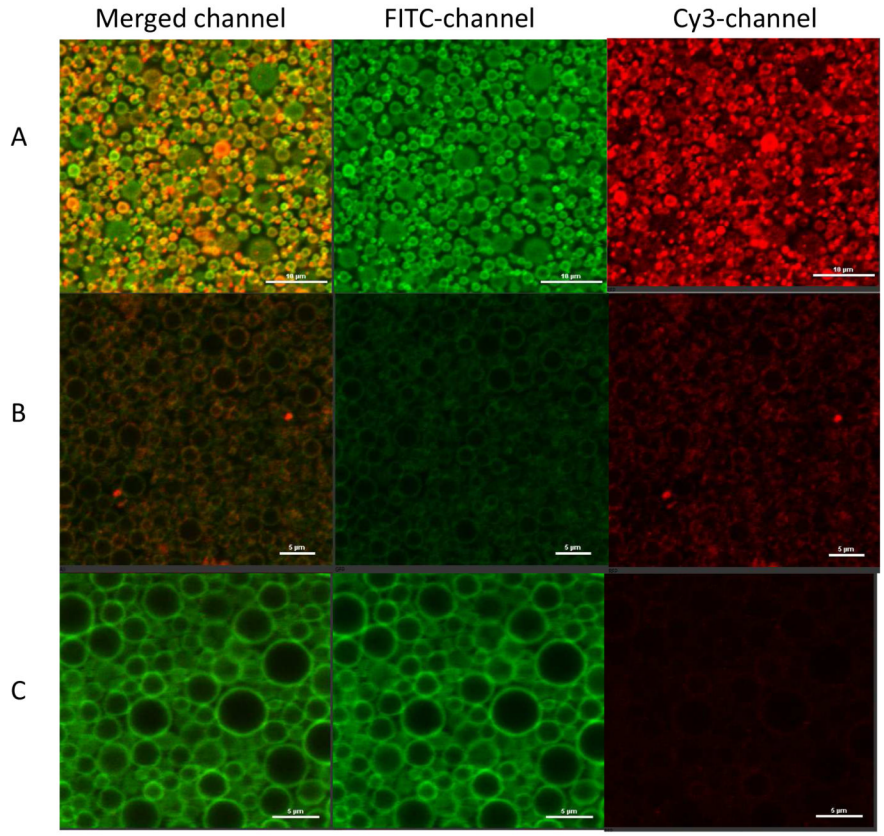


Figure 4.

Fluorescence microscopy of SAM and siRNA-MB complexes. (A) SAM complexes with FITC labeled human serum albumin (HSA) microbubble shell (FITC-channel) and Cy3 labeled polyplexes (siRNA-Cy3-ABP Cy3-channel). (B) SAM complexes with FITC labeled arginine grafted bioreducible polycation (FITC-ABP FITC-channel) and Cy3 labeled siRNA (siRNA-Cy3-ABP, Cy3-channel). (C) siRNA-MB complexes. FITC-HSA microbubble shell (FITC-channel) and Cy3 labeled siRNA (Cy3-channel). Merged channel represents FITC-channel plus Cy3-channel.

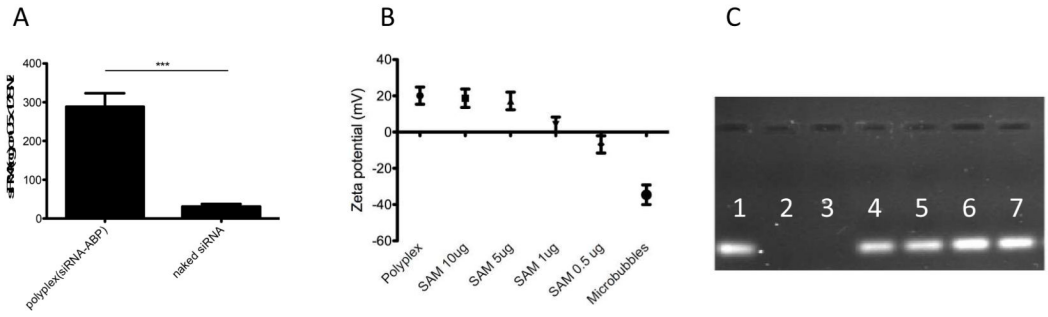


Figure 5.

SAM loading capacity, zeta potential and gel retardation assay. (A) Loading capacity of MB complexed with naked siRNA and polyplexes. 0.5×10^8 MB combined with 500 ng naked siRNA-Cy3 and 500 ng siRNA-Cy3 complexed with ABP in a 10:1 ABP:siRNA w/w ratio. (B) Zeta potential of SAM, microbubbles and polyplex (siRNA-ABP) complexes. Number next to SAM indicates siRNA amount used to form SAM complexes. (C) Gel retardation assay of SAM and siRNA-MB complexes after US exposure 1 MHz for 10 min. (1) siRNA untreated. (2) SAM untreated. (3) SAM + 1 Watt/cm^2, 50% duty. (4) SAM + 1 Watt/cm^2, 50% duty + DTT treatment 2.5 mM for 60 min in 37° C. (5) siRNA-MB untreated. (6) siRNA-MB + 1 Watt/cm^2, 50% duty. (7) SAM no US and + DTT treatment 2.5 mM for 60 min in 37° C. Data represent mean ± SD (N=3) and significance tested ($P < 0.001$) by t-test.

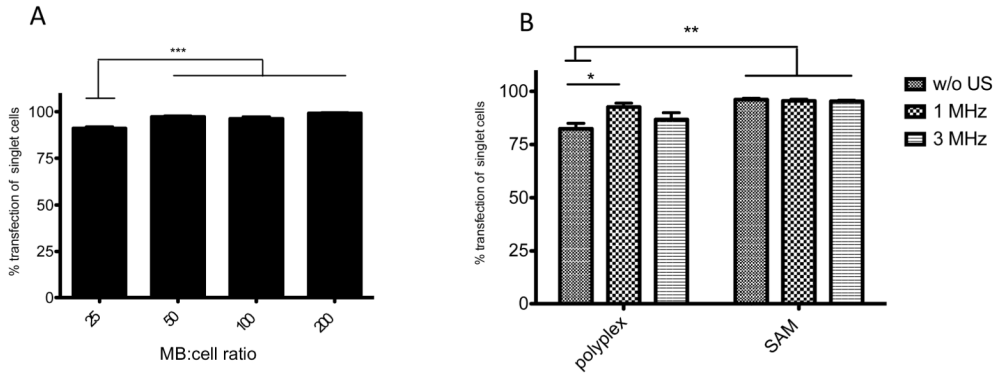


Figure 6.

Cellular uptake of siRNA determined by FACS analysis in A2780 cell line using SAM and polyplexes. (A) Intracellular delivery with 10 nM siRNA in SAM complexes using different MB:cell ratios using 1 MHz US condition with 0.5 Watt/cm^2 and 50% duty. (B) Cellular uptake with 10 nM siRNA complexed in polyplexes or SAM complexes using 1 and 3 MHz US conditions with 0.5 Watt/cm^2 and 50% duty. Data represent mean ± SD (N=3) and significance tested ($P < 0.05$) by one-way ANOVA and Tukey post test.

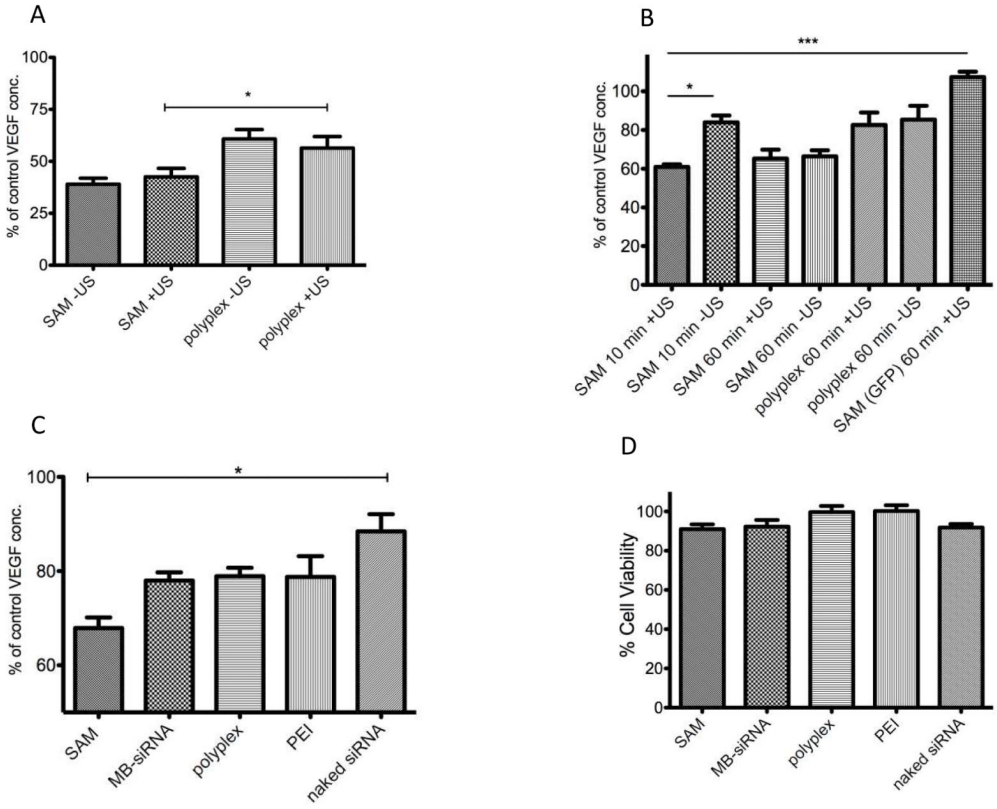
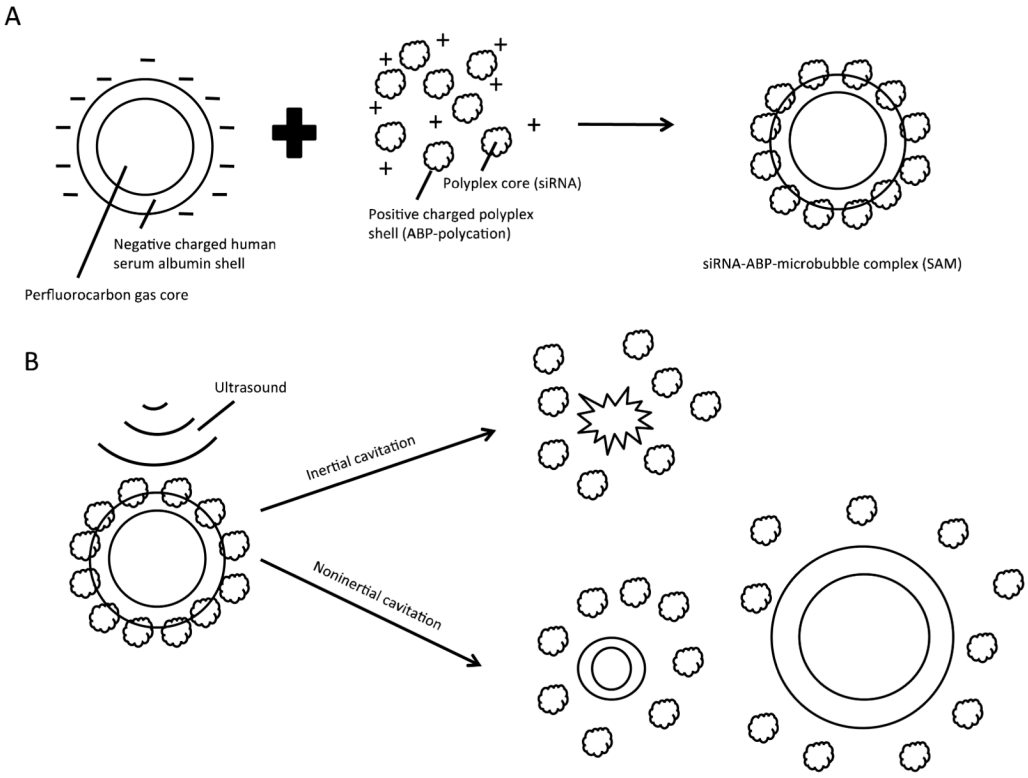


Figure 7.

Transfection efficiency of VEGF-siRNA using SAM and US. VEGF ELISA and MTT assay in A2780 cell line treated with or without US 1 MHz, 0.5 Watt/cm^2 and 50% duty. (A) VEGF concentration after transfection of A2780 cells in serum free media for 4 hours after US exposure with 50 nM siRNA targeting VEGF, complexed in SAM or polyplexes. (B) VEGF concentration after transfection of A2780 cells in serum free media for 10 or 60 minutes after US exposure. SAM (GFP-siRNA) represents a non-VEGF targeting siRNA complexed in SAM. (C) VEGF concentration after transfection of A2780 cells in serum free media for 10 minutes after US exposure with 50 nM siRNA targeting VEGF, complexed in SAM, MB-siRNA, polyplexexs (ABP-siRNA) or PEI (1:1 w/w ratio PEI:siRNA) (D) Cell viability of (C) was determined by MTT assay and expressed as relative cell viability compared to the control. Data represent mean ± SD (N=3) and significance tested ($P < 0.05$) by one-way ANOVA and Tukey post test.



Scheme 1.

siRNA-ABP-Microbubble complexes (SAM) assemble and polyplex release mechanism. (A) Microbubbles consisting of a perfluorocarbon gas core and a negatively charged HSA shell react with positively charged polyplexes (siRNA-ABP bioreducible) due to electrostatic interaction. (B) Exposure to ultrasound leads to SAM inertial or noninertial cavitation depending on the stability of microbubbles. Cavitation and/or bursting process of the microbubble will release the polyplexes from the microbubble shell by producing high enough shear forces, shock waves, jet streams and microstreaming that disrupts electrostatically forces between microbubble and polyplexes.