Covalent binding of molecules to plasma immersion ion implantation-activated microparticles for delivery into cells

Hedi V. Kruse$^{1,2}$ | Clara T. H. Tran$^1$ | Nico van Zandwijk$^3$ | Natalka Suchowerska$^2$ | David R. McKenzie$^{1,2}$ | Glen Reid$^{4,5}$

Delivery of biomolecules to target cells is an essential step in gene therapy, targeted drug delivery, and cell imaging, and can be achieved by encapsulation or conjugation to micro- or nanoparticles. For successful systemic delivery, these complexes must be stable under physiological conditions and prevent leakage of the cargo. Covalent binding of the active agent to a carrier is one-way to facilitate this stability but frequently requires several steps. Here we show that plasma immersion ion implantation (PIII) treatment can activate the surface of paramagnetic polystyrene microparticles (MPs), increasing autofluorescence and allowing conjugation of biological molecules in a single-step. Using PIII-activated MPs, we demonstrate covalent binding of therapeutically relevant payloads including antibodies (Abs) and small-interfering RNAs (siRNAs). The PIII-activated magnetic particles were actively taken up by the cells, were nontoxic, and could be visualized due to increased autofluorescence induced by the PIII treatment. Ab and siRNA were effectively conjugated simultaneously to the active surface, and these functionalized MPs were also taken up by cells. This simple PIII-based activation system has the potential to be applied to many different therapeutic approaches.

KEYWORDS
antibodies, drug delivery, magnetic particles, mesothelioma, plasma immersion ion implantation, small-interfering RNAs

1 INTRODUCTION

Delivery of biomolecules into cells is an essential step in gene therapy, targeted drug delivery, and cell imaging. Biomolecules are frequently associated with nanoparticles or microspheres for delivery to targeted tissues in the body. The particles are not only the vehicle for transporting the biomolecules but also serve to stabilize them during the delivery.

Magnetic particles (MPs) are an attractive choice as carriers for biomolecules as they can be manipulated under a magnetic field. They have been used for magnetic resonance imaging, hyperthermia treatment, drug delivery and gene
therapy, and for magnetic separation for purification and immunoassays.\(^1\)\(^2\) Superparamagnetic iron oxide nanoparticles (SPIONs) have been developed as contrast agents for magnetic resonance imaging (MRI) and several commercial SPIONs have been approved for MRI clinical applications.\(^3\) The delivery of MPs to a tumor was found to enhance hyperthermia treatment effect due to the additional hysteresis heating from an external alternating magnetic field.\(^4\) For drug delivery, magnetic particles carrying drugs administered into an experimental animal can be concentrated around a chosen target area by means of an externally applied magnetic field.\(^5\) The MPs concentrated in this way could potentially increase cellular uptake of released drug without increasing the drug dosage. MPs have already been used as nonviral transfection agents for gene delivery in cancer treatments that aim to upregulate or downregulate gene expression.\(^6\) For example, small-interfering RNA (siRNA) and microRNA (miRNA) delivery by nanoparticles have the potential to silence oncogene expression.\(^7\) As DNA and RNA half-lives are short in the bloodstream, a delivery system is required to deliver them to the tumor target site. MPs have been found to be useful carriers for this purpose due to their ability to be steered an external magnetic field.

A strong attachment of biomolecules to the surface of delivery particles is critical to avoid biomolecule loss and well as retain biomolecule activity during transport. Physical attachment is greatly dependent on the surrounding environment, and thus has low stability. Covalent strategies for binding are therefore preferred for their stability and control of the orientation of the attached molecule. Wet chemical methods for covalent binding that form suitable linker groups on the surface, such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) coupling, maleimide coupling, and click-chemistry reactions have been reviewed.\(^8\) These methods conjugate biomolecules to the particles by crosslinking reactive groups on the biomolecules (carboxylic acid, amine, or sulfhydryl) to reactive groups created on the particle surface. Depending on the strategy, the biomolecules are sometimes modified with functional groups before binding. Disadvantages of linker chemistry include the need for chemicals that could interfere with downstream applications or are unsuitable for sensitive biological systems.

In previous work,\(^9\) we have demonstrated the feasibility of plasma immersion ion implantation (PIII) to create covalent binding sites for enzymes, antibodies (Abs), and oligonucleotides on MPs. We showed that the PIII treatment method overcomes the constraints of linker chemistries for polymer surfaces by creating dangling bonds (unpaired electrons), which react directly with biomolecules upon contact in a buffer solution. The ability of PIII-treated MPs to carry a covalently bound Ab into a cell has not yet been demonstrated. We further explore the potential of PIII-treated MPs for this purpose using a mesothelioma cell line model, paving the way for future siRNA-based treatments.

## 2 | MATERIALS AND METHODS

### 2.1 | Particle composition and preparation for PIII treatment

SPHERO magnetic polystyrene microparticles (MPs) were purchased from Spherotech Inc (PMS-20-10). According to information provided by the manufacturer, the MPs were synthesized by coating a layer of iron oxide mixed with polystyrene onto monodisperse polystyrene core particles. The particles consisted of 10% to 15% iron and had a nominal size of 2.0 to 2.9\(\mu\)m in diameter. The size distribution is claimed by the manufacturer to be uniform. We confirmed using light microscopy that the particles were spherical and monodisperse. The MPs were provided by the manufacturer in aqueous solution and were washed twice with milliQ water and freeze-dried (Christ Alpha 2-4 LDplus) at \(-20^\circ\text{C}, 1.03\text{ mbar}\) for 24 hours to obtain them in dry powder form for the PIII treatment.

### 2.2 | PIII treatment for particle activation

The PIII treatment of MPs has been described in our previous work\(^9\) and is shown schematically in Figure 1. Briefly, MPs (5 mg) were placed at the bottom of a glass tube and evacuated to \(2.7 \times 10^{-4}\text{ mbar}\). The bottom of the glass tube was covered by a copper electrode (Figure 1) while the open end was connected to the pump system through a grounded metal fitting. High-purity nitrogen gas was introduced into the glass tube through a gas reservoir. The glass tube and gas reservoir were then isolated from the pump system by closing the connecting valve. The pressure inside the glass tube was adjusted to 0.4 mbar by adding nitrogen gas through a flow controller. Nitrogen plasma was generated by applying 5 kV negative pulses from a RUP6 pulse generator (GBS Elektronik GmbH, Dresden, Germany) to the copper electrode at a repetition rate of 3 kHz and a pulse length of 40 \(\mu\)s for periods of 2 minutes for a total treatment time of 20 minutes. During a pulse, nitrogen ions formed in the plasma were accelerated towards the high-voltage copper electrode area where the particles
Figure 1  Schematic diagram of the plasma immersion ion implantation treatment of microparticles. Plasma was generated inside the insulated sample container and nitrogen ions were accelerated under high-negative pulses towards the copper electrode area. The particles were suspended inside the copper area, under an external vibrator, while being bombarded by nitrogen ions.

were located and agitated by an external mechanical vibrator. The collisions of energetic ions with the polystyrene on the surface of the MPs create dangling bonds on the surface and in the subsurface which changes the surface property of the MPs after the treatment. The presence of implanted charge on an object positioned inside the electrode has been proven.10 Due to the outgassing and sputtering of materials inside the glass tube within the copper electrode area, the pressure inside glass tube started to increase. After 2 minutes, the power supply was stopped and the glass tube was reconnected to the pump system to evacuate and refreshed with nitrogen gas before the next 2 minutes period treatment. The total treatment time was 20 minutes. After the treatment, MPs were suspended in milliQ water and stored at ambient temperature until usage the same or following day.

2.3 | Immobilization of biomolecules to MPs

2.3.1 | Immobilization of a fluorescent Ab to MPs

For the immobilization of Abs to MPs (either untreated [UT] or PIII-treated), 2 μg of goat anti-mouse IgG Alexa Fluor 594 (ThermoFisher; A-11005) was incubated at room temperature with 1 mg MPs in 200 μL immobilization buffer (acetate buffer pH4, 10 mM) overnight on a rotary suspension mixer at 12 rpm. The particles were then washed three times with 1 mL phosphate-buffered saline (PBS). In order to separate the MPs from the solution, the MP-containing tubes were put into a magnetic tube rack for 1 minute before removing the supernatant. The MPs were then resuspended in fresh buffer outside the magnetic rack. The particles were then washed twice with 1 mL stringent stripping buffer (sodium bicarbonate 25 mM, 5% sodium dodecyl sulfate (SDS), and 2% Tween-20) and analyzed via fluorescence microscopy (Zeiss Axio Imager M2; ZEN2 software, blue edition). UT and PIII-treated MPs incubated in immobilization buffer without Abs were used as negative control and underwent the same washing regime.

2.3.2 | Immobilization of siRNA to MPs

A modified (tailed) siRNA was immobilized to the MPs. The siRNA sequence is specific for the proto-oncogene coding polo-like kinase 1 (PLK1) and causes growth inhibition as described previously.11 The sequence used is the PLK1-specific dicer substrate siRNA shown to have enhanced activity over standard siRNA designs.11 It is modified by the addition of a 20-nucleotide polyA tail to act as a spacer between the particle and siRNA. This is based on our previous work that showed that a polyA tail on an oligonucleotide caused the oligonucleotide to bind to PIII activated MPs via the polyA tail.12 All siRNAs and specific controls were purchased from Integrated DNA Technologies; and sequences are given in Table 1.

Transfection experiments using lipofectamine RNAiMAX showed that growth inhibitory activity of the tailed siRNA was equivalent to the original siRNA (Figure S1). To attach siRNAs to the MPs, 1 mg MPs were incubated overnight in 300 μL acetate buffer (pH4, 10 mM) containing 1.5 μM siRNA. After incubation, the MPs were magnetically separated from the supernatant as described above and washed with a series of buffers with various salt and surfactant concentrations (Table 2, Buffer A–E; 1 mL buffer per wash). Finally, the particles were resuspended in 300 μL ultra-pure RNase free water. Wash supernatants were collected and together with the MPs suspensions were analyzed by agarose gel electrophoresis and reverse transcription real-time polymerase chain reaction (RT-qPCR).
TABLE 1  PLK1-specific siRNAs and controls used in the study

| Name            | Sense sequence          | Antisense sequence         |
|-----------------|-------------------------|----------------------------|
| PLK1-Dice       | CCAUUAACGAGCUGCUAAUGACGA | UCGUCAUUAAGCAGCUGUUAUGGUU  |
| PLK1-Dice-ctrl  | CGUUUAACGUGACGCUAAUGACGA | UCGUCAUUAAGCAGCUGUUAACGCUA20 |
| PLK1-Dice-20A   | CCAUUAACGAGCUGCUAAUGACGA20 | UCGUCAUUAAGCAGCUGUUAUGGUU |
| PLK1-Dice-20A-ctrl | CGUUUAACGUGACGCUAAUGACGA20 | UCGUCAUUAAGCAGCUGUUAACGCUA20 |

“Dice” denotes dicer substrate siRNA; “20A” indicates tailed siRNAs. Sequence in red denotes DNA nucleotides, and A20 is the poly-A tail; bold indicates mutated sequences in control siRNAs.

Abbreviations: PLK1, polo-like kinase 1; siRNA, small-interfering RNA.

| Buffer         | Components                      | pH   |
|----------------|---------------------------------|------|
| Immobilization buffer | Acetate, 10 mM                  | 4.0  |
| A               | PBS (Gibco), 0.1 mg RGD         | 7.1  |
| B               | Water (UltraPure)               | N/A  |
| C               | 2.0× SCC, 0.1% SDS             | 7.7  |
| D               | 0.2× SCC, 0.1% SDS             | 7.1  |
| E               | 0.1× SCC, 0.5% TWEEN 20        | 6.5  |

Abbreviations: PBS, phosphate-buffered saline; SCC, saline-sodium citrate; SDS, sodium dodecyl sulfate.

2.4 Detection of nucleic acids

For gel electrophoresis assays, a 3% agarose gel containing 0.5 μg/mL ethidium bromide was prepared in a sodium borate buffer; and 10 μL of each washing solution or the MP suspension was analyzed. For RT-qPCR, the RT reaction was carried out with MultiScribe reverse transcriptase (ThermoFisher; 4311235) following the manufacturer’s protocol, with a stem-loop RT primer specific for the PLK1 siRNA. The stem-loop RT primer and template were mixed and heated to 70°C for 2 minutes prior to adding the reverse transcriptase master mix. KAPA SYBR FAST Universal (Sigma-Aldrich; KK4602) was used to quantify the cDNA via real-time quantitative PCR which was performed according to the manufacturer’s instructions on a ViiA7 thermocycler (ThermoFisher).

2.5 Cell culture and delivery of MPs into cells

The mesothelioma cell line MSTO-211H (ATCC; CRL-2081) was maintained in RPMI 1640 Medium (ThermoFisher; 11875) supplemented with 10% fetal bovine serum (FBS) (ThermoFisher; 10437028) and kept in 5% CO₂ and 95% relative humidity at 37°C. For MP uptake experiments, MSTO cells and MPs with or without immobilized biomolecules were coseeded at a ratio of 0.2 to 2.4 μg MPs per 1000 cells in media containing 0.75% penicillin/streptomycin (ThermoFisher; 15070063) and 1.5 μg/mL amphotericin B (Sigma-Aldrich; A2942) in plates with different well size, depending on the experiment (Table 3). The cells were then cultured for 1 to 4 days depending on the experiment. Growth inhibition assay was carried out as previously described.11

2.6 Immunofluorescence

For internalization and localization studies of MPs, MSTO cells were coseeded with MPs into 24-well plates containing glass slides. After 24 or 48 hours, the cells were washed twice with PBS and fixed with 4% paraformaldehyde/PBS for 15 minutes. For visualization of intracellular protein LAMP-1 or beta-actin, the cells were permealized with 0.2% Triton X-100 in PBS for 15 minutes followed by three washes with PBS buffer. To avoid nonspecific binding, cells were
incubated with 10% FBS in PBS at 37 °C for 1 hour. For the detection of LAMP1, the cells were incubated with a LAMP1 (D2D11) XP Rabbit mAb (#9091, Cell Signaling) in a 1:200 dilution in 1% FBS/PBS, at 37 °C for 1 hour. For the detection of beta-actin, the cells were incubated with an anti-β-Actin mouse mAb (A-5441, ThermoFisher) in a 1:400 dilution in 1% FBS/PBS, at 37 °C for 1 hour. Unbound Abs were removed by washing the cells three times with PBS. The primary Abs were visualized by incubation with secondary Abs labeled with red (ThermoFisher; A-11005) or green fluorophore (ThermoFisher; A-11008) in a 1:600 dilution in 1% FBS/PBS, at 37 °C for 45 minutes followed by 3× PBS washes. The glass slides were mounted in the presence of ProLong Diamond Antifade Mountant with DAPI (ThermoFisher; P36962).

### RESULTS

#### 3.1 Influence of PIII treatment on MP characteristics

PIII treatment led to several observable differences in the MPs. First, MPs were darker in color after the treatment, changing from bright orange for UT MPs to dark brown for PIII-treated MPs (Figure 2A). This darkening, appearing after PIII treatment, is similar to that occurring on PIII-treated polystyrene film surfaces and is attributed to the formation of a carbonized layer on the polymer surface. Second, we found that the MPs exhibited an increased autofluorescence across the entire detectable fluorescence spectrum after the PIII treatment. It was especially prominent with emission filters for wavelengths of 500 to 550 nm (Figure 2A, upper panel). Third, the treated MPs became more hydrophilic and dispersed well in water even after storage for up to 18 hours, consistent with earlier observations. PIII treatment is known to increase surface energy and hydrophilicity of polystyrene. Our previous intensive x-ray photoelectron spectroscopy and fourier transform infrared spectroscopy analysis showed the presence of unpaired electrons and oxygen containing groups on the surface of PIII-treated polystyrene. Finally, but most important, is the change of MPs surface charge after the PIII treatment. In PBS buffer at physiological pH, the zeta potential of UT and PIII-treated MPs is $-36.3 \pm 2.2$ mV and $-18.1 \pm 0.6$ mV, respectively, while in a low-salt pH4 buffer, they have opposite net charge of $-34.8 \pm 1.1$ mV and $31.7 \pm 0.4$ mV, respectively (Figure 2B). The high surface charge induces interparticle electrostatic repulsion facilitating colloidal stability of monodispersed particles in a suspension. The positive charge of PIII-treated MPs in pH4 buffer is advantageous for attachment of negatively charged biomolecules such as DNA and RNA.

#### 3.2 Covalent binding of fluorescently labeled Abs to PIII-treated MPs

The most important benefit of PIII treatment is the ability to covalently attach biomolecules to the treated surface in mild buffer solution due to the reaction of unpaired electrons. To compare the difference between PIII-treated and UT MPs, we attached fluorescently labeled Abs to the particles and evaluated the signal via fluorescent microscopy from different wash regimes. Figure 3 shows the efficiency of PBS and stripping buffer washes to remove physically bound Abs. Washing MPs with PBS was not sufficient to remove physically associated Abs from either treated or UT MPs (middle panel). We observed that those physically absorbed Abs slowly diffuse from both particle types and created a strong halo around the beads in the preparation over the course of 2 hours. After washing with a stringent stripping buffer (5% SDS, 2% Tween-20), almost all absorbed Abs were removed from the UT MPs. The remaining fluorescence signal on PIII-treated MPs can...
be assigned to covalent attached Abs. The signal was still observable after overnight incubation in stripping buffer at 37 °C (not shown). This strong attachment of Abs is consistent with covalent bonds of biomolecules on PIII-treated MPs reported in previous work.9

3.3 Covalent attachment of a tailed siRNA to PIII-treated MPs

Previously, we demonstrated the attachment of an oligonucleotide to PIII-treated magnetic particles.9 Here we extend the work to describe the immobilization of poly A-tailed siRNAs on PIII-treated MPs. An excess of siRNA molecules was incubated with UT or PIII-treated MPs overnight in a pH4 acetate buffer. This buffer was chosen to ensure that the siRNAs are physically attracted to the positive charged PIII-treated MPs to increase the covalent attachment efficiency. During siRNA incubation with MPs, we observed a critical dispersion pattern of the UT and PIII MPs in solution due to the interaction of particle surface charge and siRNA in low-ionic strength pH4 immobilization buffer. The net charge
of positively charged PIII-treated MPs in the presence of negatively charged siRNA alters towards neutral, resulting in aggregation of those particles due to the lack of electrostatic repulsion (Figure 4A). While having negative surface charge in pH4 buffer, UT MPs have no attachment to siRNA and show no change in dispersion.

A washing regime with a range of buffers with different pH and salt concentrations and detergents was used to remove noncovalently absorbed siRNAs. Gel electrophoresis and RT-qPCR studies of the immobilization buffers, washing buffers, and final particles suspensions were carried out to determine the quantity of siRNA. In gel electrophoretic analysis, we observed that siRNA retention was strongly dependent on the pH of the washing buffer (Figure 4B). As expected from the zeta potential measurement, most of the siRNAs incubated with UT MPs remained in the immobilization buffer, whereas most of the siRNAs were still associated with the PIII-treated MPs (Figure 4B, immobilization buffer lanes). The washing buffers of UT MPs contained almost no siRNA, whereas most of the siRNAs associated with the PIII-treated MPs were washed off with a PBS-based buffer (buffer A). Washing buffer C, which had the highest pH and salt concentration, released another small portion of siRNA from PIII MPs which is visible as a weak band in the gel (Figure 4B).

We used via RT-qPCR to confirm the electrophoresis results (Figure 4C). We detected siRNA in the same washing solutions and particle suspension as were found in the gel analysis, with the relative quantities measured by RT-qPCR reflecting the band intensity differences. The RT-qPCR values show that siRNA was present on the PIII-treated beads after the last washing step whereas there was no siRNA associated with the UT MPs.

### 3.4 Delivery of MPs into cancer cells

It is well known that cancer cells internalize spherical micro- and nanoparticles in vitro. In experiments with mesothelioma cells, we found that PIII treatment did not influence cellular uptake of the MPs. After a 24-hour incubation with UT or PIII-treated MPs almost every particle was found inside a cell, with cells appearing to take up any MPs in close proximity. Inside the cells, the particles accumulated almost exclusively around the nucleus (Figure 5A). No major toxic effects were observed even with particle concentrations that resulted in each cell harboring up to 30 particles.

To study the cellular uptake of Abs attached to PIII-treated MPs, we employed fluorescence and phase contrast microscopy of fixed cells 24 hours after exposure to MPs. As controls, we used UT MPs incubated with Abs and PIII-treated
FIGURE 5  Delivery of antibodies (Abs) into MSTO cells through internalization of microparticles (MPs). A, Cellular uptake and distribution of plasma immersion ion implantation (PIII)-treated MPs in MSTO-H221 cells 48 hours after coseeding with MPs (0.2 μg/1000 cells). Scale bar: 40 μm. B, Phase contrast and fluorescent microscopy analysis of cells that internalized PIII-treated (PIII) MPs with immobilized AlexaFluor 594 Abs after stripping (upper panel), untreated (UT) MPs with immobilized AlexaFluor 594 Abs after stripping (middle panel), and treated MPs without Abs (lower panel). MSTO-H221 cells were fixed and stained 48 hours after transfection with MPs (0.02 μg/1000 cells). DAPI dye was used to visualize nuclei (blue). Scale bar: 10 μm

MPs without Ab. The controls (Figure 5B; second and third rows, first and last columns) show very dim fluorescence compared to the bright red signal of PIII-treated MPs immobilized with Abs (Figure 5B; first row, first and last columns). These images confirm that it is possible to attach biomolecules to PIII-activated MPs and that these are taken up into cells. It is interesting to observe once again that the internalized particles accumulated around the nucleus (Figure 5B, merge column). This phenomenon could be useful for other applications such as radiotherapy or hyperthermia as the particles could transfer energy efficiently to the nucleus.

Although the active siRNA (see Figure S1) was successfully immobilized on PIII-treated MPs and taken up by cancer cells, we did not observe siRNA-mediated target knockdown or growth inhibition (data not shown). We investigated the intracellular fate of internalized MPs into cells by immunostaining for LAMP-1, a well-known protein marker for the lysosomal compartment.17 This showed that the majority of MPs colocalized with the LAMP-1 (Figure 6), suggesting that...
the internalized MPs followed an endocytosis pathway and were sorted into the lysosome or lysosome-like compartment for degradation.

4 | DISCUSSION

The development of micro- and nanoparticles capable of forming stable associations with biomolecules is a crucial step for a safe systemic delivery of agents to a targeted tissue. Here we show that PIII treatment offers a simple way to facilitate covalent binding of biological agents onto the surface of magnetic microspheres. Our results indicate that PIII treatment increases surface wettability and hence, facilitates a better dispersion of the particles in a buffer solution. The PIII-treated MPs also exhibited increased autofluorescence, which is a strong indicator for the effect of ion implantation on the MP surface and is useful for evaluating the effectiveness of PIII treatment.

PIII activation alters the surface charge of the particles from negative to positive in a pH 4 buffer. The positive charge facilitates the conjugation of negatively charged molecules such as nucleic acids by attracting the molecules into close proximity to the particle. Based on the results of gel electrophoresis, RT-qPCR, and zeta potential measurements, a model for the interaction between UT and PIII-treated MPs with siRNA molecules in incubation buffer is proposed as shown in Figure 7. In a pH 4 buffer, free siRNA molecules in solution are attracted to the PIII-treated MPs and some of them contact the PIII-treated surface where they are tethered by reacting with free radicals, forming a monolayer of covalently bound molecules. The covalent binding is confirmed by the detection of siRNA on the PIII-treated MPs, after several washing steps, with strong ionic detergents containing buffers that remove physically adsorbed molecules. The remainder of the siRNA molecules, although not covalently linked, is strongly held in association with the particle by electrostatic interactions. When the pH of the buffer solution changes, the surface charge of the MPs also changes, causing a release of a large portion of the electrostatically retained nucleic acids. The siRNA is then detected in the washing buffers (Figure 4C). In contrast, the negative charge of the UT MPs repels siRNA, resulting in most of the siRNA molecules remaining in the incubation buffer. The ability to scavenge RNA and DNA from solution in an acidified medium at pH 4 and then release it simply by raising the pH to physiological levels leads to applications of the PIII-treated particles in the processing of nucleic acids. For example, it could be used for RNA or DNA extraction, enrichment, or purification. In this regard, the manufacturer hints at the possibility of using the SPHERO particles for DNA extraction but does not provide a protocol.18,19 The PIII-treated SPHERO particles, we describe, could be used for this purpose. The integrity of the nucleic acids is assured as only mild buffers containing no harsh chemicals that could cause damage are used.

**FIGURE 7** Interaction of small-interfering RNA (siRNA) to microparticles (MPs) in pH 4 buffer solution. A, Negatively charged untreated MPs repel siRNA molecules. B, Positively charged plasma immersion ion implantation (PIII)-treated MPs attract siRNA. Some siRNA molecules covalently attach to the MPs while some are not covalently bound but are strongly attracted to the MPs by electrostatic forces.
The dry, one-step PIII process offers a simpler and more straightforward solution for conjugating biomolecules to polymer-coated MPs compared to the multi-step time consuming wet chemical procedures reported in Reference 20,21. The covalent nature of the bonds between the surface of the PIII-treated particles and the Ab is proven from the strong fluorescence signal of the labeled Ab after stringent washing while the Abs are removed from the UT MPs (Figure 3). Functionality and orientation of the Abs on the surface have not been studied here. However, it is expected that the Abs remain native, as previous work showed that immobilized horseradish peroxidase remains functional, indicating that the radicals tether the protein without denaturing it. The more hydrophilic surface of the PIII-treated MPs also helps to retain native protein confirmation.9 Radical binding is also not specific and may occur at different sites of the Ab leaving the antigen binding site exposed. Future studies will investigate target binding abilities of the immobilized Abs. Additional advantages of the low-temperature PIII process are the inherent particle sterilization22 and the ability to attach multiple biomolecules simultaneously.9

For targeted drug delivery and gene silencing, complex particle vehicles are needed to overcome extracellular and intracellular barriers. While smaller nanoparticles have been the preferred size for approaches such as gene therapy, there are examples of larger nanoparticles (400 nm) and MPs (>1 μm) being used.23-25 For example, our previous preclinical work24 and clinical trial in mesothelioma patients25 used bacterial minicells of 400 nm in diameter to deliver miRNA mimics to patients. Moreover, there are some advantages of using particles in the low micrometer range, especially, where localization of the therapy is desired.26 For example, injection of MPs into the pleural cavity has been found to be effective.27 While particles of smaller size may in general lead to more efficient delivery (depending on target organ), MPs in the 1 to 150 μm range have been widely studied for drug and DNA-based therapeutics.23

Even with the use of nanoparticles as a delivery vehicle, there remain many unsolved problems for in vivo delivery of biomolecular cargoes.28 First, one or more targeting moieties must be immobilized to the particle to facilitate cell surface binding.29 For example, Abs that bind to cell surface receptors such as epidermal growth factor receptor30 or substrates such as folate that bind to overexpressed folate receptors31 are critical to selectively target a range of cancer cells. Second, the attachment of a lysosomal releasing agent onto the vehicle such as cationic lipid or cationic polymer32 is required to help the particles to escape the endolysosomal compartments after uptake. Finally, an effector molecule that ensures the desired cellular response e.g. cytotoxic drugs, nucleic acids, and enzymes are to be attached and delivered. As it is able to bind many types of molecules simultaneously, PIII treatment has the potential to be further developed as a simple to use vehicle for targeted delivery. Another major advantage of using magnetic particles as a vehicle is the ability to track the particles throughout the body by magnetic particle imaging (MPI), an emerging high-resolution imaging technique. MPI using targeted magnetic particles could be used to detect tumors and metastases as well as monitor drug delivery to the malignancies.

The fluorescence signal from labeled Abs provides evidence for the delivery of covalently attached biomolecules on the PIII-treated MPs (Figure 5B). We infer that the covalently attached siRNA molecules on PIII-treated MPs were delivered into the MSTO cells; however, no gene silencing was observed. Figure 6 provides a clear evidence that the siRNA cargo is physically and continuously separated from the cytoplasmic target mRNA following endocytosis and lysosomal fusion, making them unable to enter the gene silencing pathway. Once the MPs reach the lysosome, the siRNA would be degraded by lysosomal RNases. Future studies will focus on strategies to promote the escape of the cargo from the lysosome. Possible endosomal releasing agents that could be coimmobilized with siRNA are the so-called “proton sponges” such as polyethylenimine, chloroquine,33 or verteporfin.34 Verteporfin has the advantage that the release mechanism is triggered by irradiation with red light to produce reactive oxygen species, which disrupts lysosome membranes.34,35

5 | CONCLUSION

This study has shown that PIII treatment activates the surfaces of polystyrene-coated MPs, increasing surface wettability, modifying surface charge, and generating an autofluorescent signal. The treated MPs are able to attract and associate with siRNA molecules, effectively scavenging them from solution under pH conditions which render them positively charged. Some of the associated molecules covalently bind to the MPs, allowing a cargo of biomolecules to bound strongly to the surface of the MPs in a single step and then effectively transported into cells. We have demonstrated the efficient delivery of the MPs and their cargo inside mesothelioma cells, where the particles and the molecular cargo were found to accumulate around the nucleus. Our results suggest that PIII-treated MPs will be useful in many applications such as DNA/RNA purification and therapeutic molecule delivery. The accumulation of the particles around the nucleus may also prove useful in the radio-sensitization of cells during radiation therapy. Future work will focus on surface immobilization of multiple agents for complex functions such as targeting, endosomal escape, drug delivery, and gene silencing.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Hedi Kruse, Data curation-Lead, Formal analysis-Lead, Investigation-Lead, Methodology-Equal, Writing-original draft-Lead, Writing-review & editing-Equal. Clara Tran, Investigation-Supporting, Methodology-Equal, Writing-original draft-Supporting, Writing-review & editing-Supporting. Nico Van Zandwijk, Conceptualization-Equal, Resources-Equal, Writing-review & editing-Supporting. Natalka Suchowerska, Conceptualization-Supporting, Funding acquisition-Supporting, Resources-Equal, Writing-review & editing-Supporting. David McKenzie, Conceptualization-Equal, Funding acquisition-Lead, Methodology-Supporting, Project administration-Lead, Resources-Equal, Writing-original draft-Supporting, Writing-review & editing-Equal. Glen Reid, Conceptualization-Equal, Funding acquisition-Equal, Methodology-Supporting, Resources-Equal, Writing-original draft-Supporting, Writing-review & editing-Equal.

STATEMENT OF ETHICAL CONDUCT
The authors declare that relevant protocols and procedures for the ethical conduct of research and guidelines for working with commercially sourced human cell lines have been complied with.

ORCID
Hedi V. Kruse https://orcid.org/0000-0002-6662-7549

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