Formation of Platinum Nanocrystals on Silicon Nanotubes and Corresponding Anti-Cancer Activity in Vitro

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ABSTRACT: Biodegradable porous silicon nanotubes (pSiNTs), functionalized with primary amine moieties via the use of 3-aminopropyltriethoxysilane (APTES), is demonstrated as a template for formation of platinum nanocrystals (Pt NCs) (1–3 nm). Transmission electron microscopy—energy dispersive X-ray analysis (TEM–EDX) indicates a relatively high and tunable concentration of Pt uniformly immobilized on a given nanotube (wt % Pt: 20–60%). In vitro viability and cellular uptake studies are consistent with a time-dependent toxicity of Pt NCs-pSiNTs against HeLa cells that is influenced by the degradation kinetics of the pSiNTs; internalization of the composites inside the cells exerts cellular damage in an apoptotic manner, therefore suggesting promising future applications in anticancer treatments.

Keywords: porous silicon, nanotube, platinum nanocrystals, anticancer, nanomedicine

1. INTRODUCTION

To achieve enhanced therapeutic effects, drug delivery using nanoparticle-based formulations has been extensively investigated for decades.1−3 While nanoparticles (NPs) are constructed to attain high drug loading as well as to improve bioavailability of drug molecules, biocompatibility of the vectors themselves is also imperative.2,4 An established example is the biodegradable polymer, poly(lactic-co-glycolic acid) (PLGA), which has been one of the most attractive drug carriers at present due to its ability to degrade over time into glycolic and lactic acids which are metabolized and excreted from the body.1 While various organic NPs have been studied in the delivery of multiple therapeutic organic molecules (e.g., anticancer agents such as camptothecin (CPT) and doxorubicin) to date, selected designs of inorganic NPs have also achieved considerable success.2,6,7 For nanostructures utilized as efficient drug delivery carriers, porous inorganic materials have received significant attention owing to their high surface area compared to nonporous counterparts, thereby permitting incorporation of high drug payloads, along with straightforward routes to surface functionalization (facilitating targeting and release specificity).8−10

Among possible candidates for biomedically relevant applications, nanostructured porous silicon (pSi) is especially noteworthy since its biocompatibility was first demonstrated in the mid 1990s.11−13 One of the advantages of pSi is its biodegradability in aqueous environments into biocompatible orthosilicic acid [Si(OH)4], which can be readily excreted from the body in a nontoxic manner.14,15 Recently, fabrication of a unique one-dimensional form of pSi has been demonstrated, namely nanotubes, with a relatively well-defined tunable structure (shell thickness, length, diameter, and porous morphology) via a sacrificial ZnO nanowire (NWs) template.16 Such silicon nanotubes with a porous morphology (pSiNTs) readily resorb in buffered aqueous media at physiological conditions in a similar manner to bioactive nanostructured porous silicon, thereby enhancing potential therapeutic applications of this material.16 Taking advantage of high surface area and diversity of surface functionalities of pSiNTs, this material can also readily serve as a versatile template for synthesis of a secondary nanostructure component in order to ideally transform into multifunctional nanomaterials.

With regard to possible target therapeutic functions, small-sized platinum nanocrystals (Pt NCs) (1–3 nm) with intrinsic anticancer activity have recently been recognized.17−19 It has been proposed that (surface) Pt0 metallic atoms are readily oxidized into (leached) Pt2+ within a cellular environment, thereby exerting cytotoxicity via the induction of DNA damage.18 In order to achieve high efficacy, aggregation of Pt NCs must be minimized, and targeted delivery also needs to be considered.19 To address those issues, recent reports have described multiple strategies such as encapsulation of Pt NCs within a pH-responsive polymeric matrix functionalized with targeting ligand or coating the NCs with peptides which are selectively toxic to cancer cells.17,19 In both strategies, Pt NCs exhibit high toxicity toward cancer cells and were proposed as
promising alternatives to conventional Pt-based drugs such as cisplatin.17,19

In the work presented herein, we describe fabrication of a novel nanohybrid composed of a uniform distribution of Pt NCs formed on pSiNTs that have been surface-functionalized with 3-aminopropyltriethoxysilane without addition of an extra reducing agent. We also specifically address here time-dependent cytotoxicity of the composites upon exposure to cervical HeLa cancer cells and accompanying cellular uptake studies, revealing a mechanism that relies on time-dependent resorption of the nanotube carrier to mediate cytotoxicity of the nano-Pt species, a rather novel delivery design in nanomedicine.

2. EXPERIMENTAL SECTION

2.1. Materials. The following chemicals were all used as obtained: from Mallinkrodt: zinc acetate Zn(CH3COO)2·2H2O; from Anresco: sodium hydroxide NaOH; from Pharmco: methanol MeOH; ethanol EtOH, toluene; from Praxair: 0.5% silane SiH4 in helium He (g); He, UHP grade (g) from Gelset: 3-aminopropyltriethoxysilane APTES; from Sigma-Aldrich: zinc nitrate hexahydrate Zn(NO3)2·6H2O, hexamethylenetetramine HMTA, ammonium chloride NH4Cl; potassium tetrachloroplatinate K2PtCl4, diethylamine; from Biotium: nickel(II) chloride hexahydrate NiCl2·6H2O, tetraethyl orthosilicate TEOS, Dulbecco’s modified Eagle’s medium (and related components) DMEM; from Acros Organic: hydrazine hydrate (100%); from Matheson, Coleman & Bell: potassium hexachloroplatinate (K2PtCl6); from Fisher Scientific: sodium borohydride NaBH4; from Promega: CellTiter Glo, Caspase 3/7 Glo, from MTI Corporation, Fluorine doped tin oxide (FTO) glass.

2.2. Cell Culture. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (88 U/mL), streptomycin (88 μg/mL), glutamine (0.88 mM) and 1/100 minimum essential medium (MEM) nonessential amino acids (Sigma Life Science). Cells were maintained at 37 °C with 5% CO2 in air, in a humidified incubator.

2.3. Instrumentation. Transmission electron microscope (TEM) imaging and energy dispersive X-ray analysis (TEM-EDX) were performed using a JEOL JEM-2100 electron microscope equipped with a GENESIS XM2 imaging EDX spectrometer. The EDX analysis method employed uses a pure element intensity factor (PEIF) to calculate the k-ratio, which is then corrected by ZAF to determine the composition of a sample. All EDX spectra were consistently acquired at 200 kV, 80 000 magnification, and a ~700 nm × 700 nm scanning area. Zeta potential analyses were performed using a ZetaPALS Zeta Potential Analyzer (Brookhaven), Luminescence measurements were acquired with a BMG Labtech FLUOSTar Omega fluorosence/lymencence plate reader; cell imaging was obtained using a Zeiss Axioplan 2 microscope (in brightfield mode), sonication was done using Branson Sonication; unmodified (U) pSiNTs and unmodified (U) pSiNTs were sonicated in deionized water (pH ~ 6.0) for 30 min, and zeta potential measurements were performed at room temperature.

2.4. Porous Silicon Nanotube Synthesis. pSiNTs were fabricated via a ZnO sacrificial template method.20 For synthesis of the ZnO NWs core, ZnO nanocrystals ( seeds) were prepared by stirring a mixture of Zn(CH3COO)2 (12.5 mL, 0.01 M), MeOH (25 mL), and NaOH (6 mL, 0.03 M) for 2 h at 60 °C. FTO glass substrates were washed in 10% HCl and sonicated in acetone. ZnO seeds were deposited on the glass substrates by spin coating method: the substrate surface was coated with ZnO seed solution for 25 s and was spun for 20s at 3000 rpm; this process was done twice. Then the substrates were annealed in air for 40 min at 260 °C; after samples were cooled down to room temperature, the spin coating and annealing processes described above were repeated one more time. FTO glass substrates precoated with ZnO seeds were incubated in a solution of HMTA (50 mL, 0.08 M) and Zn(NO3)2·6H2O (50 mL, 0.08 M) for 1.5 h at 95 °C. The substrates with ZnO NWs were rinsed with DI H2O and dried in air.

Chemical vapor deposition (CVD) using silane (0.5% SiH4 in He) (200 sccm) diluted further in pure (UHP) He gas (250 sccm) was performed to deposit Si on ZnO NWs. To obtain a thin porous Si sidewall morphology (10–12 nm), the samples were heated in SiH4 for 5 min at 540 °C. Finally, removal of the ZnO NW core was done by etching with NH4Cl at 500 °C for 2 h.

2.5. Functionalization of pSiNTs with 3-Aminopropyltriethoxysilane (APTES) and Zeta Potential Characterization. pSiNTs (still attached to the substrates) were immersed in 2% APTES (v/s) solution (toluene solvent) for 4 h at room temperature. The samples were then washed with toluene and acetone. For zeta potential analysis, unmodified (U) pSiNTs and APTES-pSiNTs were sonicated in deionized water (pH ~ 6.0) for 30 min, and zeta potential measurements were performed at room temperature.

2.6. Formation of Pt NCs on pSiNTs. In a typical reaction, APTES-functionalized pSiNTs (again attached to the substrate) were incubated in 2 mL of varying K2PtCl4 concentrations (0.5 mM, 1.5 mM, and 3.3 mM) for either 4 or 24 h at room temperature. Then the samples were washed with DI H2O, EtOH and dried in air.

2.7. Synthesis of Platinum Nanoparticles. As a control, small-sized blue-staining Pt NPs were synthesized using a protocol adapted from Wu’s method.20 In particular, K2PtCl6 (1 mL, 8 mM) and sodium citrate (0.5 mL, 40 mM) were added in DI H2O (18.5 mL), and the solution was stirred vigorously at room temperature for 30 min. Then NaBH4 (100 μL, 50 mM) was added dropwise into the above solution, and the resulting solution was continuously stirred for 1 h at room temperature. Pt NPs were characterized by TEM imaging and TEM-EDX.

2.8. Synthesis of Silica Nanotubes and Formation of Pt NCs on the Nanotubes. As an additional compositional control, silica nanotubes (SiO2 NTs) were synthesized using the procedure developed by Gao et al.21 In this method, silica coating on nickel-hydrargne nanorod core was performed via a sol–gel process, and the core was removed in an aqueous-phase HCl etching step. SiO2 NTs were calcined to remove organic residues and then functionalized in 2% APTES solution. Unmodified SiO2 NTs or APTES-functionalized SiO2 NTs were incubated in K2PtCl4 (2 mL, 1.5 mM) for 24 h. The product was centrifuged, washed with deionized water, and finally dried with ethanol.

The number of amino groups of APTES molecules grafted on SiO2 NTs was quantified by a Kaiser test, which was adapted from Soto-Cantu’s procedure with some minor modifications.22 In this method, a ninhydrin solution [0.35% (w/v)] was prepared in ethanol. Functionalized SiO2 NTs (2 mg in 0.5 mL deionized water) was mixed with EtOH (3.5 mL). The sample was briefly sonicated in an ice bath for 10 s, and ninhydrin solution (1 mL) was added into the vial. The mixture was heated in an oil bath at 65 °C for 30 min. After the sample was cooled down for 15 min to room temperature, it was centrifuged, and the absorbance of the supernatant was measured at 574 nm. To construct a calibration curve, a glycine stock solution prepared in deionized water was utilized. Under a standard set of nanoparticle fabrication conditions (length = 200 nm; inner diameter = 20 nm; Shell = 7 nm), these results yield a value of (2.73 ± 0.89) × 1016 NH2 moieties/nanotube, or with the mean structural parameters of a given nanotube experiment, 0.767 ± 0.025 NH2 groups/nm2.

2.9. Evaluation of Cell viability with Pt NCs-pSiNTs. HeLa cells were seeded at a density of 2000 cells/well in a 96-well plate, and were incubated at 37 °C, 5% CO2 for 24 h. The next day, cells were treated with Pt NCs-pSiNTs, which were dispersed in complete medium, and the final concentrations of Pt NCs-pSiNTs were 35 μg/mL and 50 μg/mL. Cell viability was measured after 48 h, 72 h, and 96 h at 37 °C, 5% CO2. To assess cell viability, CellTiter Glo assays were performed. Specifically, after incubation, the medium was removed, and cells were washed with PBS (100 μL) to remove any excess nanoparticles. Fresh complete medium (50 μL) followed by

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CellTiter-Glo reagent (50 μL) were added into each well. The sample was mixed using an orbital shaker and incubated at room temperature for 5 and 10 min, respectively. The samples were transferred into a black 96-well plate, and luminescence was recorded. For the control, HeLa cells were treated with unmodified pSiNTs at the following concentrations: 17.5 μg/mL, 25 μg/mL, and 35 μg/mL.

2.10. Evaluation of Cell Viability with Pt NPs and K₂PtCl₄. HeLa cells were seeded and incubated in the same conditions as described above. After 24 h, cells were treated with different concentrations of Pt NPs or K₂PtCl₄ (10 μg/mL, 17.5 μg/mL, 25 μg/mL, and 35 μg/mL). Cell viability after 48 h, 72 h, and 96 h were evaluated using CellTiter-Glo assays.

2.11. Evaluation of Caspase 3/7 Activity in Cells Treated with Pt NCs-pSiNTs. HeLa cells were seeded and treated with Pt NCs-pSiNTs as described in viability assays in Section 2.9. Caspase 3/7 Glo assays were performed to evaluate caspase 3/7 activity in HeLa cells. After incubation, the medium was removed, and cells were washed with PBS (100 μL) to remove any excess nanoparticles. Fresh complete medium (50 μL) and Caspase 3/7 Glo reagent (50 μL) was added into each well, and the sample was briefly mixed using an orbital shaker for 30 s. Then the plate was incubated at room temperature for 1 h and covered with aluminum foil to be protected from light. The samples were transferred into a white 96-well plate, and luminescence was recorded. For the control, HeLa cells were treated with unmodified pSiNTs.

2.12. Tracking Degradation of Pt NCs-pSiNTs Composites in the Growth Medium. The medium obtained from cell samples after incubation with Pt NCs-pSiNTs was saved, centrifuged (5 min, 10,000 rpm) and washed with DI H₂O to collect the remaining Pt NCs-pSiNTs. The samples were transferred into a white 96-well plate, and luminescence was recorded. It should be noted that caspase activity was normalized based on the cell number determined from cell viability. For the control, HeLa cells were treated with unmodified pSiNTs at the following concentrations: 25 μg/mL and 35 μg/mL.

2.13. Characterization of Cellular Uptake of Pt NCs-pSiNTs Composites. Cellular uptake of Pt NCs-pSiNTs composites was evaluated by two methods: nonfluorescent labeling and fluorescent labeling.

2.13.1. Nonfluorescent Labeling Method. Cells were seeded at a density of 1 × 10⁴ cells/well in a 24-well plate and incubated at 37 °C with 5% CO₂ in air, in a humidified incubator for 24 h prior to treatment. Next day, cells were treated with Pt NCs-pSiNTs (35 μg/mL), and were incubated for 24 h, 48 h, and 72 h. After incubation, the medium was removed and washed with PBS (1 mL) to remove excess composites, fresh complete medium (500 μL) was added into the well, and cells were observed using visible light (brightfield mode) microscopy.

2.13.2. Fluorescent Labeling Method. Prior to cell studies, Pt NCs-pSiNTs were labeled with Alexa Fluor 594 by incubating the composites, which remained attached to the substrate, in a solution of excess Alexa Fluor 594 overnight at 4 °C. Samples were washed with DI H₂O and stored at 4 °C for cell studies.

Cells were seeded at a density of 2 × 10⁴ cells/mL on a coverslip placed in a well of a 6-well plate for 30 min, and complete medium (1 mL) was added into the well. Cells were incubated and treated with Pt NCs-pSiNTs in a similar manner described previously in Section 2.9. After incubation, CellTracker Green BODIPY dye (0.5 mL, 10 μM) was added into each well and incubated at 37 °C for 30 min. After incubation, cells were washed with PBS 3 times to remove excess dye. The coverslip was mounted on a glass slide, and cells were observed using a confocal fluorescence microscope. In conjunction to staining cells with Green BODIPY, cell samples without Green BODIPY dye labeling were also analyzed.

3. RESULTS

3.1. Characterization of pSiNTs and APTES-Functionalized pSiNTs. The structure of pSiNTs prepared by the ZnO sacrificial template method can be sensitively adjusted by modulating ZnO growth and Si deposition conditions. Particularly in our studies presented here, by restricting growth time of ZnO NWs to 1.5 h, pSiNT length was limited to less than 1 μm, which is optimal for efficient cellular uptake. Under this condition, inner diameter of the resulting pSiNTs ranged from 35 to 50 nm. In addition, sidewall structure of pSiNTs is strongly dependent on deposition conditions. By limiting deposition temperature to 540 °C and reaction time to 5 min, Ostwald-type coalescence of Si into Si islands occurred, thereby producing a thin Si sidewall that creates a uniform porous morphology (10–12 nm thickness) (Figure 1A and B).

Prior to synthesis of Pt NCs-pSiNTs composites, the pSiNT surface was first functionalized with APTES. These functionalized pSiNTs were characterized using zeta potential analysis, which evaluates charges of the surface functional groups. For the unmodified pSiNTs, since hydrogen ions tend to dissociate from the surface silanol group at pH ~ 2, the particle surface was negatively charged (ζ = −20.09 ± 0.56 mV) at the operating pH (~5.5–6). In contrast, functionalized pSiNTs possessed a positively charged surface (ζ = +24.03 ± 0.53 mV) due to the tendency of the amino group to protonate at pH values lower than 9.6, thereby suggesting successful APTES functionalization. In terms of structural changes, wall thickness of the functionalized pSiNTs was slightly thicker (2–3 nm) than unmodified pSiNTs probably as the results of formation of a thin APTES-derived silica layer (Supporting Information (SI) Figures S1 and S2).

3.2. Formation of Pt NCs on pSiNTs. From TEM and TEM-EDX characterization, incubation of functionalized pSiNTs in K₂PtCl₄ yielded uniform coverage of Pt species.
(black spots) with sizes ranging from 1 to 3 nm (Figure 2A and SI Figure S3). High resolution imaging (HR-TEM) also revealed lattice spacing of the spots that were associated with the (111) index of crystalline Pt (d = 0.21 nm) (Figure 2C). By varying K₂PtCl₄ concentration and incubation time, the concentration of Pt deposited on the nanotubes can be modulated ranging from 20% to ∼60% by weight (Wt%, Table 1). Samples prepared using a total reaction time of 24 h yield a greater coverage of Pt species as indicated by higher Wt% Pt obtained from TEM-EDX. In an examination of the crystallinity of the resulting Pt NCs, HR-TEM revealed a relatively high density of crystalline domains and 1−3 nm crystal size in a 24 h sample. For 4 h samples, due to a lack of crystallinity of Pt species reflected in the Fast Fourier Transform (FFT) associated with a given HR-TEM image, it is unclear whether it is a consequence of an ultrasmall crystal size or alternatively, an amorphous structure.

For unmodified (U) pSiNTs (as a control), a negligible amount of elemental Pt species is deposited randomly on the nanotube surface (SI Figure S4). An additional control using APTES-functionalized SiO₂NTs yielded a more uniform coverage of significant amount of Pt NCs than that of unmodified SiO₂NTs (SI Figure S5). Such results suggest the use of this amine-functionalized platform as facile method to synthesize Pt NCs.

3.3. Cell Viability of HeLa Cells after Treatment with Pt NCs-pSiNTs. In order to evaluate the cytotoxicity of Pt NCs-pSiNTs composites, we selected two composites with different % Pt (by wt) ranges: 30−35% (1.5 mM/4 h) and 45−50% (1.5 mM/24 h) (Table 1). CellTiter-Glo viability assays were performed, quantifying the number of viable cells based on the amount of ATP present in the metabolically active cells. The luminescence intensity measured from the sample after incubation with the assay reagents directly correlates to the amount of ATP (e.g., the higher the luminescence intensity, the greater the viable cell number).

For the control, the unmodified pSiNTs had negligible influence on HeLa cell viability at all the investigated nanotube concentrations after 96 h (Figure 3). In contrast, treatment with both types of Pt NCs-pSiNTs composites at 35 μg/mL concentration gradually reduced cell viability to 60−80% after 48 h and significantly decreased to less than 50% both after 72 and 96 h. At a higher dose (50 μg/mL), cell viability was slightly lower but statistically not significant (Figure 4). In comparison to the control, our results suggested that overall toxicity of the composites stemmed from the presence of Pt NCs rather than the template pSiNTs themselves. Normalization of cytotoxicity based on the amount of Pt NCs present
in the composites suggested Pt NCs formed after 4 h incubation were more toxic than the 24 h NCs (SI Figure S6).

To explicitly evaluate the effects of Pt$^{2+}$ and Pt$^{0}$ species on cell survival, a series of control experiments was performed to examine relative cellular viability after treatment with K$_2$PtCl$_4$ and citrate-capped Pt NCs (3.5 ± 1.1 nm) (SI Figure S7). When comparable amounts of Pt NCs deposited on pSiNTs prepared in 1.5 mM K$_2$PtCl$_4$ (4 h), Pt salt precursor (K$_2$PtCl$_4$) exerted lower cytotoxicity, while free-standing Pt NCs significantly reduced cell viability to ∼50% after 72 h, thereby confirming the cytotoxic species in the Pt NC-pSiNT composite as Pt NCs (SI Figures S8A and B).

3.4. Evaluation of Caspase 3/7 Activity in HeLa Cells Treated with Pt NCs-pSiNTs.

In order to assess whether cell death induced by Pt NCs-pSiNTs was the result of apoptosis, the activity of caspase 3/7 enzymes activated during programmed cell death pathway was evaluated. Our data suggest that unmodified pSiNTs did not significantly affect caspase 3/7 activity. On the contrary, from the 48 h time period when cytotoxicity began to be detectable, cells treated with both Pt NCs-pSiNT composite exhibited elevated caspase 3/7 activity ranging from a 1.5 to a 2-fold increase compared to cells without treatment. This suggests cell death was induced via an apoptotic pathway (Figure 5).

3.5. Dissolution of Pt NCs-pSiNTs in Growth Medium.

Analyses of both Pt NCs-pSiNTs (1.5 mM/4 h and 1.5 mM/24 h) collected from the growth medium after 24 h, 48 h, and 96 h exposure to cells in culture revealed gradual transformation of the morphology and composition of the materials as a function of incubation time. For TEM-EDX analysis, the large standard deviations observed in 48 and 72 h samples were due to small amounts of the nanotubes remaining in the medium. For both types of Pt NCs-pSiNTs, most of the composites remained intact within the first 24 h, with some slight change in composition due to partial dissolution in the medium. On the other hand, significant decomposition was observed after 48 h; this entails a loss of discrete nanotube morphology, in addition to elevated levels of Pt relative to a reduction in Si content, as expected with the dissolution of the template and the insoluble Pt species remaining behind (Figure 6 and Table 2).

3.6. Cellular Uptake of Pt NCs-pSiNT Composites.

Based on the results of the cytotoxicity and caspase activity assays, we reasoned that cellular uptake of the composites should occur to enable oxidation of some Pt$^{0}$ to Pt$^{2+}$, which are presumably responsible for cellular damage events. To examine this hypothesis, in vitro cellular uptake of Pt NCs-pSiNTs was analyzed by brightfield and fluorescence microscopies. Our results suggest accumulation of the Pt NC-pSiNT composites within the cytoplasm of a measurable number of cells after 24 h, and a continuous increase in cellular uptake after 48 and 72 h (Figure 7 and SI Figure S9).

4. DISCUSSION

One of the most unique aspects of the approach described above lies with respect to the synthetic route employed in the fabrication of the platinum nanocrystals anchored to the silicon nanotube framework. The most common synthetic strategy for Pt NPs necessitates the use of reducing agents, such as NaBH$_4$ and ascorbic acid, to facilitate reduction of Pt$^{2+}$ to Pt$^{0}$. In order to maintain dispersity in aqueous media as well as long-term storage, stabilization of such freestanding nanocrystals is often achieved by capping agents, such as the surfactant cetyltrimethylammonium bromide (CTAB) and polymeric stabilizers such as polyvinylpyrrolidone (PVP). For the synthesis of ultrasmall Pt NCs (<3 nm), even more carefully controlled routes are required to sensitively provide a narrow crystal size distribution as well as minimize aggregation of the
resultant NCs; the latter effect, of course, reduces the cellular uptake of the Pt in a therapeutic context. Some strategies reported to date, such as phase transfer method(s) or use of templates (e.g., dendrimers), are capable of synthesizing Pt NCs as small as 1 nm. In our approach presented here, we have developed a facile route to synthesize very small Pt NCs (1–3 nm) on pSiNTs functionalized with APTES as a template. The goal here is to rationally disperse the nanocrystals on another nanoscale solid support that slowly resorbs over time in a biocompatible manner, for the explicit purpose of mediating the release of the Pt-based therapeutic species. From the TEM images shown, there is a relatively uniform coverage of ultrafine Pt NCs preferentially formed on

| Table 2. Elemental Composition of Pt NCs-pSiNTs from Dissolution Studies |
|---------------------------------|-----|-----|-----|-----|
| Initial 44.1 ± 3.8 16.5 ± 2.3 38.2 ± 3.9 1.3 ± 0.1 |
| 24 h 40.0 ± 5.5 15.6 ± 2.3 43.3 ± 3.9 1.0 ± 0.6 |
| 48 h 14.5 ± 8.2 12.8 ± 3.0 70.6 ± 10.1 2.1 ± 0.9 |
| 72 h 20.6 ± 15.2 13.8 ± 4.7 64.2 ± 18.5 1.4 ± 0.5 |
| Initial 38.8 ± 4.8 14.4 ± 3.3 45.4 ± 4.8 1.2 ± 0.9 |
| 24 h 38.1 ± 5.7 18.0 ± 3.9 34.8 ± 5.1 9.0 ± 2.0 |
| 48 h 15.7 ± 11.7 8.4 ± 3.2 73.9 ± 13.6 1.9 ± 0.6 |
| 72 h 14.7 ± 12.4 8.7 ± 2.8 74.3 ± 13.5 2.3 ± 1.3 |

Figure 6. TEM images of Pt NCs-pSiNTs after dissolution in the DMEM growth medium showing gradual change in the morphology of the composites over incubation period. 30–35 wt % Pt: A: initial; B: 24 h; C: 48 h; D: 72 h; 40–45 wt % Pt; E: initial; F: 24 h; G: 48 h; H: 72 h.

Figure 7. Cellular uptake of Pt NCs-pSiNTs after 48 h incubation in growth medium. A–C: 1.5 mM/4 h (30–35 wt % Pt); D–F: 1.5 mM/24 h (40–45 wt % Pt) (notes: A and D HeLa cells under brightfield of fluorescence microscope (nonfluorescent method); B and E: Accumulation of Alexa dye-stained composites around nuclei of the unstained HeLa cells (fluorescent method); C and F: Accumulation of Alexa dye-stained composites in Green BODIPY-stained HeLa cells) (fluorescent method).
APTES-pSiNTs without addition of any reducing agents, implying the important role of primary amine moieties in the generation of these Pt NCs.

In addition to (1) these –NH₂ species (arising from the APTES) contributing to Pt ion reduction, other possibilities exist and must be considered: (2) Si–Si bonds; and (3) silanols (e.g., polyol species). For (2), Si–Si bonds (along with Si–H species) have been exploited by Ogata et al. and the Sailor group as efficient reducing agents in immersion plating methods to effectively deposit various metals (e.g., Ag, Cu, Pt) onto porous silicon. However, in our case, control experiments with unmodified pSiNTs result in the random deposition of negligible amounts of Pt NPs (SI Figure S4), suggesting that any exposed Si–Si bonds play an insignificant role in the formation of Pt NCs. In order to assess the role of polyols in nanocrystal formation (commonly from glycolic solvents in metal NP synthesis) as possible reducing agents, that is, silanols in this case, silica nanotubes (SiO₂ NTs) served as another control. Again, trace amounts of Pt NCs were observed on unmodified SiO₂ NTs, and significantly higher Pt concentrations were only detected in the case of APTES-functionalized SiO₂ NTs (SI Figure S5), thereby confirming the role of the -NH₂ group of APTES as the dominant contributor to the formation of Pt nanocrystals on pSiNTs. Not only does literature precedent exist with regard to the use of, amine-containing molecules as capping agents, but amines such as oleyl amine and triethylamine have been reported as efficient reductants in the synthesis of Au NPs. Hence, we propose that the primary amino groups (emanating from APTES) not only coordinate with PtCl₄⁻ on the nanotube surface but also serve as a reducing agent that converts Pt²⁺ into Pt⁰, in the form of nanosized clusters without any additional reducing agent. Owing to the porous morphology of pSiNTs, the resulting Pt NCs were effectively stabilized and isolated from each other, hence preventing undesirable cross-fusion. This is a unique attribute of our particular form of nanoporous Si in nanotube morphology.

While Pt-based anticancer agents such as cisplatin have been effectively used to trigger programmed cell death, clear disadvantages exist, including resistance in some cancer cell lines (e.g., hepatocellular carcinoma, HCC), along with a lack of specificity and selectivity, which have diminished enthusiasm for use of this chemotherapeutic drug. Presumably owing to high surface area, oxygen adsorption increases and thus facilitates corrodibility of Pt NCs. In the acidic environment of endosome and lysosome, corrosive Pt is then readily dissolved to form soluble Pt²⁺ species which subsequently leaches out from the surface of the particles and exerts damages within cells via DNA platination. Previous X-ray photoelectron spectroscopy (XPS) experiments on sub-10 nm diameter Pt nanocrystals (NCs) confirm the presence of surface Pt(II) species in NCs of this size regime; furthermore, a clear increase in Pt(II) concentration with decreasing particle size is observed. Recent studies have successfully demonstrated ultrasmall Pt NCs (1–3 nm) can reduce cell viability and even effectively overcome chemoresistance in HCC, thereby demonstrating Pt NCs as a potentially alternative approach to the use of cisplatin. In order to achieve high therapeutic activity of Pt NCs, not only should aggregation be minimized but also accumulation of high concentration of NCs within cells must be achieved. In our method presented above, ultrasmall Pt NCs uniformly distributed on pSiNTs qualify these requirements, hence implying this novel composite as a possible candidate for anticancer studies.

Our results obtained from in vitro viability assays using Pt NCs-pSiNTs with varying Pt content [Pt Wt%: 30–35% (1.5 mM/4 h) and 45–50% (1.5 mM/24 h)] in HeLa cells suggests a Pt dependent cytotoxicity of each of the two above composites from the presence of Pt NCs since unmodified pSiNTs had no significant impact on cell viability even at the highest nanotube concentration (35 μg/mL). To elucidate possible mechanisms that trigger cell death, we evaluated caspase 3/7 activity, which is associated with apoptosis, to distinguish it from necrosis—an abnormal and harmful unprogrammed cell death. Apoptosis is a programmed cell death pathway that arises via activation of initiator caspases (e.g. caspase 8 and 9) followed by activation of the effector caspases (caspase 3 and 7), which subsequently trigger apoptotic events. Therefore, by quantifying the amount of activated caspase 3/7, the mode of cell death occurring in Pt NC-pSiNT-treated cells can be identified. Consistent with viability assays, our data confirmed biocompatibility of the unmodified pSiNTs as they exhibited a negligible effect on caspase activity. Interestingly, for cells treated with both types of composites, elevated caspase 3/7 levels range from a 1.5 to 2-fold increase and were not significantly influenced by the concentrations tested in the assay, thereby indicating the ability of Pt NCs-pSiNTs to promote apoptotic events. Further mechanistic details have yet to have been elucidated for our composites discussed here, but activity related to peroxidase-like character (known for Pt NC systems) is one possibility. To clarify the impact of Pt species on cell viability, K₂PtCl₄ and citrate-capped Pt NCs were examined as controls. As mentioned above, previous studies suggested Pt²⁺ are responsible for toxicological relevance of Pt NCs; hence, in these control experiments we evaluate possible Pt concentrations assuming an upper limit based on the comparable amount of Pt in the NCs (30–35 wt % Pt). By introducing K₂PtCl₄ (25 μg/mL) with a comparable amount of Pt species (35 wt %), K₂PtCl₄ surprisingly exerted a significantly lower toxicity (65–70% viability) even after 96 h, therefore implying low bioavailability of K₂PtCl₄ to the cellular compartment. On the other hand, treatment of HeLa cells with citrate-capped Pt NCs (17.5 μg/mL) yields a reduction in viability is similar to that of Pt NCs-pSiNTs composites at comparable Pt concentrations. Hence, we propose that cellular internalization of Pt NCs is necessary for exerting damages by leaching high concentrations of Pt²⁺ once inside the cells.

In an attempt to elucidate further details associated with the cytotoxicity of Pt NCs-pSiNT, dissolution studies and cellular uptake experiments of the two composites (Pt Wt%: 30–35% and 45–50%) were performed. While most of the composites remain intact after 24 h of incubation in the growth medium, significant structural evolution of nanotube morphology was particularly observed after 48 h with decreasing Si and increasing Pt concentrations, respectively; thereby confirming biodegradability of the pSiNTs template in the biological medium. Interestingly, cellular uptake of the composites was observed after 24 h and accumulation within the cytoplasm continuously escalated after 48 and 72 h as indicated in both
bright-field and fluorescence confocal microscopy analyses. Since Pt NCs-pSiNTs remained mostly intact due to minimal dissolution of pSiNTs templates and Pt NCs in the extracellular environment during the first 24 h, it is assumed that most composites localized within the cells were initially in the intact form. It should be noted that once internalized within cells, we expect the composites would accumulate within acidic organelles (e.g., lysosome) in order to exert toxicity via the leaching of Pt\textsuperscript{2+}\textsuperscript{15,18}. We also speculate that the dissolution rate of pSiNTs should be reduced in a lower pH environment.\textsuperscript{14} Based on both viability and apoptosis assays we propose a “Trojan horse” mechanism in which a high concentration of Pt NCs was internalized within cells assisted by pSiNTs in the first 24 h followed by the subsequent release of Pt\textsuperscript{2+} in this acidic environment and assisted by dissolution of the nanotube matrix. In other words, an enhancement in the bioavailability of Pt\textsuperscript{2+} was achieved via Pt NCs-pSiNTs.

Interestingly, upon normalizing cytotoxicity data based on the amount of Pt NCs formed within different reaction times (4 and 24 h), 4 h formed Pt NCs appeared to be more toxic than the 24 h counterpart. To understand the different toxicity profiles of the two composites, HR TEM was employed. Upon analysis of HR-TEM images of the NC structure, although a relatively high degree of crystallinity was observed in the case of Pt NCs formed after a 24 h incubation, no distinctive crystalline structure was detected in samples prepared using a 4 h reaction time, presumably due to either smaller crystal size or amorphous morphology of Pt species formed after a shorter reaction time. The results therefore suggested the Pt NCs of the 4 h samples released more Pt species in aqueous solution in a given time period, resulting in higher toxicity compared to the crystalline counterpart.

5. CONCLUSION
The studies presented herein demonstrate a facile synthetic method for the formation of ultrasmall Pt NCs uniformly deposited on pSiNTs templates. In vitro investigations of anticancer properties of the composites suggested the immobilized Pt NCs elicited potent toxicity against HeLa cells via inducing apoptosis. Future work will involve investigating addition moieties to pSiNT surface for therapeutic targeting.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.9b00719.

Detailed TEM images of unmodified and APTES-functionalized pSiNTs (along with selected graphical analyses), along with additional images of Pt NCs formed on various NT surfaces (as controls); Cell viability assay results (Pt NC containing as well as controls); TEM images of Pt NC control samples; optical images of Pt NCs-pSiNTs internalized in HeLa cells (PDF)

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N.T.L. and J.L.C. conceived and planned the experiments; N.T.L. prepared all samples, performed electron microscopic characterization, carried out in vitro studies and related controls; N.T.L., G.R.A., and J.L.C. analyzed the data and wrote the manuscript.

Notes
The authors declare no competing financial interest.

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