Streamlined plug-in aerosol prototype for reconfigurable manufacture of nano-drug delivery systems

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ABSTRACT

The significant advances in nano-drug delivery systems (NDDS) for anticancer agents have led to the development of computational techniques, such as machine learning and neural networks to identify the optimal architectural and compositional design in a wide variety of therapeutic nanoformulations. On the other hand, few studies have examined downsized plug-in reaction-ware embodied in an autonomous platform for the instant reconfigurable production of engineered nanomaterials to guide optimal NDDS designs and delivery strategies. This paper describes an on-demand system for an electrically operable, continuously processible material produced by sequential spray pyrolysis and vibrating spray for single-pass NDDS assembly. In particular, a mild chemotherapeutic NDDS consisting of amorphous boron nitride (α-BN; a stable base material for loading), doxorubicin (DOX; an anticancer drug), and folic acid-chitosan conjugate (FACHI; a targeting and antiopsonic agent), called α-BN-DOX@FACHI, was fabricated using the developed system. α-BN-DOX@FACHI was assessed for the pH-responsive release of DOX, targeting of the folate receptor, and its resistance to opsonization and macrophage phagocytosis. α-BN-DOX@FACHI was found to be a mild cancer chemotherapeutic with reasonable biosafety. Integrating a metal ablation device with the developed on-demand system enabled the reconfiguration of NDDS from α-BN-DOX@FACHI to α-BN-Au-DOX@FACHI or α-BN-Pt-cisplatin@bovine serum albumin to add a photothermal effect with a range of architectures and compositions.

1. Introduction

Engineering nano-drug delivery systems (NDDS) to secure the desired physicochemical properties for carrying medical agents to diseased cells or tissue efficiently is a critical part of recent cancer nanomedicine [1,2]. Significant advances have been made in the development of NDDS with various loadings, targeting, treating, and excreting functions with biosafety and biocompatibility, as well as the key parameters for optimally tailoring NDDS formulation [3,4]. Previous studies proposed a concept to derive the autonomous targeting of a biological site and adjust the dose or release rate of therapeutic agents by constructing micro/nanoscale delivery bots [5,6]. On the other hand, the optimal design to carry NDDS to a specific cell or tissue and interpret biological mechanisms for translatable cancer therapeutics is unclear [7]. Moreover, the use of a developed anticancer agent with NDDS requires more than 10 years of research and development efforts and immense expense [8,9]. Consequently, the extensive exploration of the optimal designs of NDDS based on computational approaches, such as machine learning and neural networks for the high-throughput identification of nano-bio interactions across numerous types of NDDS in different cells and tissues [10–15], has become a major focus. Computing-based NDDS development may reduce the translational gap between the bench and bedside significantly [16].

Although recently introduced computational strategies can reduce the high cost and effort to screen many different candidate formulations, identifying the best design of NDDS still requires laborious flask-based experiments for fabricating various formulations and understanding the interactions between the candidate NDDS and molecules, cells, or tissues [3]. To reduce the time, cost, and effort as well as the risk of failure, the reconfiguring or repurposing of clinically approved
anticancer drugs and generally recognized as safe excipients have been proposed to secure translatable NDDS with complimentary delivery and therapeutic functions for safer and efficacious treatments [17]. This suggests that the presence of an integratable platform for the rapid reconfigurable manufacture of lead candidates to determine structure-therapeutic relationships may accelerate the realization of an autonomous NDDS design and testing process [18].

With regards to adaptable manufacture for computing-based NDDS screening, an electrically operable platform that can reconfigure the assembly of NDDS needs to be connected to a computational system where the modulations of the base material and the therapeutic and targeting agents for building candidate NDDS formulations can be conducted to provide desired architectures and compositions readily in an on-demand manner [19]. A conceptual layout of the digitally implementable equipment for downsized additive manufacturing of a range of candidate NDDS has already been introduced to tune the size, shape, surface chemistry, and chemical composition to guide future NDDS designs and delivery strategies conveniently despite the absence of an autonomous flow system suited to a reconfigurable NDDS assembly [20]. As a smart modular flow assembly, an autonomous robotic experimentation was introduced to tailor colloidal quantum dots intelligently in a continuous-flow manner [21]. Owing to this stepping stone technique, a downsized platform for the automated manufacture of noble metal-based NDDS, even possessing photoinduced excretion, was introduced [22]. Nevertheless, the development of an electrically operable system for scalable and low-cost manufacturing is needed to install a system in a realizable autonomous NDDS assembly platform.

This study developed a plug-and-play serial device for the reconfigurable scalable manufacture of chemotherapeutic NDDS through in-flight self-assembly to be embodied in an autonomous manufacturing-screening platform. As shown in Fig. 1, the pyrolytic manufacture of a base material and subsequent encapsulation with anticancer and targeting agents were achieved through streamlined aerosol processing by turning on electrically operated reaction ware (vibrating nozzles, an electrically heated tube reactor, a photocharger, and flow controllers). Amorphous boron nitride (a-BN) was selected as the base material for constructing the NDDS to avoid unwanted side effects and high toxicity [23–27], as well as the infeasible processability, biodegradability, and scalability [28–33]. In this system, mist droplets of an a-BN precursor solution from a vibrating nozzle entered an electrically heated tubular reactor using a nitrogen carrier gas for the pyrolysis and subsequent condensation to a-BN nanoplatelets. Despite several reports of the feasibility of hexagonal BN (h-BN) as a nanocarrier for anticancer therapies because of the better biocompatibility and cost-effectiveness than graphene-based NDDS [34–39], there are no reports on the use of a-BN from a continuous-flow process to secure easy accessibility for clinical applications. The ejection of surface electrons from the platelets was induced by exposure of a-BN-laden flow to 185-nm UV irradiation because of the higher work function of photons (6.2 eV) than a-BN (~4 eV) [40–42]. This resulted in substitutional electrostatic interactions between the a-BN surfaces and adjacent negatively charged groups (e.g., OH− from photocharging of H2O) of the reactant gas [43,44]. These interactions enabled the attachment of positively charged groups (e.g., protonated NH2) of doxorubicin (DOX) to the a-BN surfaces after dispersal in a DOX solution [45–47]. The a-BN-DOX dispersion and folic acid-chitosan (FACHI) conjugate solution was mixed continuously and injected into another vibrating nozzle to produce all-in-one droplets containing a-BN, DOX, and FACHI. The FACHI conjugate (35:65 of FA:CHI in mass) was selected to promote tumor accumulation with minimal opsonization of a-BN-DOX through active targeting of the folate receptors (FRs) [48–51]. This conjugate was prepared in the presence of N-(3-dimethylanilinopropyl)-N′-ethylcarbodiimide (EDC) hydrochloride as a crosslinking agent (refer to Fig. S1A) for coupling between the γ-carboxyl groups of FA and the primary amine groups of CHI (confirmed through Fourier transform infrared [FTIR; around at 1604 cm−1 in Fig. S1B] [52] and nuclear magnetic resonance [NMR; around at

Fig. 1. Schematic diagram of the aerosol prototyping consisting of spray pyrolysis and vibrating spray for the single-pass assembly of a-BN-DOX@FACHI NDDS and an assessment of the physicochemical property and anticancer efficacy. A BN precursor solution was sprayed by passing through a vibrating nozzle into an electrically heated tube reactor under nitrogen gas flow, leading to the in-flight formation of pyrolytic a-BN nanoplatelets. These suspended platelets were then exposed to 185-nm UV to detach the surface electrons to combine electrostatically with adjacent OH− groups. The resulting platelets were immersed in a DOX solution as a suspension where positive charges in DOX were attached electrostatically to negatively charged surfaces of the platelets. This suspension was mixed with a FACHI solution upstream of another vibrating mesh to generate all-in-one droplets to be a-BN-DOX@FACHI NDDS after diffusional drying. The resulting NDDS was used in both in vitro and in vivo chemotherapeutic models after physicochemical characterization.
2.4 ppm in Fig. S1C) spectroscopy [48] to retain affinity to FRs [53]. The all-in-one droplets were dried with a nitrogen carrier gas by passing through a silica gel-packed hollow tube reactor, resulting in the formation of a-BN-DOX@FACHI (FACHI passivated a-BN-DOX) NDDS. The physicochemical properties of the collected NDDS were characterized to validate the developed technique. The bioassays were performed in vitro (human colon [HT-29] and lung [A549] carcinoma cells) and in vivo (HT-29 [FR overexpressing] xenograft tumor-bearing mice) models to examine the potential of targeted mild cancer chemotherapy with the biosafety of instantly manufactured NDDS. A reconfiguration of aerosol prototyping was further attempted to provide NDDS with near-infrared (NIR) laser-induced photothermal effects (-60 °C temperature elevation) as well as different architectures (ultrasmall Au included a-BN-DOX@FACHI; a-BN-Au-DOX@FACHI) and compositions (ultrasmall Pt included, cisplatin [cisPt] and bovine serum albumin [BSA]-loaded instead of DOX and FACHI; a-BN-Pt-cisPt@BSA).

2. Experimental

2.1. Preparation of a-BN precursor and FACHI

An a-BN precursor solution workable for inert gas flow was prepared. Briefly, boric acid (B0394, Sigma-Aldrich, USA) and melamine (240818, Sigma-Aldrich, USA) were dissolved in hot deionized water at a 2:1 M ratio with magnetic stirring. After cooling the mixture solution, the resulting paste was filtered using a microfiltration unit (33980-300, PYREX®, USA) and dispersed in deionized water (×10 dilution) to be supplied to a vibrating nozzle.

To prepare FACHI, FA (F7876, Sigma-Aldrich, USA) was conjugated with CHI (42344, Sigma-Aldrich, USA) in the presence of EDC hydrochloride (E1769, Sigma-Aldrich, USA) as a crosslinking agent. In particular, 100 mg of FA and 65.144 mg EDC hydrochloride were first dissolved in 10 mL (1:1.5 M ratio) of dimethyl sulfoxide (DMSO; 276855, Sigma-Aldrich, USA) to activate the carboxylic groups in FA. The activated FA solution was added dropwise to the CHI solution (pH 4.7 acetate buffer basis) with magnetic stirring for 16 h in the dark. After setting the pH of the solution to 9.0 by adding a NaOH solution, the solution was dialyzed against PBS (pH 7.4) using a Spectra/Por® 7 dialysis membrane (MWCO = 3.5 kDa) for three days in the dark to remove the unbound FA and isolate the FACHI conjugates. The yellow FACHI was finally harvested by lyophilization for storage.

2.2. Plug-in manufacture of NDDS

As shown in Fig. 1, electrically operable reaction ware, spray pyrolysis, photocharging, and vibrating spray, were connected serially under nitrogen gas (99.9999% purity) flow to secure the single-pass assembly. The a-BN precursor solution was supplied to the reservoir of a lab-made vibrating nozzle (array of 4 μm holes) using a peristaltic pump (323DSu/D, Watson Marlow Bredel Pump, USA). Thus, mist droplets were generated by passing the solution through the nozzle at a vibration frequency of 128 kHz. These droplets were carried by nitrogen gas (1 L min⁻¹) to an electrically heated tubular reactor operated at a wall temperature of 650 °C to form pyrolytic a-BN nanoplatelets using the following reaction:

\[
\text{B}_2\text{O}_3 + \text{CO(NH}_2)_2 \rightarrow 2\text{BN} + \text{CO}_2 + 2\text{H}_2\text{O}
\]

where \(\text{B}_2\text{O}_3\) and \(\text{CO(NH}_2)_2\) are the pyrolyzed components of boric acid and melamine in the droplets. The platelet-laden flow was mixed with 2 L min⁻¹ of room temperature nitrogen gas and exposed to 185-nm UV to detach the surface electrons of the platelets (6.2 eV photons attack the surfaces of a-BN platelets with a work function of ~4 eV, [40–42]) for electrostatic combination with the adjacent \(\text{OH}^+\) groups in the reacted gas through charge recombination (positively charged a-BN + \(\text{OH}^+\) groups), resulting in the formation of negatively charged a-BN platelets. These platelets were then dispersed in a DOX solution, where the positive charges in the DOX molecules were attached electrostatically to the negatively charged surfaces of the platelets. This suspension was mixed with a FACHI solution using a peristaltic pump before the mixture solution entered another vibrating nozzle to generate the all-in-one droplets. These droplets were carried by nitrogen gas (3 L min⁻¹) to enter a diffusion dryer to extract the solvent from the droplets, resulting in a-BN-DOX@FACHI NDDS. The resulting NDDS were harvested, characterized, and dispersed in buffered saline for use in both in vitro and in vivo chemotherapeutic models.

2.3. Size distribution, morphology, and surface chemistry

The in-flight formation of a-BN and the incorporation of DOX@FA-Chi on a-BN were identified using an SMPS (3936, TSI, USA), where a-BN- or a-BN-DOX@FACHI-laden nitrogen gas flow (0.3 L min⁻¹) was sampled directly for electrostatic size classification and optical particle counting and sizing in real-time.

The morphologies of the base a-BN and its DOX or DOX@FACHI incorporated configurations were analyzed by depositing the resulting particles on a carbon-coated copper grid (Tedpella, USA) using an aerosol tube sampler (Ecomesure, France) with a vacuum pump (3033, TSI). The resulting particles on a copper grid were observed by transmission electron microscopy (TEM, Tecnai G2 F20 S-TWIN, FEI, USA).

The zeta potentials of the base a-BN and its DOX or DOX@FACHI incorporated configurations were obtained at 25 °C using a zetasizer (Nano-S90, Malvern Instruments, UK) after the resulting particles were dispersed in deionized water. The formation of FACHI conjugates was examined by proton nuclear magnetic resonance (\(^1\)H NMR, 600 MHz, Varian Inc., USA) and FTIR (Spectrum 100, PerkinElmer Life and Analytical Sciences, USA) spectroscopy. For NMR analysis, FACHI and CHI were dissolved individually in deuterated acetic acid (CD\(_3\)COOD)-d\(_4\)/deuterium oxide (D\(_2\)O) (1/4 v/v) while FA was dissolved in deuterated DMSO, d\(_6\)(CD\(_3\)SO) for the spectroscopic comparison. The FTIR spectra of the FACHI, including the individual FA and CHI, were obtained and compared in the range, 4000–600 cm⁻¹. In addition, the FA content in FACHI was estimated using a UV–Vis spectrophotometer (U-2800, Hitachi, Japan) by a comparison between the analytical calibration curve and the sample data points at 363 nm.

2.4. LC, EE, and DOX release

The LC and EE of DOX in a-BN-DOX and a-BN-DOX@FACHI were measured using an ultrafiltration method (MWCO = 10 kDa; Amicon®, Millipore, USA) under centrifugation with 5000 rpm for 10 min to separate the unbound DOX. The fraction of unbound DOX was estimated by comparing the UV–Vis absorption spectra from the control and a-BN-DOX or a-BN-DOX@FACHI at a wavelength of 481 nm. The time profiles of DOX release from a-BN-DOX@FACHI NDDS were recorded at pH 5.0 (ABS) and pH 7.4 (PBS). The amount released from the NDDS was estimated at predetermined times using a UV–Vis spectrophotometer at the same wavelength.

2.5. Cytotoxicity

The biocompatibility of a-BN and a-BN@FACHI was assessed. HDF cells were purchased from the American Type Culture Collection and cultured in DMEM. HDF cells (1 × 10⁴) were seeded in each well of a 96-well plate and incubated overnight. The cells were then treated with different concentrations ranging from 5 to 200 μg mL⁻¹ and incubated at 37 °C in a 5% CO₂ atmosphere for 24 h or 48 h. For an in vitro anticancer study, HT-29 and A549 cells were purchased from Korea Cell Line Bank and cultured in Rosewell Park Memorial Institute 1640 medium with 1-glutamine (300 mg L⁻¹). 25 mM hydroxymethyl piperazinethanesulfonic acid supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin
antibiotics. Approximately 1 × 10⁶ cells were seeded in each well of a 96-well plate, incubated for 24 h, and then treated with a-BN-DOX@FACHI (0.01–100 μg mL⁻¹), including free DOX and a-BN-DOX for comparison for 24 h.

The treated cells were washed twice with Dulbecco’s PBS (DPBS) and incubated with MTT solution for 4 h in the dark. The absorbance of the dissolved formazan crystals (formed by live cells) in DMSO was measured using a microplate reader (Multiskan EX, Thermo Scientific, USA) at 570 nm. In addition, the IC₅₀ of the two carcinoma cell lines treated with a-BN-DOX@FACHI was calculated using GraphPad Prism 7 (GraphPad Software, USA).

2.6. ROS generation

ROS generation from the base a-BN exposure was assessed by seeding HDF cells (2 × 10⁴ cells per well) and incubating them overnight on a 12-well plate. The cells were treated with a-BN for 24 or 48 h at a fixed concentration (200 μg mL⁻¹). The incubated cells were collected, stained for 30 min with 30 μM of DCFDA, and examined using a flow cytometer to determine the ROS levels in the treated cells.

2.7. Cellular uptake

A CLSM (Leica Microsystems, Germany) was used to observe the localization of a-BN-DOX@FACHI NDDS in HT-29 and A549 cells. Briefly, 2 × 10⁵ cells per well were seeded separately on coverslips placed on 12-well plates and incubated for 24 h. The cells with or without the FA pretreatment (for 1 h at 10 μg mL⁻¹ well⁻¹) were treated with a-BN-DOX@FACHI for 1 h at 5 μg mL⁻¹ well⁻¹ and stained with LysoTracker® Green (100 ng mL⁻¹, Thermo Fisher Science, USA) for 10 min to trace the lysosome within the cells. The cells were fixed with 4% paraformaldehyde, mounted on a glass slide, and sealed with glycerin for CLSM observations.

FACS (Calibur™, BD Biosciences, USA) analysis was conducted by sedding 2 × 10⁵ cells per well and incubating them for 24 h in a six-well plate. The cells with or without the FA pretreatment were treated with a-BN-DOX@FACHI (5 μg mL⁻¹ well⁻¹) for 30, 60, and 90 min (time-dependent analysis). The treated cells were washed twice with ice-cold DPBS after incubation, trypsinized for collection, centrifuged, washed to obtain cell pellets, and dispersed in 1 mL ice-cold DPBS before the FACS analysis. The uptake of a-BN-DOX@FACHI by RAW 264.7 macrophages was also examined using the procedure mentioned above.

2.8. Hemolysis

The hemocompatibility of base a-BN, and a-BN-DOX@FACHI, free DOX, and a-BN-DOX were examined using Sprague Dawley (SD) rat blood. Fresh whole blood from SD rats was collected in heparinized tubes, centrifuged (3500 rpm for 10 min), washed three times with DPBS to remove the plasma, and dispersed in DPBS. Different a-BN (1–100 μg mL⁻¹) or DOX (5–20 μg mL⁻¹ in a-BN-DOX and a-BN-DOX@FACHI) concentrations were added to the plasma free blood (4%), and the samples were then incubated for 1 h at 37 °C. The erythrocytes were centrifuged at 4 °C, and the absorbance of the supernatants was measured by UV–Vis spectrophotometry at 540 nm. The negative control was treated with the DPBS supernatant, while the positive control was the hemolysis caused by deionized water.

2.9. Apoptosis

FITC Annexin V/7AAD staining (BD Biosciences, USA) was used to assess cellular apoptosis in HT-29 and A549 cells treated with a-BN-DOX@FACHI as well as free DOX and a-BN-DOX for 24 h. The treated cells were harvested, washed, and dispersed in DPBS using trypsin. The cell pellets were then stained with 2.5 μL of FITC Annexin V and 2.5 μL of 7AAD for 15 min in the dark and finally dispersed in the binding buffer of Annexin V for flow cytometry.

2.10. Biodistribution

The in vivo localization of the prepared NDDS was assessed by replacing DOX in the NDDS with Cy5.5 to form a-BN-Cy5.5@FACHI (Base a-BN was also labeled to form a-BN-Cy5.5 for comparison.) for intravenous injection into the mice with tumors ~250 mm² in size. The fluorescent images of the mice were acquired using the Fluorescence-labeled Organism Bio-imaging Instrument system (NeoScience, South Korea) at predetermined times (1, 4, 8, 24, and 36 h postinjection). The mice were sacrificed 36 h postinjection. The resulting ex vivo fluorescent distribution of the harvested tumors and major organs was recorded; the fluorescent intensity was quantified using NEO image software.

2.11. In Vivo antitumor study

The xenograft tumor model was developed by a subcutaneous injection of 1 × 10⁷ HT-29 cells into the right flanks of six-week-old female BALB/c nude mice. The mice were distributed into four groups (five mice per group) when the tumors reached ~100 mm². The mice were treated four times (three-day intervals) with a-BN-DOX@FACHI, including free DOX and a-BN-DOX for comparison at an equivalent dose (5 mg kg⁻¹). The tumor volumes, body weights, and digital images, were recorded at predetermined times to plot the time profiles. The tumor diameters were measured using digital Vernier calipers to estimate the tumor volumes.

2.12. Immunohistopathological analysis

The treated mice were euthanized, and the histopathological and histomorphometric changes in the resulting tumors and major organs were observed by H&E staining. The changes in the immunoreactivity of the tumors against apoptotic (cleaved caspase-3 and cleaved PARP), angiogenesis (CD31), and tumor cell proliferation (Ki-67) markers were determined using the primary antisera and avidin-biotin-peroxidase complex immunohistochemical staining.

2.13. Statistical analysis

The findings are expressed as the mean ± standard deviation. A student t-test for group pairs and one-way variance analysis for multiple groups were used to assess the statistical significance between the groups. The results were considered statistically significant at *p < 0.05.

All mouse experiments were approved and carried out in accordance with the instructions and guidelines of the Institutional Animal Ethics Committee, Yeungnam University, Republic of Korea.

3. Results and discussion

The production of a-BN and subsequent incorporation of DOX and FACHI was examined. The size distributions of floating particles on a nitrogen gas flow were obtained using a scanning mobility particle sizer (SMPS) by direct suction of a part of the reacted flow. Fig. 2A presents the size plots of a-BN (downstream of the photocharging sector) and a-BN-DOX@FACHI (downstream of the diffusional drying sector) configurations corresponding to two Gaussian distributions. The geometric mean diameters (GMD) of a-BN and a-BN-DOX@FACHI were 96.6 and 102.1 nm, respectively. These matched different circular distributions of the samples, where the incorporation of DOX and FACHI increased the particle size of the base a-BN. No significantly protruded and distorted parts were observed in the distributions representing a unimodal Gaussian distribution. The unimodal character even for a-BN-DOX@FACHI suggests that the DOX and FACHI components are incorporated equivalently on the base a-BN to avoid the generation of free DOX@FACHI particles during the single-pass assembly. A negative picompare
level current (obtained using a Faraday cup electrometer) of the photocharged a-BN as well as the −COOH groups in the FACHI conjugates may induce the intercalation of DOX molecules between a-BN and FACHI through electrostatic complexation (a-BN-DOX and DOX-FACHI) [54–56] that facilitates the successive layering (increasing GMD) of DOX and FACHI on the base a-BN without detectable dislocations.

The assembled morphologies of a-BN-DOX and a-BN-DOX@FACHI, including the microstructure of base a-BN were observed by placing the resulting particles on carbon-coated copper grids by electrostatic precipitation for transmission electron microscopy (TEM) observations. The first two panels in Fig. 2B show low- and high-magnification TEM images of the base a-BN. The size distribution histograms of the specimens (200 particles per specimen) with lower magnification TEM images are also depicted in Fig. S1D, which match the size distributions obtained from the SMPS. The individual platelets were distributed on a grid, and the high-magnification images clarified the laminates with a turbostratic structure of the resulting platelets. The particles in the low-magnification image match the size distribution from the in-flight a-BN measurement, while the high-magnification images and the SAED pattern prove the amorphous nature of the resulting a-BN nanoplatelets. The images for a-BN-DOX and a-BN-DOX@FACHI showed no significant changes in appearance despite the increase in size. (C) Zeta potentials of a-BN-DOX@FACHI as well as individual a-BN and a-BN-DOX. The incorporation of DOX on 185-nm UV exposed a-BN nanoplatelets caused a potential shift from negative to positive, which was intensified further after FACHI incorporation on a-BN-DOX. (D) FTIR spectra of a-BN-DOX@FACHI as well as individual a-BN, FACHI, and a-BN-DOX. The characteristic bands of base a-BN at 600-1000 cm⁻¹ and 3100-3600 cm⁻¹ were changed by incorporating DOX and FACHI. (E) LC and EE of DOX for a-BN-DOX and a-BN-DOX@FACHI. Both LC and EE of a-BN-DOX were enhanced by the addition of FACHI. (F) DOX release profiles of a-BN-DOX@FACHI at pH 7.4 (dispersed in PBS) and 5.0 (dispersed in ABS). The cumulative release of DOX for 24 h reached approximately 95% and 70% at pH 5.0 and pH 7.4, respectively, exhibiting pH-dependence.

Fig. 2. Size distribution, morphology, surface charge—chemistry, LC—EE, and pH-responsive DOX release of a-BN-DOX@FACHI NDDS. (A) In-flight size distribution of a-BN-DOX@FACHI from diffusional drying of the all-in-one droplets, including base a-BN for comparison. There was only an increase in GMD from the combination with free DOX and FACHI without significant distortion of the distribution and the generation of satellite peaks. (B) Representative TEM images of a-BN-DOX@FACHI as well as individual a-BN and a-BN-DOX. The first two panels exhibit low- and high-magnification TEM images with a SAED pattern of base a-BN. The particles in the low-magnification image match the size distribution from the in-flight a-BN measurement, while the high-magnification images and the SAED pattern prove the amorphous nature of the resulting a-BN nanoplatelets. The images for a-BN-DOX and a-BN-DOX@FACHI showed no significant changes in appearance despite the increase in size. (C) Zeta potentials of a-BN-DOX@FACHI as well as individual a-BN and a-BN-DOX. The incorporation of DOX on 185-nm UV exposed a-BN nanoplatelets caused a potential shift from negative to positive, which was intensified further after FACHI incorporation on a-BN-DOX. (D) FTIR spectra of a-BN-DOX@FACHI as well as individual a-BN, FACHI, and a-BN-DOX. The characteristic bands of base a-BN at 600-1000 cm⁻¹ and 3100-3600 cm⁻¹ were changed by incorporating DOX and FACHI. (E) LC and EE of DOX for a-BN-DOX and a-BN-DOX@FACHI. Both LC and EE of a-BN-DOX were enhanced by the addition of FACHI. (F) DOX release profiles of a-BN-DOX@FACHI at pH 7.4 (dispersed in PBS) and 5.0 (dispersed in ABS). The cumulative release of DOX for 24 h reached approximately 95% and 70% at pH 5.0 and pH 7.4, respectively, exhibiting pH-dependence.
a-BN-DOX@FACHI), confirming the successive layering of DOX and FACHI on the negatively charged a-BN. This layering of a-BN-DOX@FACHI and individual a-BN (after photocharging), FACHI, and a-BN-DOX was examined by FTIR spectroscopy (Fig. 2D). The spectrum of a-BN exhibited two intense bands at 1287 cm⁻¹ and 748 cm⁻¹, which matched the in-plane B–N stretching and out of plane B–N–B bending vibrations, respectively [46], supporting the pyrolytic formation of the BN structure. Another band at approximately 3250 cm⁻¹ vibrations, respectively [46], supporting the pyrolytic formation of the matched the in-plane B–N–B bending vibration), 990 cm⁻¹ (–C=O and –C=O–H, 1100 cm⁻¹ (–C–O stretch), 1300 cm⁻¹ (–C–H), and 3300 cm⁻¹ (–OH/asymmetric stretch –NH₂ groups) [25,46,67], proving the successive layering of DOX and FACHI on the base a-BN by a comparison with the characteristic bands for a-BN-DOX and FACHI.

The DOX loading capacity (LC) of a-BN with and without FACHI was 7.52 ± 0.32% and 6.24 ± 0.58%, respectively. The encapsulation efficiency (EE) was estimated as the ratio of the amount between the DOX and a-BN by a comparison with the characteristic bands for a-BN-DOX and FACHI. Therefore, the EE values of DOX in a-BN-DOX@FACHI and a-BN-DOX were 77.39 ± 1.00% and 64.15 ± 2.50%, respectively (Fig. 2E). The results further supported the DOX layering on a-BN through electrostatic complexation as well as π–π auto-stacking of aromatic rings in DOX upon the first conjugation of DOX molecules on the a-BN surface. The higher EE value of a-BN-DOX@FACHI may be due to interactions between the protonated NH₂ groups in DOX and the negatively charged groups of FACHI [54], leading to an enhanced EE of DOX [49]. The time-dependent (in dialysis membranes for 24 h) profiles of DOX release were obtained at pH 7.4 (in phosphate-buffered saline [PBS] to simulate physiological conditions) and pH 5.0 (in acetate buffered saline (ABS) to simulate endolysosomal conditions) (Fig. 2F) [34] According to the differences in the release profile, the intercalation of DOX between a-BN and FACHI conferred a pH-responsive release of DOX, where the enhanced release from a-BN-DOX@FACHI NDDS at pH 5.0 may be due to protonated –NH₃⁺ in DOX, which increases its hydrophilicity [56]. In addition, the better solubility of CHI at acidic pH accelerated the release of complexed DOX, suggesting the pH-responsive activity of the NDDS for site-selective release of DOX.

To examine the stealth property of the FACHI conjugates, the resulting a-BN-DOX@FACHI, including free DOX and a-BN-DOX for comparison, were incubated with RAW 264.7 macrophages (as model phagocytes). The uptake activity (phagocytosis) of the macrophages as

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Fig. 3. Uptake assessment of a-BN-DOX@FACHI NDDS by macrophages (in comparison with free DOX and a-BN-DOX) and carcinoma cells (with and without FA pretreatment) to assess the NDDS localization. (A) Representative confocal images of RAW 264.7 macrophages treated with free DOX, a-BN-DOX, and a-BN-DOX@FACHI. Significant differences in DOX (red) fluorescence were observed between a-BN-DOX@FACHI and comparative samples (free DOX and a-BN-DOX). (B, C) Flow cytometry and MFI profiles of the macrophages treated with free DOX, a-BN-DOX, and a-BN-DOX@FACHI. These profiles matched the fluorescent intensities from the treatments of macrophages. (D) Representative confocal images of A549 and HT-29 cells incubated with a-BN-DOX@FACHI (5 μg mL⁻¹ per each well) for 1 h in the absence (–) and presence (+) of an FA pretreatment (10 μg mL⁻¹ per each well). (E) Time MFI plots (30–90 min) of A549 and HT-29 cells treated with a-BN-DOX@FACHI in the absence (upper panel) and presence (lower panel) of the FA pretreatment (*p < 0.05 and **p < 0.01). The FA untreated cells exhibited greater DOX fluorescence, while significant differences between the cell lines were observed in the absence of the FA pretreatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
DOX fluorescence was visualized and quantified by confocal laser scanning microscopy (CLSM) and fluorescence-activated cell sorting (FACS), as shown in Fig. 3A–3C. A representative CLSM image for a-BN-DOX@FACHI revealed weaker DOX fluorescence than that of free DOX and a-BN-DOX, confirming that the FACHI lowers the phagocytic uptake and opsonization process of the NDDS significantly [68–70]. The FACS profiles and their quantified plots (mean fluorescence intensity [MFI]) support the resistance of a-BN-DOX@FACHI to the macrophage uptake, which may be useful for promoting systemic circulation and interactions between the tumor cells and NDDS. The CLSM images (Fig. 3D) of A549 (normal FR expression) and HT-29 (FR overexpression, Fig. S2C) cells incubated with a-BN-DOX@FACHI in the absence (noted as -) and presence (noted as +) of the FA pretreatment were obtained to identify the intracellular localization of DOX from a-BN-DOX@FACHI NDDS. LysoTracker Green was used to label the highly acidic compartments and lysosomes. Because of the FR overexpression property, the FA pretreatment suppressed the internalization of the NDDS for HT-29 cells due to receptor saturation, while no notable differences in DOX fluorescence between - and + were observed for the A549 cells (Figs. 3E and S3). Thus, similar fluorescent intensities for A549 and HT-29 cells were induced in a time-dependent manner, representing FR-mediated endocytosis of the NDDS for targeted DOX delivery [71]. On the other hand, caveolae- and clathrin-mediated endocytosis could also be involved in the internalization of the NDDS [72].

The induction of apoptosis for anticancer activity was determined by assessing the ROS in NDDS-treated HT-29 and A549 cells using FACS with fluorescein isothiocyanate (FITC) Annexin V/7-aminoactinomycin D (7AAD) staining kit. As shown in Fig. 4A, the fraction of early apoptosis in HT-29 cells treated with a-BN-DOX@FACHI was significantly greater (30.2%) than the other configurations (a-BN-DOX (18.4%) and free DOX (11.2%)), even A549 cells (9.59%) treated with a-BN-DOX@FACHI. The A549 cells treated with a-BN-DOX@FACHI exhibited slight enhancement in early apoptosis compared to those treated with a-BN-DOX and free DOX. The highest level of early apoptosis in HT-29 cells (FR overexpression) treated with a-BN-DOX@FACHI reflected the FR-mediated endocytosis that implicates the enhanced cellular uptake and subsequent promotion of ROS generation in the cells from DOX release. In the MTT assays of the treated HT-29 and A549 cells (Fig. 4B), the viability profiles revealed concentration-dependent cytotoxic effects on both cell lines. The profiles for HT-29 cells treated with a-BN-DOX@FACHI revealed greater cytotoxicity than a-BN-DOX and free DOX, which matched the enhanced early apoptosis FITC Annexin V/7AAD assay. This is because higher ROS levels eventually facilitate the destruction of the cellular compartments (e.g., nucleic acids, proteins, lipids, and membranes). In A549 cells, however, the profile for a-BN-DOX@FACHI coincided with those from a-BN-DOX and free DOX in line with the ROS profiles for A549 cells, reflecting the enhanced internalization in HT-29 cells through the incorporation of FACHI conjugates for FR-mediated endocytosis. The half-maximal inhibitory concentrations (IC50) of a-BN-DOX@FACHI were calculated by fitting the viabilities to the four-parameter logistic model. The IC50 values for the treated HT-29 and A549 cells were 14.036 and 43.004 μM, respectively, substantiating the validity of FR-mediated endocytosis for enhancing cancer chemotherapeutics [72,73]. Owing to these workable properties from plug-in manufacture, a hemolysis assay was conducted to assess the biosafety of a-BN-DOX@FACHI in blood circulation. Compared to the hemolytic fraction of base a-BN (Fig. 5A, exhibiting comparable hemolytic percentages with previous studies) [74,75], the incorporation of DOX and DOX-FACHI on a-BN (Fig. 5B) did not alter the hemolysis of free DOX (5–20 μg mL⁻¹) or exceed that of the a-BN at 100 μg mL⁻¹ compared to the negative control (PBS). Hence, the plug-in manufactured a-BN nanoplatelets and their incorporated forms (a-BN-DOX and a-BN-DOX@FACHI) to be embodied in an autonomous anticancer platform undergo <10% hemolysis to ensure hemocompatibility in the blood circulation for assessing the targeted cancer chemotherapeutics.

The in vivo localization of the NDDS was examined using a fluorescence in vivo imaging system by labeling cyanine 5.5 (Cy5.5) to form a-BN-Cy5.5@FACHI and subsequent intravenous administration into HT-29 xenografted tumor-bearing mice to evaluate the tumor-targeting ability for 36 h (Fig. 5A). a-BN-Cy5.5 was included for comparison. For ex vivo imaging, the tumors and major organs were excised for observations. No significant accumulation of the NDDS was observed in the tumor region until 1 h postinjection compared to the other organs. On the other hand, more intense signals were observed for both NDDS (a-BN- and a-BN-Cy5.5@FACHI) at the other time points. Thirty-six hours after the intravenous injection, the a-BN-Cy5.5@FACHI-injected mice exhibited the most intense fluorescence in the tumor region, suggesting

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**Fig. 4.** Generation of apoptosis and cytotoxicity in human carcinoma cell lines after treatment with free DOX, a-BN-DOX, and a-BN-DOX@FACHI. (A) FACS quantification of HT-29 and A549 cells from FITC Annexin V/7AAD staining to assess the induction of cellular apoptosis. The percentage differences between the treatments in the early apoptosis (lower-right panel) were clearer for HT-29 cells than for A549 cells. (B) Cytotoxicity profiles of the cells treated for 24 h as a function of the DOX concentration (0.01–100 μg mL⁻¹). The incorporation of a-BN or a-BN@FACHI on DOX was only significant for HT-29 cells.
the effectiveness of FACHI conjugates for targeted delivery. In contrast, significant fluorescence was observed in the kidneys and liver of the α-BN-Cy5.5-treated mice. This targeting activity attenuated the distribution in the other organs substantially as a reversal by the progressive FR-mediated localization of α-BN-Cy5.5@FACHI in the tumor site (Fig. 5B). Furthermore, the reduced opsonization by the CHI moiety in FACHI may increase the circulation half-life of the NDDS that prolongs their interactions with the tumor. Therefore, the off-target uptake of α-BN-Cy5.5 is related to opsonization and subsequent accumulation by the organs (heart, liver, lung, and kidneys) with abundant macrophages because of the absence of the CHI moiety [76,77]. The proton sponge effect of CHI can induce the escape of NDDS from reticuloendothelial system uptake, which prolongs circulation in vivo by minimizing the process of opsonization [78,79]. Although the resulting biodistribution needs to be compromised for repurposing or further reducing the accumulation in other organs, it is vital to demonstrate the workability of the plug-in manufactured NDDS by inhibiting its retention in the vital organs for targeted cancer chemotherapeutics.

An in vivo antitumor study was designed to examine the systemic effects of α-BN-DOX@FACHI, as shown in Fig. 6A, which included a
comparison with free DOX and α-BN-DOX. No significant differences in tumor growth were observed between free DOX and α-BN-DOX treated mice (inset digital image of Fig. 6B). Combining the α-BN platelets with DOX@FACHI improved the inhibition of tumor progression significantly, which may be due to the prolonged circulation for ensuring the selective tumor accumulation of α-BN-DOX@FACHI and providing targeted DOX release (the targeted chemotherapeutics). The targeted DOX release was provided despite the initial burst release of DOX from α-BN-DOX@FACHI NDDS under in vitro conditions. This might be because of protein corona formation on the NDDS as a buffer layer between the NDDS and biological fluid for suppressing the burst DOX release [80] because the protein can be formed from adsorbed relevant biomolecules on the surface of the injected NDDS [81,82]. The bodyweight of the mice was also recorded throughout the treatment period to monitor the systemic toxicity of α-BN-DOX@FACHI, free DOX, and α-BN-DOX. The free DOX induced a marked decrease in body weight after eight days post-treatment (Fig. 6C), probably because of its inherent cardiotoxicity and myelosuppressive properties [83]. In contrast, there were no significant decreases in body weight for the α-BN-DOX and α-BN-DOX@FACHI treatments, supporting the reduced systemic toxicity by incorporating DOX and FACHI with α-BN or even only loading DOX on α-BN. All mice treated with α-BN-DOX@FACHI, including free DOX and α-BN-DOX, were alive throughout the antitumor analysis period (26 days).

Tumor sections were extracted from the mice treated with free DOX, α-BN-DOX, and α-BN-DOX@FACHI. The mice showed alterations during treatments to examine apoptosis, angiogenesis, proliferation, and necrosis of tumor cells (Fig. 6D). The apoptotic markers (cleaved caspase-3 and poly(ADP-ribose) polymerase [PARP]) were observed in the order of α-BN-DOX@FACHI > α-BN-DOX > free DOX (Table S1). In contrast, Ki-67 and CD31, as markers of proliferation and angiogenesis, were inversely correlated, clarifying the correlation between the treatments and biological processes. The biosafety profiles of the α-BN-DOX@FACHI treatment, including free DOX and α-BN-DOX were examined further in the heart, liver, spleen, lung, and kidney through a histopathological examination (Fig. S5). No signs of toxicity were observed in the excised organs from the mice treated with α-BN-DOX@FACHI, even those from free DOX and α-BN-DOX treatments (Table S2), highlighting the validity of the plug-in manufactured NDDS to minimize the toxicity from the blood circulation and accumulation in the organs.

When chemotherapeutic and photothermal effects are required for multimodal cancer treatments, applying an inorganic nanoparticle (enabling for surface plasmon resonance heating) generation device combined with the developed plug-in system showed strong potential. Fig. 7A presents the downsized integrated system. The system included an Au or Pt metal ablation device (Figs. S6A and S6B; drawing and digital image of the device) for continuous ultrasmall (~3 nm in diameter) Au or Pt particles (Fig. S6C; representative TEM images) generation before the electrically heated tubular reactor for incorporation with the BN precursor droplets from a vibrating nozzle, in which Au or Pt particles were attached to the droplets of the BN precursor solution and passed through the heated tubular reactor for the formation of α-BN-Au (or -Pt). The in-flight size distributions measured using an SMPS showed no significant differences between Au or Pt and α-BN-Au (or α-BN-Pt) (Fig. S6D), demonstrating the equivalent incorporation of the particles on α-BN platelets. The representative low- and high-magnification TEM images of α-BN-Au-DOX@FACHI NDDS (upper panels of Fig. 7B) exhibited face-centered cubic (fcc) Au dots on base α-BN from device integration, proving the validity of the addition of a metal ablation device to the developed plug-in system. Replacing Au with Pt for ablation...
as well as DOX and FACHI with cisplatin (cisPt) and bovine serum albumin (BSA) resulted in the formation of α-BN-Pt-cisPt@BSA NDDS (lower panels of Fig. 7B). Integration of the metal ablation device can enable an increase in the NIR laser-induced temperature (>60 °C at 1 W cm⁻² irradiation intensity for 3 min) of the NDDS (Fig. 7C) for chemotherapeutic thermal effects. These modifications of the developed plug-in product provide a proof-of-concept for a built-to-order supply of candidate NDDS with different architectures and compositions for ensuring autonomous cancer therapeutics (Fig. 7D).

4. Conclusions

As an integratable on-demand system with an autonomous platform, electrically operable spray pyrolysis and vibrating spray, including 185-nm photocharging, were combined serially for the single-pass assembly of α-BN-DOX@FACHI NDDS (~200 nm lateral dimension) in the aerosol state. The flows from the three solution reservoirs for base α-BN, DOX, and FACHI were digitally distributed to be combined as all-in-one droplets by passing through a vibrating spray nozzle for subsequent solvent extraction, resulting in the formation of α-BN-DOX@FACHI NDDS. In particular, mist droplets of an α-BN precursor solution entered an electrically heated tubular reactor. The droplets were then pyrolyzed and condensed into a turbostratic BN structure because of a short residence time (11 s) in the presence of ambient nitrogen gas flow. The DOX molecules were then layered on the base α-BN through electrostatic attractions, where negatively charged α-BN nanoplatelets due to 6.2 eV photon attack induced the attachment of positively charged groups of DOX on the surface of the platelets after dispersing the platelets in a DOX solution. A vibrating spray of a mixture of the platelet-dispersed DOX and FACHI solutions induced FACHI overlaying on α-BN-DOX and finally formed platelet-like α-BN-DOX@FACHI (12 mg min⁻¹ per single channel) after diffusion drying. The developed on-demand system could functionalize the stable-based materials and be workable for the tight conjugation of anticancer and targeting-antiangiogenic agents without significant alteration in the shape of the base material. For fundamental bioassays, the base platelets were amorphous to provide negatively charged sites for conjugation with no significant adverse effects on biosafety. The in vitro and in vivo experiments showed that the incorporation of DOX and FACHI onto the base platelets led to enhanced targetability to the folate receptors, decrease in opsonization and phagocytosis, pH-responsive DOX release, and the rapid induction of apoptosis compared to free DOX and α-BN-DOX while retaining the systemic safety to major organs because of the mild chemotherapeutic effects from the nontoxic and stable base material. An intervening electrical Au or Pt ablation device as well as replacing DOX and FACHI with cisPt and BSA in the on-demand system provided further photo-thermal temperature elevations (>60 °C) and different NDDS structures (α-BN-Au-DOX@FACHI [14 mg min⁻¹ per single channel] and α-BN-Pt-cisPt@BSA [15 mg min⁻¹ per single channel]). Therefore, the described streamlined electrically operable reaction ware could provide a reconfigurable, digitally realized design for candidate NDDS to be embodied in an autonomous platform for the desired delivery and therapeutic efficacy.

Credit author statement

Srijan Maharjan: Experiment, Analysis, and Writing – original draft.
Milan Gautam: Conceptualization, Experiment, and Analysis. Kishwor Poudel: Experiment and Analysis. Chul Soon Yong: Conceptualization, Analysis, and Editing. Saew Kang Ku: Experiment and Analysis for In Vivo. Jong Oh kim: Conceptualization, Analysis, Writing & review & editing, Supervision, and Funding acquisition. Jeong Hoon Byeon: Conceptualization, Investigation, Analysis, Writing – original draft, Writing – review & editing, Data curation, and Supervision.

Data availability

The raw data required to reproduce these findings are available to download from the supplementary information.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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