Endogenously Triggerable Ultrasmall-in-Nano Architectures: Targeting Assessment on 3D Pancreatic Carcinoma Spheroids

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Supporting Information

ABSTRACT: Several nanomaterials rely on the passive accumulation in the neoplasm target because of enhanced permeability and retention effect. On the other hand, directing nanomaterials to the target by employing the targeting agents may lead to a pivotal improvement in the efficacy of the treatment for a number of cancers. However, targeting moieties often lose their functionality upon injection in the bloodstream, leaving questions on their efficiency. Here, we assessed using a significant in vitro 3D model of pancreatic carcinoma the targeting efficiency of passion fruit-like nanoarchitectures (NAs) incorporated with a peptide that can recognize transferrin directly in the medium, thereby modulating protein solvation. NAs are biodegradable ultrasmall-in-nano platforms that combine the most appealing behaviors of noble metal nanomaterials with organism excretion of the building blocks by the renal pathway. Although the confocal images did not illustrate the significant differences in the targeting efficiency of the peptide-modified NAs, an improved internalization was quantitatively observed by inductively coupled plasma-mass spectrometry analysis. Our findings demonstrate that the peptide conjugation of NAs might be considered to enhance their theranostic potentials for this type of neoplasm.

INTRODUCTION

Accurate delivery of therapeutics to the site of interest is pivotal for the treatment of a number of diseases, among which are neoplasms, in order to enhance the efficacy while decreasing the adverse side effects. On the one hand, nanomaterials have commonly demonstrated an intrinsic ability to passively accumulate in a number of neoplasms because of the enhanced permeability and retention (EPR) effect. On the other hand, an increasing number of investigations have shed light on the fact that the EPR is a complex phenomenon that may not accurately represent the biological dynamics in humans. Thus, despite some drawbacks such as complex postprocessing procedures, additional production cost, and uncertain efficiency, a number of targeting agents have been developed and conjugated to nanomaterials. An elegant approach to enhance the targeting efficacy of nanomaterials is through modulation of their protein corona composition, which leads to an endogenously triggered selectivity. Meanwhile, besides localized accumulation of nanomaterials to the target, other concerns have hampered the translation to clinical practice of almost all the candidates developed in the last 30 years. This is especially true for noble metal nanoparticles (NPs), which still remain confined to the bench-side because of the concerns on undefined persistence, despite a number of appealing features for theranostics. The combination of the behaviors of NPs with the metal excretion from the renal pathway was recently demonstrated by the platforms developed within the ultrasmall-in-nano approach. Passion fruit-like nanoarchitectures (NAs) are promising examples of nature-inspired ultrasmall-in-nano platform that may bring again NPs to the forefront of cancer theranostics by overcoming the key question of regulatory agencies regarding the metal persistence after the designed action. NAs are biodegradable silica nanocapsules comprising gold ultrasmall NPs (USNPs) embedded in a polymeric functional matrix. In cellular environment, NAs disassemble to biocompatible and renal clearable building blocks in 48 h: silicic acid, polymers, and endogenous glutathione (GSH) coated gold USNP. Furthermore, the impressive versatility and robustness of the production protocol was already demonstrated together with some of their potential applications as drug delivery vehicles and dual photoacoustic/ultrasound imaging contrast agents, pointing out that all the components of NAs are intrinsically essential.

Here, we utilized the straightforwardly modifiable surface of NAs to perform a click chemistry conjugation of the NPs to a customized peptide. The employed peptide (Tf2, sequence:...
CGGGHKYLRW) binds with transferrin ($K_d = 0.90 \pm 0.25 \mu M$) and was designed to target cells overexpressing the transferrin receptor. Moreover, Tf2 is capable of endogenously triggering an enhanced accumulation of NAs in neoplasms by the protein corona modulation. The cell targeting and internalization performance of peptide-functionalized NAs were quantitatively assessed on significant in vitro three-dimensional (3D) models of pancreatic carcinoma. We have developed 3D spheroids because of their closer resemblance to in vivo models compared to conventional two-dimensional (2D) monolayer cell cultures. The advantages of using 3D spheroids include the establishment of tumor microenvironment, physiochemical gradients, and cell−cell/matrix interactions. In addition, in vitro 3D models are in agreement with the development and promotion of alternatives to animal experiments according to the "3Rs concept."20

RESULTS AND DISCUSSION

The 3D cultures of MIA PaCa-2 were prepared by a modified hanging method and maintained in an incubator with an orbital shaker. It was worth noticing that the orbital shaking was crucial for the maintenance of the 3D cultures to avoid monolayer proliferation. The 3D cell cultures can be maintained for up to 1 month, and the diameter of the spheroids range from 650 $\mu m$ to about 1 mm. In order to improve the consistency of our investigation, the 3D spheroids were maintained for 12−14 days, reaching an average diameter of 950 $\mu m$. In Figure 1A, the bright-field microscopy image of a typical 3D MIA PaCa-2 spheroid is reported, demonstrating the concentric arrangement of viable and necrotic cells. Moreover, the 3D spheroids were observed to be a composite of spherical cells with an average size of 17.1 $\pm$ 2.9 $\mu m$. In Figure 1B,C, the orthogonal view (Figure S1A) illustrated the spherical construction of the cells more evidently. For comparison, we also cultured MIA PaCa-2 cells in a standard 2D monolayer fashion (Figure 1D,E), demonstrating an epithelial-like growth. The transmission electron microscopy

Figure 1. (A) Bright-field microscopy image of the 3D MIA PaCa-2 cell culture prepared through the hanging method (scale bar: 100 $\mu m$). Zoomed-in bright-field (B) and confocal (C) microscopy images of the MIA PaCa-2 cultured in 3D (scale bars: 10 $\mu m$). Bright field (D) and confocal (E) microscopy images of MIA PaCa-2 cultured in 2D (scale bar: 10 $\mu m$). The nuclei (blue) and the cell membranes (green) were stained with Hoechst 33342 and CellMask green plasma membrane stain, respectively.

Figure 2. (A) Scheme of the synthesis of NAs-647−Tf2. Gold USNPs were embedded in AlexaFluor-647-modified poly(L-lysine). The polymeric aggregates were employed as templates to form hollow silica nanocapsules (NAs) by a modified Stöber method. The surface of NAs was modified by silane−PEG−maleimide linkers. Finally, NAs were click-chemical functionalized by the peptide Tf2, and GSH was added to block the excess maleimide ends. (B) Background-subtracted UV−vis spectrum of standard NAs (the continuous black line) and NAs-647 (the red dashed line). Measurements were performed in PBS. (C) TEM images of NAs (left), NAs-647 (middle), and GSH-treated NAs-647−Tf2 (right). The Gray matrix is due to a not-completely-dry sample during the imaging. Scale bar: 50 nm.
(TEM) ultrastructure analysis confirmed that the morphology of the MIA PaCa-2 cells was similar in both 2D and 3D cultures (Figure S2A,B). Interestingly, cells composing the 3D models were interconnected mostly by an early tight junction (Figure S2C,D). Signs of mild hypoxia were also observed (Figure S2C).

Passion fruit-like NAs were produced by employing the standard protocol.13 The general synthesis scheme to obtain about 100 nm NAs (Figure S3A) is illustrated in Figure 2A. To track NAs during the cellular investigations, commercial AlexaFluor-647 was covalently linked to the polymer [poly-(l-lysine); 15−30 kDa] prior to its use to synthesize the dye-loaded NAs (NAs-647).15 As expected, the absorbance spectrum of NAs-647 (Figure 2B) showed the presence of bands because of the dye and the localized surface plasmon resonance (LSPR) of gold USNPs. Only the absorbance due to LSPR was observed on the spectrum of the standard NAs. Furthermore, previous surface-enhanced Raman spectroscopy investigations have demonstrated that the dyes were comprised in the cavity and not on the surface of NAs.13 The surface modification of NAs-647 was performed employing a 2 kDa silane−poly(ethylene glycol) (PEG)−maleimide linker in order to (i) incorporate a thiol-reactive group on the surface of NAs through a single postprocessing step and (ii) utilize the benefits of PEGylation on nanoparticle-based theranostics.22 Then, the click chemistry conjugation of Tf2 peptide to NAs was performed through a maleimide−thiol reaction. The potential free-maleimide groups left on the surface of NAs were blocked by employing GSH. The changes in the zeta potential values after each step confirmed the success of the procedure (Figure S3B). Indeed, the zeta potential for NAs-647 was −21.3 ± 0.6 mV and increased to −7.23 ± 0.41 mV for NAs-647−Tf2 because of the presence of the peptide. GSH blocking slightly decreased the zeta potential to −8.09 ± 0.53 mV, confirming the presence of some free-maleimide groups after the reaction with Tf2, to which GSH subsequently reacted with. All cellular experiments were performed using GSH-treated NAs-647−Tf2. Remarkably, the TEM images (Figure 2C) highlighted that the postprocessing steps did not affect the physical structure of the NAs. NAs, NAs-647, and NAs-647−Tf2 maintained the “passion fruit-like arrangement,” and no significant structural modifications were recognized. To further ensure the success of the functionalization procedure, the maleimide−thiol reaction was also performed using a thiol-modified Atto-425 instead of Tf2 (NAs-647−SH-Atto425). The same dye containing a carboxylic acid functional group was employed to serve as the control sample (NAs-647/Atto425-COOH). The confocal images after incubation of 3D MIA PaCa-2 models with both samples (Figure S4) confirmed through Pearson’s coefficient the higher colocalization of NAs-647 and Atto-425 in NAs-647−SH-Atto425 compared to the control NAs-647/Atto425-COOH.

NAs-647 and NAs-647−Tf2 were incubated for 2 h with 3D MIA PaCa-2 models, and confocal images were collected from 0 to 72 h postincubation (Figure 3A). At t = 0 h, both NAs attached on the cell membranes resulting in individual dots of similar size (inset of Figure 3A). After 24 h, the signals from both types of NAs appeared more aggregated and more colocalized inside the cells, highlighting an internalization process. Overall, no qualitative differences on the targeting efficiency were distinguished between NAs-647 and NAs-647−Tf2. Interestingly, the intensity of the signals from the dye increased for both samples at 48 and 72 h time points. This can be related to the changes in the structures of NAs-647 and NAs-647−Tf2, implying their biodegradation. Indeed, at time 0 and 24 h, the NAs were intact, and the dye was quenched...
because of the proximity to the gold USNPs, leading to reduced emission intensity.14 On the other hand, at later time points, NAs disassembled, allowing the dye to increase the distance from gold USNPs surfaces. This observation was further supported by the decreasing amount of gold quantified through inductively coupled plasma-mass spectrometry (ICP-MS). It is worth noticing that the imaging quality in confocal microscopy is dependent on the sample depth. Cells and NAs located in the inner part of the 3D cell constructs were not clearly imaged. Therefore, analytical instruments, such as ICP-MS, provide complementary data that take into consideration the total gold content of the whole 3D cell construct, for a quantitative analysis of the samples. Furthermore, the collected data can be normalized for both the gold loaded in the nanomaterials and the protein content of the 3D cell constructs, in order to obtain a more accurate comparison of the targeting and internalization efficiencies of nanomaterials. Indeed, the collected amount of gold from both NAs-647 and NAs-647–Tf2 was double-normalized to the total protein content and to the starting amount of gold on each NAs where the 3D spheroids were incubated (Figure 3B). NAs-647–Tf2 resulted to an average of 1.5× higher internalization with respect to NAs-647. Overall, the surface functionalization and peptide conjugation of NAs may not have generated a substantial improvement in the targeting efficiency of NAs but led to an increased particle cell internalization. On the other hand, the significant internalization of nontargeted NAs-647 may be due to the ability of the MIA PaCa-2 cells to uptake materials from the environment even if organized in 3D models. Apart from enhanced internalization, NAs-647–Tf2 also demonstrated intracellular retention. This effect can be related to the transferrin receptor-mediated endocytosis, which may also have a different intracellular fate compared to the nonspecific endocytosis of unfunctionalized particles.53

■ CONCLUSIONS

In summary, we have evaluated the targeting and internalization efficiency of the peptide-functionalized passion fruit-like NAs on 3D pancreatic carcinoma models. The confocal microscopy images confirmed the cell localization and internalization of both targeted and nontargeted NAs after 2 h of incubation. Furthermore, we have quantified an increased cell internalization of the peptide-conjugated NA. Thus, this approach can be considered to improve nanoparticle internalization for this kind of neoplasm, regardless of the concerns with targeting moiety conjugation in terms of additional procedures and cost.

■ EXPERIMENTAL SECTION

Materials. Alexa-647 was purchased from Invitrogen, and silane–PEG–maleimide (2 kDa) was from Nanocs. All other chemicals were purchased from Sigma-Aldrich, unless otherwise specified. All chemicals were used as received.

Synthesis of Passion Fruit-Like NAs. Synthesis of Dye-Modified Poly(lysine). Poly(lysine) hydrobromide (15–30 kDa) was dissolved in Milli-Q water to a final concentration of 20 mg/mL. From this stock solution, 150 μL was added with AlexaFluor-647-NHS ester (200 μg) in phosphate-buffered saline (PBS) (200 μL). The solution was stirred overnight at room temperature and used without further purification.

Synthesis of Gold USNPs. Gold USNPs with a diameter of approximately 3 nm were prepared by mixing Milli-Q water (20 mL), poly(sodium 4-styrenesulfonate) (70 kDa, 30% aqueous solution), and HAuCl4 solution (10 mg/mL in Milli-Q). The solution was stirred vigorously while adding 200 μL of 8 mg/mL NaBH4. The solution was allowed to further mix for 2 min and aged for another 10 min.

Synthesis of Gold Nanoparticle Arrays. Poly(lysine) (200 μL), plain or dye-modified, was added to the solution containing the gold USNPs. The solution was stirred for 20 min. The gold aggregates were collected through centrifugation at 13 400 rpm for 3 min. The product was resuspended in Milli-Q water (2 mL) and sonicated for a maximum of 4 min.

Synthesis of NAs or NAs-647. The synthesized gold nanoparticle arrays (2 mL in Milli-Q) were added to a solution containing absolute ethanol (70 mL), ammonium hydroxide solution (2.4 mL, 30% in water), and tetraethyl orthosilicate (40 μL, 98%). The solution was allowed to gently shake for 3 h. The products, NAs or NAs-647, were collected by centrifugation at 4000 rpm for 30 min. Particles were washed twice with ethanol to remove unreacted precursors. A short-spin centrifugation was done to separate particles with the diameter greater than 200 nm. The supernatant was then collected and centrifuged again at 13 400 rpm for 2.5 min. The final product was stored in 1 mL of ethanol.

Surface Modification and Functionalization of NAs-647. NAs-647 was added to ethanol (5 mL) with a silane–PEG–maleimide linker (1 mL, 400 μg/mL). The volume was adjusted to 10 mL using ethanol, and the solution was stirred vigorously for 15 min. The particles were recovered by centrifugation at 13 400 rpm for 5 min and washed with ethanol twice. Then, the particles were resuspended in N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES) buffer (1.25 mL, 20 mM, pH 7.2) and sonicated. The Tf peptide was then added to the solution elsewhere.6 The peptide (250 μL, 400 μg/mL in HEPES buffer) was added, and the solution was stirred for 2 h. t-GSH (500 μL, 200 μg/mL in HEPES buffer) was then added to the solution to react with the excess of maleimide groups. The reaction was stirred for another hour. NAs-647–Tf2 was recovered by centrifugation at 13 400 rpm for 3 min. Particles were washed twice in ethanol. The final product was stored in ethanol to maintain sterility and sonicated for 5 min.

UV–Vis Spectrophotometry. The absorption spectra were obtained using a double-beam Jasco V-550 spectrophotometer. The samples in PBS (1×) were placed in quartz cuvettes with a 1.5 mm path length.

Electron Microscopy. The TEM observations of particles and cells were performed in ZEISS Libra 120 and operated at an accelerating voltage of 120 kV. The colloidal solutions of the NPs (5 μL) were deposited to a 300-mesh of carbon-coated copper grids.

Ultrastructure Analysis of 3D MIA PaCa-2. The MIA PaCa-2 3D spheroids in the suspension have been fixed in 2% glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.4) for 1 h at room temperature and then treated for resin embedding as previously reported.54 Briefly, the recovered spheroids were kept in a new fixative solution (overnight at 4°C), then the cells were postfixed 1 h (2% OsO4 in sodium cacodylate buffer; 0.1 M pH 7.4), and stained with 3% solution of uranyl acetate in 20% ethanol. Finally, they were dehydrated and embedded in epoxy resin (Epon 812, Electron Microscopy Science, Hatfield, PA, USA). Polymerization has been performed for 48 h at 60°C. In order to perform ultrastructural analysis, 90 nm

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sections of the treated samples were cut by using UC7 (Leica Microsystems, Vienna, Austria).

Zeta Potential Measurement. The zeta potential measurements were performed on Malvern Zetasizer nano ZS90 using a DTS 1060 standard capillary cell. During measurements, the NPs were resuspended in PBS (2 mL) at pH 7.4 and sonicated for 5 min. The reported values are the average of five consecutive measurements.

3D Cell Culture. Human pancreatic carcinoma cells (MIA PaCa-2) were purchased from the American Type Culture Collection. The cells cultured in 2D were maintained in a Dulbecco’s modified Eagle medium from Invitrogen (Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 4 mmol/L l-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). 3D cell constructs were prepared as reported elsewhere, with slight modifications. B briefly, cells cultured in 2D were washed with PBS and detached from the culture plate using trypsin–ethylenediaminetetraacetic acid (Invitrogen). Cells were settled by centrifugation at 200g for 5 min. Fresh culture medium was added. The cells were counted, and the concentration was adjusted to 1 × 10^6 cells/mL. After homogenizing, 10 μL of cell suspension drops were placed on the lid of a 35 mm cell culture dish. The lid was flipped into the chamber containing 2 mL of PBS. The cells were allowed to settle at the bottom of the drops and were transferred to a 35 mm suspension/nontreated culture dish after 24 h. The cells were finally placed inside a CO_2 incubator with an orbital shaker (90 rpm).

Cell Incubation in NAs. In a 500 μL plastic vial, a 200 μL aliquot of NAs-647 or NAs-647−Tf2 stored in ethanol was added. The particles were collected by centrifugation, resuspended in a complete medium (200 μL), and sonicated for 5 min. NAs were then added to a 2 mL plastic vial containing 800 μL of the complete medium and the 3D models, which were transferred using 1 mL pipet tips. The vials were incubated for 2 h at 37 °C and 5% CO_2. After that, the cells were washed with PBS twice. All the 3D models were transferred to a 35 mm nontreated culture dish with 3 mL of the complete medium. The cells evaluated on different time points were taken from the same culture dish.

Confocal Microscopy. In a 2 mL plastic vial, four to six cell constructs were collected using 1 mL pipet tips. The cells were settled and washed in PBS, whereas in 100 μL of PBS, Hoechst 33342 (Invitrogen; 80 μg/mL) and CellMask green plasma membrane stain (Invitrogen; 10 μg/mL) were added, and the cells were incubated at 37 °C and 5% CO_2 for 15 min. Afterward, the cells were washed twice with PBS, transferred to a LabTek 8-well chambered cover glass, and added with a 1:1 solution of 70% glycerol and FBS. Imaging was performed on an Olympus FV1000 inverted confocal laser scanning microscope equipped with a thermostat chamber set at 37 °C and 5% CO_2. The lasers for excitation were 405, 488, and 633 nm. The imaging was done 0, 24, 48, and 72 h after incubation. All images were analyzed using Fiji-ImageJ software version 1.51s.

Bradford Assay. In a 2 mL plastic vial, four to six cell constructs were collected using 1 mL pipet tips. The cells were settled and washed three times with cold PBS. For the final washing, the cells were centrifuged at 13 400 rpm, 4 °C for 10 min. The supernatant was removed, and the cells were added with 50 μL of lysis buffer. The samples were agitated for 1 h. The lysates were separated by centrifugation at 13 400 rpm, 4 °C for 10 min. The pellets were saved for gold quantification. Bradford assay samples were prepared in 1:5 dilution in PBS. The calibration curve was prepared using bovine serum albumin standards. Samples, calibration solution, and blank (3 μL) were placed on 96-well plates, and 150 μL of Bradford assay solution (Thermo Fisher Scientific) was added. The plate was incubated for 10 min at room temperature, and the absorbance at 595 nm was recorded.

ICP-MS Analysis. Lysates and pellets were combined, dissolved in 1 mL ICP-MS-grade HNO_3, and digested by microwave irradiation (200 °C/15 min) in borsilicate glass vessels. An aliquot (200 μL) of the digested sample solution was diluted to 2 mL with ICP-MS-grade water. The content of gold was determined by ICP-MS analysis against a standard calibration curve.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01719.

Orthogonal view and bright-field microscopy image of 3D MIA PaCa-2, TEM ultrastructure of 3D MIA PaCa-2; size histograms and zeta potential values of NAs-647, NAs-647−Tf2 without GSH, and NAs-647−Tf2/GSH; and confocal microscopy images of 3D MIA PaCa-2 employed for double-color analysis (PDF).
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