Drug Delivery

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Reactive Oxygen Species-Responsive Protein Modification and Its Intracellular Delivery for Targeted Cancer Therapy**

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Dedicated to Professor George M. Whitesides on the occasion of his 75th birthday

Abstract: *Herein we report a convenient chemical approach to reversibly modulate protein (RNase A) function and develop a protein that is responsive to reactive oxygen species (ROS) for targeted cancer therapy. The conjugation of RNase A with 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzyl carbonate (NBC) blocks protein lysine and temporarily deactivates the protein. However, the treatment of RNase A–NBC with hydrogen peroxide (one major intracellular ROS) efficiently cleaves the NBC conjugation and restores the RNase A activity. Thus, RNase A–NBC can be reactivated inside tumor cells by high levels of intracellular ROS, thereby restoring the cytotoxicity of RNase A for cancer therapy. Due to higher ROS levels inside tumor cells compared to healthy cells, and the resulting different levels of RNase A–NBC reactivation, RNase A–NBC shows a significant specific cytotoxicity against tumor cells.*

Precise and spatiotemporal modulation of protein function inside cells is an appealing tool for both analytic and therapeutic applications.[1] Several genetic[2] and chemical engineering[3] approaches have recently been developed for the noninvasive modulation of protein functions within cells. Among them, chemical protection and deprotection strategies have served as efficient methods to design stimulus-responsive proteins and precisely control protein function.[4] For example, the chemical conjugation of luciferase with *ortho*-nitrobenzyl groups generated photoresponsive proteins, thereby enabling the fast activation of intracellular protein function using UV light irradiation.[5] Similarly, propargyloxycarbonyl-mediated deactivation of proteins allowed palladium-triggered protein reactivation in living cells.[6] Though both of these approaches can control protein activity with high efficiency and specificity, each has limited clinical applications, owing to the poor tissue penetration of UV light and the inherent toxicity of heavy metal ions. Therefore, the development of new approaches to modulate protein functions without external stimulus is necessary, particularly modulations responsive to inherent pathological microenvironments for disease treatment.

Mounting evidence suggests that, compared with their normal counterparts, many types of tumor cells and tissues have increased levels of reactive oxygen species (ROS).[7] These increased levels of ROS provide a biochemical basis for developing novel therapeutic strategies exploiting ROS for targeted cancer treatment.[8] In this report, we successfully modified the cytotoxic protein ribonuclease A (RNase A) as part of such a strategy. Our chosen chemical modification of RNase A is designed to be reversible in the presence of high levels of ROS. Therefore, cells with high levels of ROS (e.g., cancer cells) can restore the biological function of modified RNase A. We further demonstrated that the delivery of ROS-responsive RNase A, using synthetic cationic lipid-like nanoparticles enabled cell killing in response to the elevated ROS level inside cancer cells, whereas remaining nontoxic in healthy cells.

RNase A can cleave RNA and induce cytotoxic effects when taken up by cells.[9] It has entered clinical trials as an alternative protein therapeutic approach for treating cancer patients refractory to traditional modalities of treatment, including surgery, radiation therapy, and chemotherapy.[10] RNase A was therefore selected as a model drug to demonstrate the potential advantages of ROS-responsive chemical modification of proteins for targeted cancer therapy. In our approach, RNase A was first modified with 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (NBC), through which the protein's lysine residue was conjugated with an aryl boronic ester through a covalent carbamate linker (Scheme 1a). The conjugated boronate ester is unstable in aqueous solutions, and is quickly hydrolyzed into aryl boronic acid,[11] yielding boronic acid modified RNase A, referred to RNase A–NBC. To illustrate the key role of the aryl boronic acid in designing a ROS-responsive protein, a similar approach was used to prepare RNase A–NC, which lacks the boronic acid moiety (Scheme 1b). The treatment of RNase A–NBC with hydrogen peroxide (H_2O_2, one major intracellular ROS) triggers a self-immolative reaction, releasing lysine and restoring protein function (Scheme 1a).[8a]

It has been reported that lysine residues of RNase A are essential to its activity;[9b] therefore, we hypothesized that lysine conjugation with NBC and subsequent ROS-triggered NBC cleavage will reversibly regulate the biological function of RNase A. Furthermore, the conjugation of RNase A with aryl boronic acid decreases the isoelectric point (pI) of RNase A, further potentiating its electrostatic interactions

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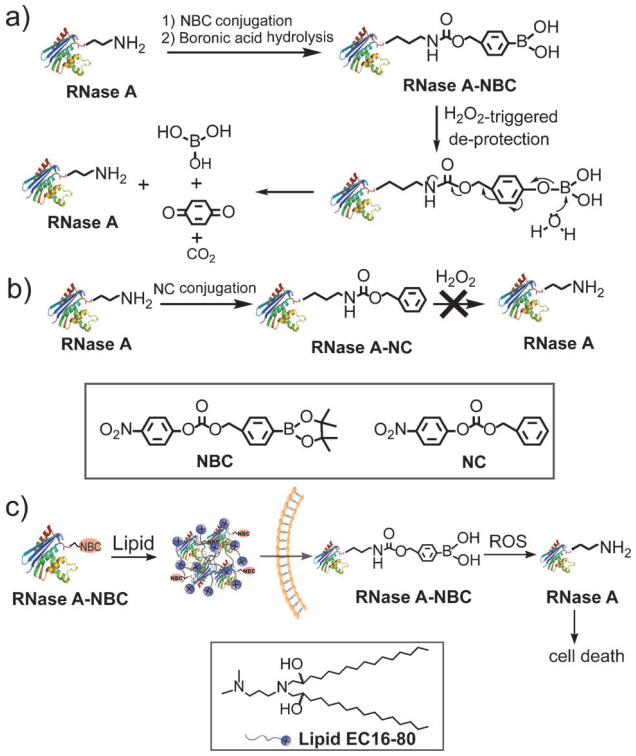

Scheme 1. a) RNase A lysine modification with NBC for ROS-responsive and b) with NC for nonresponsive protein engineering. c) Encapsulation of RNase A–NBC into cationic lipid-like nanoparticles for intracellular protein delivery.

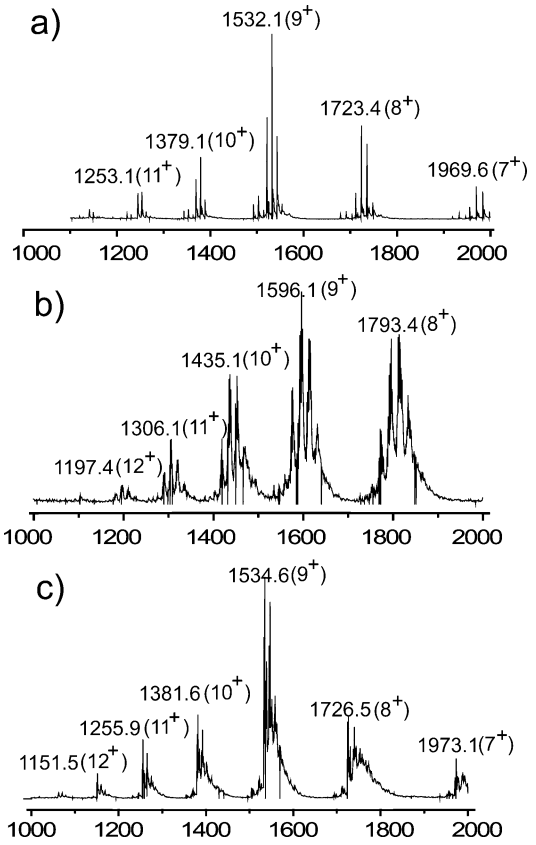

Figure 1. ESI mass spectra of a) native RNase A, b) RNase A–NBC with H_2O_2 treatment, and c) RNase A–NBC without H_2O_2 treatment.

with positively charged lipid nanoparticles (Scheme 1c) for enhanced intracellular delivery.[12] We hypothesized that after entering cancer cells, RNase A–NBC will be released from the nanoparticles and reactivated by intracellular ROS to inhibit tumor cell proliferation.

We prepared RNase A–NBC and RNase A–NC by reacting native RNase A with an excess amount of either NBC or NC (benzyl 4-nitrophenyl carbonate), respectively, in sodium bicarbonate buffered solutions (0.1M, pH 8.5), followed by ultrafiltration to purify the modified proteins (see experimental details in the Supporting Information, SI). The conjugation reactions provided both RNase A–NBC and RNase A–NC with a mean amount of seven NBC/NC conjugations per protein molecule, as calculated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure S1, SI). The ROS-responsive behavior of RNase A–NBC was investigated and confirmed using electron spray ionization mass spectrometry (ESI-MS) analysis. As shown in Figure 1, mass peaks ascribable to seven to eleven plus fragments were observed for native RNase A. NBC (Figure 1) or NC (Figure S2, SI) conjugation increased the RNase A molecular weights, as evidenced by the right-shifted mass peaks of RNase A–NBC and RNase A–NC. After H_2O_2 treatment, ESI-MS of RNase A–NBC yielded m/z values corresponding to native RNase A (Figure 1a), indicating that the H_2O_2 treatment cleaved NBC conjugation and restored RNase A–NBC. However, no mass peak shifts were observed when RNase A–NC was similarly treated with H_2O_2 (Figure S2, SI), suggesting RNase A–NC is not ROS-responsive, and demonstrating the particular utility of NBC conjugation for the design of ROS-responsive proteins.

The efficiency of both aryl boronic acid conjugation to RNase A and H_2O_2-triggered NBC cleavage was further confirmed and analyzed by a fluorescence assay based on Alizarin red S (ARS), which binds aryl boronic acid through its catechol moiety, generating a fluorescent complex.[13] As shown in Figure 2a, the addition of RNase A or RNase A–NC (0.25 mg mL^{-1}) to an ARS solution (0.0025% w/v) had no effect on the fluorescence of ARS, which was an expected result due to the lack of boronic acid present in native RNase A or RNase A–NC. With the addition of RNase A–NBC, ARS emission was enhanced up to 18-fold, indicating the conjugated NBC group in RNase A–NBC complexes with ARS, enhancing ARS fluorescence. However, pretreatment of RNase A–NBC with H_2O_2 eliminated ARS emission enhancement, indicating that ARS did not bind with treated RNase A–NBC, due to boronic acid elimination. Combined with the previous MS analysis, the conjugation of RNase A with NBC was confirmed to be ROS-responsive; modified protein RNase A–NBC is restorable to RNase A with H_2O_2 treatment.

The enzymatic activity of RNase A–NBC and RNase A–NC was studied to evaluate whether the RNase A function could be reliably regulated by NBC or NC conjugation and subsequent H_2O_2-triggered NBC cleavage. The conjugation of

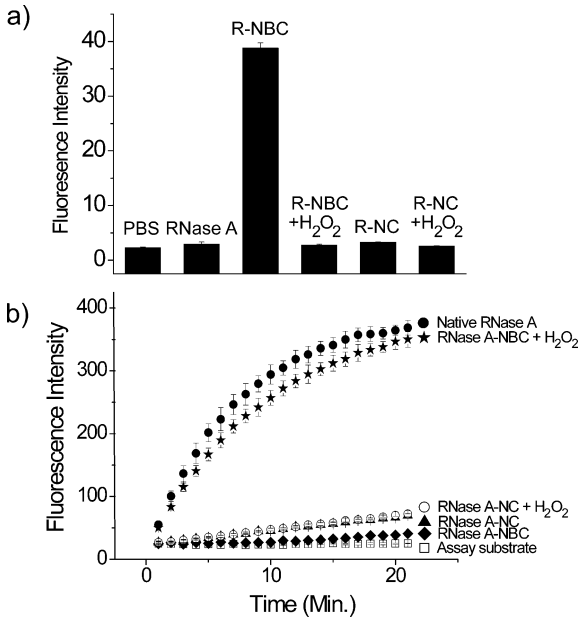

Figure 2. a) Fluorescence of Alizarin red S (ARS, 0.0025% w/v) in the presence of PBS, native RNase A, RNase A–NBC (R-NBC), RNase A–NC (R-NC), and the above proteins pretreated with 5 mM H_2O_2. b) RNase A activity assay of native RNase A, RNase A–NBC and RNase A–NC with and without H_2O_2 (5 mM) treatment.

RNase A with NBC or NC decreased the enzymatic activity to 5% or 15% of the native protein (Figure 2b), respectively, due to the blocking of lysine residues essential to RNase A activity. Treatment of RNase A–NBC with H_2O_2 restored the protein activity to 95% of its native counterpart (Figure 2b). However, no activity restoration was observed when RNase A–NC was exposed to H_2O_2 under the same conditions. Additionally, RNase A–NBC has a high selectivity toward H_2O_2 activation over other biologically relevant ROS, including *tert*-butyl hydroperoxide (TBHP), hypochlorite (ClO^-), and nitroxide radical (NO·). As shown in Figure S3 (SI), when RNase A–NBC was treated with varied types of ROS before an enzyme activity assay, only H_2O_2 treatment significantly restored the RNase A activity. In contrast, other selected ROS as well as H_2O_2 in the presence of catalase (a common biological peroxidase) had little to no effect on the RNase A–NBC activation. Thus, the RNase A activity assay suggested that the chemical conjugation of RNase A lysine with NBC effectively deactivated RNase A, which could subsequently be reactivated by H_2O_2-triggered NBC deprotection.

The ROS-responsive nature of RNase A–NBC prompted an examination of whether RNase A–NBC could be activated by high levels of ROS inside tumor cells to specifically inhibit cell proliferation, thereby serving as a tool for targeted cancer therapy. We have recently reported the efficient intracellular delivery of RNase A for cancer therapy by chemical modification: acid-reversible *cis*-aconitic conjugation facilitated RNase A complexation with cationic lipid-like nanoparticles by electrostatic interactions.[12d] We hypothesized that NBC conjugation would operate in a similar fashion: RNase A lysine conjugation with NBC would reverse the positive charge of RNase A, potentiating the electrostatic binding of RNase A–NBC with cationic lipid nanoparticles (Scheme 1c) for intracellular protein delivery. The net surface charge of RNase A before and after NBC conjugation was measured and confirmed by capillary isoelectric electrophoresis focusing (cIEF) analysis, a fast and high-resolution, isoelectric point(pI)-based technique to analyze proteins.[14] The pI value of native RNase A was determined to be 9.6 by cIEF analysis, close to the reported and theoretical values.[15] NBC conjugation, however, significantly decreased the pI of the RNase A–NBC conjugate to a wide distribution between 3 and 6, due to varying NBC conjugation intensity of RNase A molecules (Figure S4, SI). The cIEF study suggests substantial charge conversion of RNase A after NBC conjugation.

Complexes of RNase A–NBC and cationic lipid-like nanoparticles were characterized by dynamic light scattering (DLS) to determine nanoparticle size and surface charge. Cationic lipid EC16-80 (Scheme 1c), which we have recently identified as a leading lipid-like materials for protein delivery from a combinatorial library,[12d] was selected for chemically modified ROS-responsive protein delivery in this study. The addition of RNase A–NBC or RNase A–NC into empty EC16-80 nanoparticles had negligible effect on nanoparticle sizes: all had nanoparticle sizes around 150 nm in diameter (Figure S5, SI). However, the zeta-potential of EC16-80 nanoparticles was decreased from 38.9 ± 6.2 mV to 20.9 ± 2.4 mV or 13.1 ± 1.9 mV with the addition of RNase A–NBC or RNase A–NC, respectively. The EC16-80 nanoparticle surface charge changes confirmed that the complexation of RNase A–NBC or RNase A–NC with EC16-80 nanoparticles was mainly through electrostatic interactions, lowering the positive charge of EC16-80 nanoparticles. The encapsulation of RNase A–NBC into EC16-80 nanoparticles was further confirmed by ARS fluorescence assay, as protein–lipid complexation embedded aryl boronic acid and prevented its binding with ARS. As shown in Figure S5 (SI), EC16-80 nanoparticles had negligible effect on enhancing ARS emission, due to the lack of aryl boronic acid in EC16-80 to bind ARS. However, the strong emission of RNase A–NBC/ARS complex was also quenched when RNase A–NBC was precomplexed with EC16-80. This finding suggests that RNase A–NBC is efficiently encapsulated into EC16-80 nanoparticles, preventing the binding of NBC moieties to ARS.

Successful intracellular delivery of RNas A–NBC, along with an assessment of its capability to inhibit tumor cell proliferation, was investigated by treating murine melanoma cells (B16F10) with various EC16-80/protein nanoparticles. The efficient delivery of RNase A–NBC and RNase A–NC nanocomplexes into B16F10 cells was examined by treating cells with fluorescein isothiocyanate (FITC)-labelled protein/EC16-80 complexes, followed by an uptake efficiency measurement using flow cytometry analysis. As shown in Figure S6 (SI), B16F10 cells treated with FITC-labelled EC16-80/RNase A–NBC and EC16-80/RNase A–NC complexes had comparable uptake efficiency, with more than 90% of the cells taking up FITC-labelled protein. However, cells treated with fluorescent EC16-80/RNase A nanoparticle had only 40% uptake efficiency, arising from the lack of efficient

complexation of unmodified RNase A with lipid for intracellular delivery.[12d] The viability of B16F10 cells following different treatments was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and compared to that of untreated controls. As shown in Figure 3a, single-component treatment of EC16-80 (at a concentration of 2.6 μg mL^{-1}), RNase A, RNase A–NC, or RNase A–NBC (all at 1.3 μg mL^{-1}) resulted in a viability

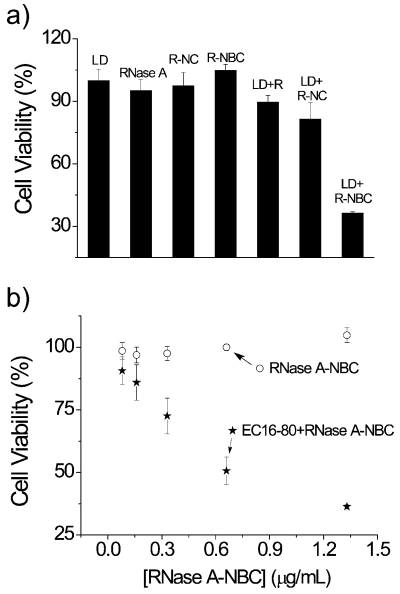

Figure 3. a) Viability of B16F10 cells treated with EC16-80 (LD), naked RNase A, RNase A–NBC (R-NBC), RNase A–NC (R-NC), or the three proteins precomplexed with lipid nanoparticles EC16-80. b) Cytotoxicity of EC16-80/RNase A–NBC nanoparticles against B16F10 cell proliferation in dependence on the protein concentration.

greater than 90%, demonstrating the low cytotoxicity of both empty lipid nanoparticles and free protein against cell proliferation. EC16-80/RNase A complex had no ability to deliver RNase A and inhibit tumor cell growth due to the lack of efficient protein encapsulation for delivery. Treatment of cells with the EC16-80/RNase A–NBC complex, however, decreased the cell viability down to 36%, with significantly higher efficiency than that of EC16-80/RNase A–NC complex, which only decreased the viability to 80% (Figure 3a). Bearing out these preliminary results, EC16-80/RNase A–NBC nanoparticles inhibited B16F10 proliferation in a protein-concentration-dependent manner (Figure 3b). We ascribe the improved ability of RNase A–NBC to inhibit cell proliferation over RNase A–NC to differential protein activation inside tumor cells. After entering cells, RNase A–NBC was reactivated by high levels of intracellular H_2O_2 and thus regained cytotoxicity. RNase A–NC, however, is not ROS-responsive and was not efficiently activated in the same intracellular environment, leading to a significantly less inhibited cell proliferation.

To further confirm the key role of intracellular ROS in activating RNase A–NBC inside cells, the effect of the intracellular ROS level on the cytotoxicity of EC16-80/RNase A–NBC was studied. To this end, B16F10 cells were treated with phorbol 12-myristate 13-acetate (PMA) to accelerate the generation of ROS, followed by 2′,7′-dichlorofluorescin diacetate (DCFDA) staining and flow cytometry analysis to measure their intracellular ROS level.[16] As shown in Figure S7 (SI), the treatment of B16F10 cells with PMA (200 nM) significantly increased the intracellular ROS level, compared to cells without PMA treatment. With 4 h incubation of EC16-80/RNase A–NBC nanoparticles (1.3 μg mL^{-1} of protein), the viability of B16F10 cells with further PMA (200 nM) exposure was decreased down to 50% of untreated controls, compared to 75% cell viability without PMA exposure (PMA treatment alone had no effect on prohibiting B16F10 cell proliferation (Figure 4a). When the concentra-

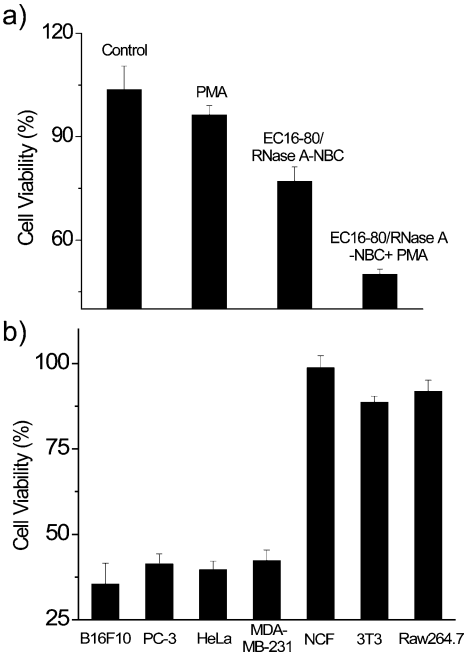

Figure 4. a) Viability of EC16-80/RNase A–NBC pretreated B16F10 cells with and without the second PMA (200 nM) treatment. b) EC16-80/RNase A–NBC delivery selectively prohibited tumor cell proliferation.

tion of RNase A–NBC administered to cells was increased from 0.08 to 1.33 μg mL^{-1}, PMA-treated cells continued to have lower viability compared to cells without PMA treatment (Figure S8, SI). The higher cytotoxicity of RNase A–NBC against PMA-treated B16F10 cells suggests that enhanced intracellular ROS level activated RNase A–NBC in an enhanced fashion, which subsequently induced an increased cytotoxicity.

Finally, to demonstrate the potential of ROS-responsive RNase A–NBC as a novel approach for targeted cancer therapy, the capability of EC16-80/RNase A–NBC nanoparticles to selectively inhibit tumor cell proliferation was studied by testing a panel of noncancerous and cancer cell lines. In this study, three noncancer cell lines, including NIH-3T3 (embryonic fibroblast cells), Raw264.7 (murine macrophages), and NCF (neonatal rat cardiac fibroblast) were

exposed to EC16-80/RNase A–NBC nanoparticles under the same conditions as a panel of cancer cell lines (HeLa cervical cancer cells, B16F10 melanoma cells, PC-3 prostate cancer cells, and MDA-MB-231 breast cancer cells). The cytotoxicity of the EC16-80/RNase A–NBC complex against the proliferation of all seven cell lines was compared using an MTT assay. As shown in Figure 4b, the four types of cancer cells decreased in viability to less than 40% after EC16-80/RNase A–NBC exposure (1.3 $\mu g\,mL^{-1}$ of protein) and 24 h of treatment; however, all noncancerous cells retained over 90% viability with the same treatments. The growth inhibition of noncancer (Raw264.7) cells was only observed when the cells were exposed to a 4-fold higher concentration of protein (up to 5.2 $\mu g\,mL^{-1}$) and up to three-fold longer exposure time (Figure S9, SI). These results suggest that RNase A–NBC treatment is highly selective in inhibiting tumor cell proliferation, and this selective cytotoxicity of RNase A–NBC was further studied and explained by measuring the intracellular ROS level and RNase A–NBC uptake efficiency in all cell lines. The general ROS level inside these cells, expected to track closely with hydrogen peroxide levels, was first measured and compared by CellROX@Green staining. As seen in Figure S10 (SI), the four cancer cell lines all have higher ROS level than the noncancerous cell lines. With respect to the uptake of EC16-80/RNase A–NBC nanoparticles, all seven cell lines have comparable uptake efficiency, with more than 85% of cells efficiently taking up RNase A–NBC (Figure S11, SI). These results indicate that the selective cytoxicity of RNase A–NBC against cancer cells is attributed to the higher level of ROS inside tumor cells, activating RNase A–NBC at higher intensities than in noncancerous cells.

In summary, we report the design of a ROS-responsive protein and its intracellular delivery as a tool for targeted cancer therapy. The chemical conjugation of lysine residues with aryl boronic acid (NBC) temporally deactivated proteins, and ROS-triggered NBC cleavage restored protein function in a highly selective manner. The EC16-80/RNase A–NBC nanoparticles prohibited tumor cell growth in dependence on both the protein concentration and the intracellular ROS level. Moreover, the delivery of RNase A–NBC selectively prohibited tumor cell growth and had minor cytotoxicity against noncancer cells. In this paper, the ROS-responsive modification of RNase A restricts RNase A effects to cancer cells; thereby, increased dosages of RNase A could be tolerated, and the improved complexation with lipid nanoparticles could increase the proportion of drug delivered to cells, causing a multiplicative effect in efficacy. We believe that the application of ROS-triggered lysine deprotection chemistry to reversibly modulate protein function could be further extended to temporal and spatially specific activation of cellular processes, and the development of additional protein-based pharmaceuticals for targeted cancer therapy.

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