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Developing Chemically Modified Redox-responsive Protein As Smart Therapeutics

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Abstract

The conditional control of protein function in response to the physiological change of diseased cells is essential to develop smart protein therapeutics. Herein, we report a redox-responsive chemical modification of protein by conjugating an intracellular glutathione (GSH)-cleavable ligand, NSA onto protein residue. We demonstrated that the NSA conjugation of Ribonuclease A (RNase A) enabled the control of protein function by GSH in aqueous solution and living cells, with extended application for targeted cancer therapy using a lipid nanoparticle-based intracellular protein delivery strategy.

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The chemically modified redox-responsive RNase A-NSA could be selectively activated by up-regulated GSH in live cells for targeted cancer therapy.

Protein is the key regulator of cell signalling and intramolecular communications,¹ the conditional control of protein activity in response to the physiological change of diseased

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Conflicts of interest

There are no conflicts to declare.

cells is essential to study the function of protein and to develop smart protein therapeutics.² In this context, the chemical modification of protein that combines synthetic organic chemistry and molecular biology provides an effective strategy to regulate protein function in biological process.³ Moreover, chemically modified protein could display physiochemical properties and functionality that are not available in the individual protein or in the chemical moiety tag added protein,^{4, 5} further making the chemical modification of protein a valuable tool for modulating biological process. Over the past several years, the strategy of designing stimulus-responsive chemistry has shown efficacy to develop prodrug⁶ and nanocarriers for spatiotemporally-controlled drug delivery,^{7, 8} while the effectiveness of such an approach for controlling protein function, particularly in response to the endogenous environment of diseased cells remains very limited.^{9, 10} This dilemma is mostly due to the delicate protein structure and the low stability of protein that challenge an efficient chemical modification of protein. Moreover, the lack of diverse chemistry to reversibly modify protein in response to diseased cells further limits a convenient chemical modification for smart protein therapeutics.¹¹ For example, cancer cells usually showed distinct environments compared to normal cells, and this character has been harnessed to develop spatiotemporally controlled protein delivery systems.¹² Despite the success of delivering protein to targeted location using these nanocarriers,^{13, 14} the design of protein constructs which could be regulated by diseased cell environments as smart protein therapeutics remains very limited.^{15–17}

Herein, we report a proof of concept study of designing redox-responsive protein conjugate that allows an effective and selective protein activity regulation by glutathione (GSH) in the aqueous solution and in living cells. GSH is the most abundant non-protein thiol inside cells (1–10 mM in cytoplasm while 2–10 μ M extracellularly), which plays critical roles in maintaining cellular redox homeostasis.¹⁸ The abnormal regulation of intracellular GSH is known to be associated with various diseases, including cancers and Parkinson's diseases (PD), and the up-regulated GSH has previously been used to design GSH-responsive nanocarriers,^{7, 8} while redox-responsive protein that shows GSH-regulated function as smart therapeutics remains very limited.^{9, 19} Using Ribonuclease A (RNase A) as a model protein, we demonstrated that the chemical modification of RNase A lysine with a disulfide bond containing redox-responsive ligand, 3-((2(((4-nitrophenoxy) carbonyl)-oxy) ethyl) disulfaneyl) propanoic acid (NSA), afforded a redox-responsive protein construct that could be regulated by intracellular level of GSH. The RNase A-NSA could be reversed to unmodified RNase A by GSH through a disulfide bond exchange mechanism, and subsequent via a self-immolative reaction (Scheme 1a).^{6, 19} Importantly, the chemical modification not only renders the physiochemical property of RNase A, but also significantly cages the enzyme activity of RNase A, due to the essential role of lysine residues on maintaining RNase A function.²⁰ The treatment of RNase A-NSA with intracellular level of GSH, however, effectively and selectively rescued RNase A-NSA activity. Moreover, it was demonstrated that RNase A-NSA showed an increased negative charge density compared to native RNase A, due to the shielding of the positive charge after lysine conjugation and the addition of negative charge by attaching carboxylic acid into RNase A-NSA. This physiochemical property change of RNase A-NSA facilitates its electrostatic interaction with cationic lipid nanoparticle carriers for intracellular delivery (Scheme 1b). Using bio-reducible lipid nanoparticles that we have recently developed for

RNA and protein delivery,^{21, 22} we demonstrated that an efficient delivery of RNase A-NSA, and subsequent activation of RNase A-NSA in living cells can selectively prohibit tumor cells growth, due to the capability of RNase A to hydrolyse RNA inside cells to induce cytotoxicity.²⁰ Although RNase A has been evaluated as antitumor agent in clinical trials,²³ the control of RNase A in response to reductive intracellular environment for targeted cancer therapy has not been reported yet.

The redox-responsive RNase A-NSA was prepared by directly reacting RNase A and NSA (Scheme 1a) in a sodium bicarbonate buffered solution (pH = 8.0) at a protein to NSA weight ratio of 1: 6. The resulted RNase A-NSA was isolated using an ultrafiltration purification after 8 h of reaction. Meanwhile, to confirm the essential role of disulphide bond-containing NSA conjugation to afford redox-responsive protein construct, we modified RNase A with a control ligand, 7-(((4-nitrophenoxy) carbonyl) oxy) heptanoic acid (NA) that lacks a disulfide bond under the same conditions. The efficient conjugation of RNase A-NSA and RNase A-NA was first confirmed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry (Figure S1). It was showed that each RNase A molecule was modified with seven NSA or NA groups on average. Enzyme activity assay of RNase A-NSA and RNase A-NA showed a very weak ribonuclease activity (Figure 1a), this is mostly due to the chemical modification of RNase A with either NSA or NA blocked the essential lysine of RNase A and therefore significantly reduced its enzyme activity. The treatment of RNase A-NSA and RNase A-NA with GSH, the most abundant reductive species in living cells, however, showed quite different protein activation and RNase A activity restoration. The treatment of RNase A-NSA (14mM) with GSH (4 mM) showed recovered ribonuclease activity up to 10-folds compared to that without any GSH treatment, and 60 % of native RNase A due to the incomplete removal of NSA group from RNase A-NSA (Figure S1). Nevertheless, no such enzyme activity restoration was observed when RNase A-NA was similarly incubated with GSH (Figure 1a), confirming the necessity and essential role of conjugating GSH-responsive NSA moiety into RNase A to produce redox-responsive protein. With the GSH treatment time increased from 2 h to 6 h or GSH concentration increased from 0.5 mM to 2 mM, the ribonuclease activity of RNase A-NSA was increased accordingly (Figure S2, S3). In a sharp contrast, no such enzyme activity restoration was observed when RNase A-NSA was only incubated with PBS, further indicating the high stability of RNase A-NSA and its selectivity toward GSH activation. The RNase A-NSA activation after GSH treatment was also confirmed and characterized by MALDI-TOF (Figure S1). The GSH treatment efficiently removed NSA groups from RNase A-NSA, but no such NA moiety cleavage was observed when RNase A-NA was similarly treated (Figure S1). Interestingly, we found that RNase A-NSA showed a high selectivity toward GSH activation in the presence of other reductive species at intracellular concentrations. Among the reductive species, including ascorbic acid (AA), cysteine (Cys), nicotinamide adenine dinucleotide (NADH), homocysteine (Hcy), sodium sulfide (Na₂S) and GSH, only GSH treatment showed effectiveness to activate RNase A-NSA (Figure 1b). The selective RNase A-NSA activation by GSH could be of great potential to develop smart protein therapeutics by harnessing the higher concentration of GSH in diseased cells to activate RNase A-NSA in situ, as to be discussed below.

An effective RNase A-NSA activation in living cells is greatly limited by the cell impermeability of protein, therefore the delivery of RNase A-NSA into cells is required to fully realize its smart therapeutic potential. To this end, we developed an effective RNase A-NSA delivery by screening a library of bioreducible cationic lipid nanoparticles that we have recently designed for small interfering RNA (siRNA)²⁴ and genome-editing protein delivery.²¹ Over the past few years, we have developed a very efficient protein delivery strategy by designing a combinatorial library of cationic lipids, combining with a screening strategy to identify the most efficient vehicles for delivering the protein of interest, and to study the relationship between chemical structure and protein delivery efficiency further.^{25–27} This strategy has been proved to be very useful and invaluable for delivering protein that shows different physiochemical properties, which usually challenges the effective self-assembly of lipid/protein nanoparticles for intracellular delivery. Herein, we generated a pool of twelve bioreducible lipids in total (Scheme S2), which were synthesized through the parallel Michael addition reaction between head amines and 2-(dodecylsulfaneyl) ethyl acrylate (O16B). The NSA modification increases the negative charge density of RNase A-NSA, and therefore facilitates the electrostatic interaction between cationic lipid nanoparticles and RNase A-NSA for intracellular delivery. To identify effective RNase A-NSA delivery vehicles, RNase A-NSA (200 nM) and different lipids (4.0 µg/mL) was mixed in phosphate buffer solution (25 mM, pH = 7.2) to form nanocomplexes before adding to HeLa cells. The viability of cells with treatments of different lipid/protein nanoparticles or lipid alone was determined 48 h post protein delivery, and normalized to that without any treatment to evaluate protein delivery efficiency. The effective delivery of RNase A-NSA, and subsequent RNase A-NSA activation by GSH inside cells restoring its ribonuclease activity to induce cytotoxicity induce cell apoptosis. As shown in Figure 2a, the screening results indicated that among the twelve lipid nanoparticles, two of them (11 and 12, termed as PDPA-O16B and PBPA-O16B, respectively, (Scheme 1) efficiently delivered RNase A-NSA into cells and decreased cell viability down to 20% compared to that of non-treated cells (Scheme S2). Meanwhile, neither lipid nor protein alone treatment showed obvious cytotoxicity against HeLa cells, confirming the effectiveness and necessity to use these lipid nanoparticles for RNase A-NSA delivery and activation in living cells. One of the leading lipids for RNase A-NSA delivery, PBPA-O16B was selected for detailed study in the following.

The cellular uptake and subcellular localization of PBPA-O16B/RNase A-NSA nanoparticles was studied by treating HeLa cells with fluorescently labeled PBPA-O16B/FITC-RNase A-NSA complex, followed by confocal laser scanning microscopy (CLSM) imaging (Figure 2b). FITC-RNase A-NSA (250 nM) treated cells showed minimal cellular internalization of protein, mostly due to the cell impermeability of free protein. PBPA-O16B/FITC-RNase A-NSA treatment, however, resulted in significant accumulation of fluorescence in the cytosol of HeLa cells. Moreover, HeLa cells with endosome staining using LysoTracker@Red also indicated a low ratio of endosome entrapment of PBPA-O16B/RNase A nanoparticles, indicating that the bioreducible lipid nanoparticles can not only deliver RNase A-NSA into cells, but also efficiently releasing protein in response to the reductive intracellular environment.

The electrostatic interaction between PBPA-O16B and RNase A-NSA or RNase A-NA assembled well-dispersed nanoparticles with size around 240 nm in average, as revealed by

the dynamic light scattering analysis and SEM imaging (Figure S4). Zeta-potential measurement indicated a significant decrease of nanoparticle surface charge, mostly due to the electrostatic interaction between negatively charged RNase A-NSA and cationic PBPA-O16B partly neutralized the charge of lipid. The comparable self-assembled nanoparticle structure of PBPA-O16B/RNase A-NSA and PBPA-O16B/RNase A-NA enabled the study of specific activation and cytotoxicity of RNase A-NSA for targeted protein therapy. It was noticeable that the delivery of RNase A-NA into HeLa cells only showed lower efficiency to prohibit cell growth than that of RNase A-NSA in a wide range concentration of protein (Figure 3). For instance, HeLa cells with the treatment of 50 nM PBPA-O16B/RNase A-NSA nanoparticle decreased cell viability down to 25% of non-treatment, while RNase A-NA nanoparticle treatment showed minimal cytotoxicity under this condition.

The enhanced cytotoxicity of RNase A-NSA should be ascribed to the specific and efficient GSH-triggered protein activation in HeLa cells. We further studied the potential of delivering PBPA-O16B/RNase A-NSA nanoparticles into cells with varied intracellular GSH level to demonstrate the potential of RNase A-NSA for targeted disease treatment. It has been previously reported that cancer cells in general show higher intracellular GSH level, we therefore hypothesized that the delivery of RNase A-NSA into cancer cells could be selectively activated for targeted cancer therapy. To this end, we selected four different cell lines, including SiHa and HeLa cancerous cells, 3T3 mouse fibroblast cells and HEK-293T human embryonic kidney cells that show lower level of intracellular GSH, for studying RNase A-NSA delivery. The intracellular GSH level of these cells was first determined using a reported cellular GSH assay method²⁸ (Figure 4a), and it was found that both HeLa and SiHa cells showed four-times higher GSH level than that of non-cancerous 3T3 and HEK-293T cells. The delivery of PBPA-O16B/RNase A-NSA nanoparticles into cancerous SiHa and HeLa cells (at a protein concentration of 200 nM) significantly decreased cell viability, while the delivery of RNase A-NSA into non-cancerous 3T3 and HEK-293T cells showed minimal cytotoxicity (Figure 4b). It is notable that the delivery of fluorescent labeled PBPA-O16B/FITC-RNase A-NSA nanoparticles into the four cell lines showed comparable efficiency (Figure S5), and the treatment of these cells with PBPA-O16B/RNase A-NA nanoparticles did not show obvious cytotoxicity (Figure 4b), indicating that the selective cytotoxicity of RNase A-NSA was due to its effective activation in cancer cells.

In conclusion, we reported herein a new chemical approach to design redox-responsive protein construct by modifying RNase A using a GSH-responsive ligand, NSA. We have further demonstrated the high efficiency of delivering redox-responsive RNase A-NSA into cells using bioreducible lipid nanoparticle for selective protein activation in cancerous cells, and its potential for targeted cancer therapy. Altogether, the principle of conjugating protein via redox-responsive chemistry could further be extended to other functional proteins, with an *in situ* protein activity modulation for precise cellular function manipulation and targeted disease treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

a) Ribonuclease activity assay of RNase A-NSA and RNase A-NA with and without GSH treatment. b) Selective RNase A-NSA activation by GSH in the presence of other reduced species. 14 mM protein was incubated with GSH (4mM) or other concentration of intracellular reductive species for 6 h before ribonuclease activity assay.

Fig. 2.

a) Identify effective lipid nanoparticles for RNase A-NSA delivery. HeLa cells were treated with lipid/RNase A-NSA complexes at a lipid concentration of 4.0 $\mu\text{g/mL}$ and a protein concentration of 200 nM. b) CLSM images of HeLa cells treated with free FITC-RNase-NSA (250 nM) or its nanocomplex with PBPA-O16B. Scale bar: 20 μm .

Fig. 3.
The viability of HeLa cells treated with free RNase A-NSA, or RNase A-NA, their complex with PBPA-O16B at indicated protein concentrations.

Fig. 4.

Selective RNase A-NSA activation in cancer cells for targeted cancer therapy. a) The comparison of intracellular GSH level of four different type of cells. b) the viability of cells treated with PBPA-O16B/RNase A-NA or PBPA-O16B/RNase A-NSA nanocomplexes.

Scheme 1.

Illustration of (a) the chemical principle for RNase A-NSA modification and activation; (b) intracellular delivery of RNase A-NSA using bio-reducible lipid nanoparticle.