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Authors: Jinsong Ren, Enguo Ju, Faming Wang, Zhenzhen Wang, Chaoying Liu, Kai Dong, Fang Pu, and Xiaogang Qu

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Modular AND Gate Controlled Delivery Platform for Tumor Microenvironment Specific Activation of Protein Activity

Enguo Ju,[a] Faming Wang,[a] Zhenzhen Wang,[a] *Chaoying Liu,*[*b] Kai Dong,[a] Fang Pu,[a] Jinsong Ren,*[a] and Xiaogang Qu [a]

Abstract: *Protein therapeutics has inspired intensive research interest in a variety of realms. It is still urgently required to avoid premature or unexpected activation of therapeutic proteins to achieve great specificity for therapy. Herein, we reported a modular AND gate-controlled delivery platform for tumor microenvironment specific activation of therapeutic protein activity based on biomineralization of molecular glue adhered protein enzyme. The AND gate integrates the specific microenvironment of tumor tissues (acidic pH and a certain concentration of ATP) as inputs and activates the therapeutic activity of protein only when both inputs are active. More importantly, the activity of therapeutic protein would not be activated either at acidic pH or in the presence of ATP, which could greatly avoid the deleterious effect on normal tissues. Besides, this AND gate can be modular design and suitable for a variety of therapeutic proteins and nucleic acids.*

Since the groundbreaking discovery of insulin as the first recombinant protein in diabetes treatment, protein therapeutics has inspired intensive research interest in a variety of realms, including vaccination, cancer therapy, regenerative medicine, and loss-of-function genetic diseases.[1] Most protein pharmaceuticals elicit their biological activity by targeting cell surface ligands or extracellular domains.[2] Nevertheless, the efficiency of protein therapeutics was limited by the intrinsic shortcomings of protein including low stability and high immunogenicity. Their biological activity would be compromised when they were exposed to a proteolytic environment or recognized by the host immune system.[3] Additionally, they also suffered from the lack of capability of targeting disease tissue with high therapeutic margins of safety while sparing normal tissue the side effects.[4] Therefore, the development of delivery systems capable of protecting the active agents from proteolytic degradation and decreasing the related toxicity to normal tissues is of great importance.

Recently, a number of synthetic nanomaterials, including liposomes, polymers, and inorganic nanoparticles, have been designed for affording protein therapeutics with maximized efficacy and minimized side effects.[5] Especially, exploiting the physiological and biochemical differences between normal and pathological conditions allows researchers to elaborate stimuli-responsive delivery systems to release the therapeutic protein in a spatially, temporally and dosage-controlled manner. Typical biological stimuli include vascular abnormalities, hypoxia, acidic pH, redox potential, and altered metabolic states.[6] Of these stimuli, adenosine-5'-triphosphate (ATP) has attracted extensive attention as the growing evidence of increased levels of ATP in many pathological processes.[7] It has been reported that intratumoral ATP concentrations are up to 10^4 times higher than those of interstitial ATP in normal tissues.[8] Based on this finding, DNA aptamers,[9] phenylboronic acid,[10] and metallic complex[11] have been previously reported to construct ATP-responsive delivery systems for enhanced therapeutic efficiency. Though promising, they still face challenges considering the ubiquity of ATP in plasma and normal tissues. In other words, even small amounts of ATP could also activate the biological activity of protein drugs, which would be inevitably deleterious to normal tissues.

The aforementioned concerns encourage us to create a programmable delivery system for activating the biological activity of the therapeutic protein by ATP in tumors tissues other than normal tissues or blood. As one of the basic Boolean logic gate, AND gate can increase the sensing specificity by integrating multiple signals to identify a specific environment. Various biomolecules, such as aptamers,[12] deoxyribozyme,[13] proteins,[14] transcriptional effectors,[15] and nanoenzyme[16] have been successfully applied for building functional logic AND gates. Especially, the stepwise logic AND gate can avoid premature or unexpected activation of therapeutic proteins, which greatly increases the specificity to improve the therapeutic efficiency and decrease the side effects.[17] Herein, we reported the biomineralization of molecular glue adhered enzyme as an acidic pH "AND" ATP logic gate for selective and controlled activation of therapeutic protein in the tumor microenvironment. As shown in Figure 1, the activity of the therapeutic protein (trypsin as an example in this study) was temporarily suppressed by molecular glues through multiple salt bridges between guanidinium ion and oxyanionic groups. The formed agglomerates were further surface engineered with pH-sensitive calcium phosphate. Benefiting from unique properties of the tumor microenvironment, the intrinsic activity could only be retrieved upon decomposition of CaP shell at acidic pH and liberation of protein from molecular glue in the presence of ATP with concentration as much as that in the tumor tissue. More importantly, the activity of therapeutic protein would not be activated either at acidic pH or in the presence of ATP, which could greatly avoid the deleterious effect on normal tissues. Therefore, the limited control of specificity and efficacy for protein therapy can be overcome by constructing a logic gate, which integrates the specific microenvironment of tumor tissue as inputs and activates the therapeutic activity of protein only in the tumor tissue. Besides, this AND gate is based on the multiple salt bridges between guanidinium and oxyanionic

[a] E. Ju, F. Wang, Z. Wang, K. Dong, F. Pu, Prof. J. Ren, Prof. X. Qu
Laboratory of Chemical Biology and State Key Laboratory of Rare Earth Resource Utilization
Changchun Institute of Applied Chemistry
Chinese Academy of Science, Changchun, Jilin 130022, P.R
E-mail: jren@ciac.ac.cn;
E. Ju and F. Wang contribute equally to this work.
[b] C. Liu
Department of Respiratory Medicine, First Affiliated Hospital, Jilin University, Changchun 130021, P. R. China
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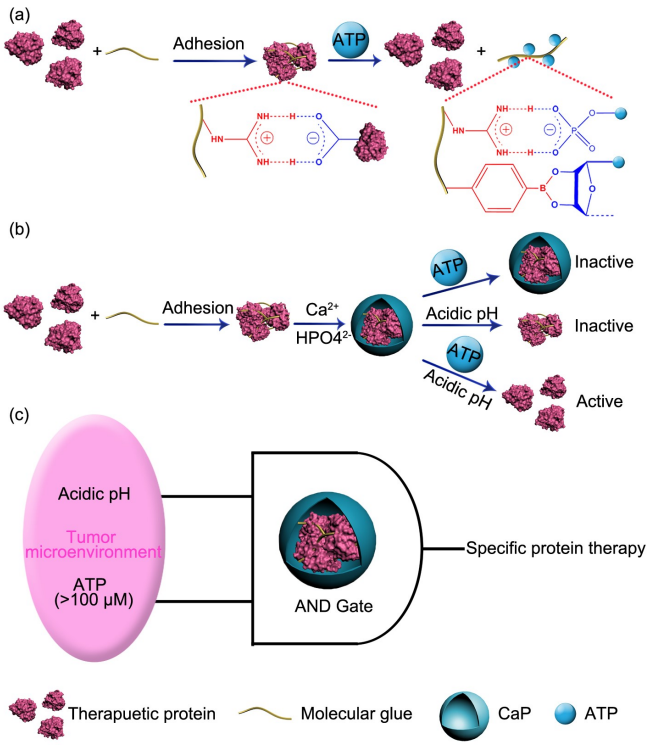

Figure 1. Schematic illustration of the modulation of therapeutic protein with molecular glue and ATP. (a) The molecular glue can adhere to the surface of protein to form aggregates, leading to suppressed enzyme activity. Addition of ATP leads a competitive binding of ATP to molecular gel, which liberates the protein and restore its activity. (b) Design of biomineralized molecular glue adhered protein for acidic pH and ATP dual-stimuli-responsive activation of therapeutic protein. (c) Construction of AND gate delivery system for tumor tissue specific protein therapy.

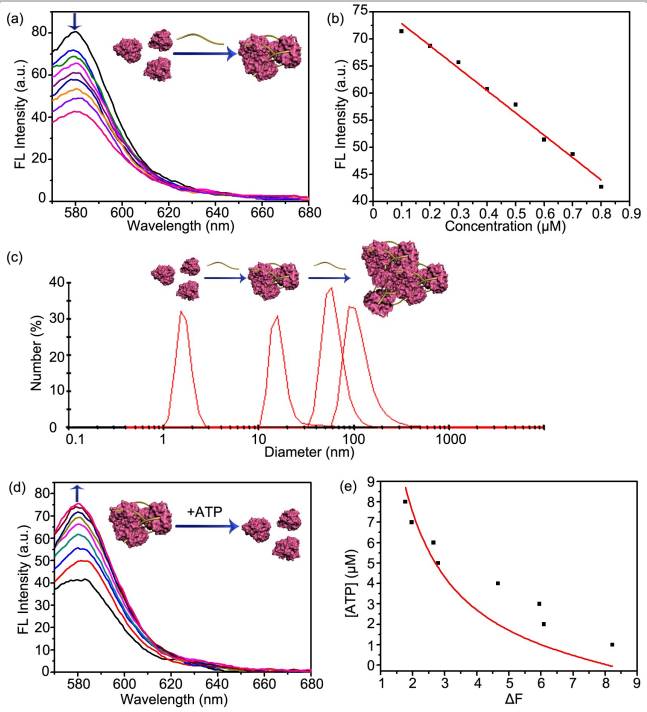

Figure 2. (a) Fluorescence titration experiments to measure the binding affinity between TRP (0.5 μM) and molecular glue. The TRP was labeled with sulforhodamine B for monitoring the fluorescence changes. (b) The relationship between fluorescence intensity at 580 nm and the concentration of molecular glue. (c) Dynamic light scattering measurement of the size of TRP (0.5 μM) with increased amount of molecular glue(0, 0.5 μM, 1 μM, and 5 μM). (d) Fluorescence intensity of the mixture of sulforhodamine B-labeled TRP (0.5 μM) and molecular glue (1 μM) with the titration of ATP. (e) The associate constants between ATP and TRP/Glue could be calculated from the the fluorescence changes against ATP concentrations based on competitive binding model.

groups, which can be modular design and suitable for many therapeutic proteins and nucleic acids.

The molecular glue was firstly synthesized through "thio-yne" click chemistry between alkyne-appended monomers containing guanidinium ion and boronic acid units, and a dithiol monomer[10] (Figure S1-S5). The molecular glue was incorporated with guanidinium ion and boronic acid. The cooperated effect of guanidinium ion/phosphate ion salt bridge and covalently bound boronic acid/1,2-diol can result in a high affinity of molecular glue toward ATP than protein. The number-average molecular weight of molecular glue was calculated to be about 8, 000 by quantification of the thiol termini through Ellman's assay. To confirm the molecular glues could adhere to the surface of trypsin to form agglomerates, we labeled trypsin with a fluorescence probe sulforhodamine B and conducted titration experiments with molecular glue. As shown in Figure 2a, the addition of molecular to trypsin could quench the fluorescence emission at 580 nm, which was attributed to the aggregation of trypsin caused by the strong adhesion of molecular glue with proteins. The association constant for the binding between molecular glues and trypsin was calculated to be $1.7 \times 10^6\ M^{-1}$ by fitting fluorescence changes to the quadratic equilibrium binding equation (Figure 2b). Dynamic light scattering measurements revealed the addition of molecular glue in trypsin could result in increased hydrodynamic sizes, which further supported that molecular glues could result in the agglomerates of trypsin (Figure 2c). In addition, more amount of molecular glue could yield bigger agglomerates. We then chose the ratio of trypsin to molecular glue as 1:2 with a hydrodynamic size of 60 nm for the following biomineralization.

Subsequently, to demonstrate the molecular glue that adhered to trypsin could be detached by the competitive interaction of ATP, we monitored the fluorescence intensity changes of sulforhodamine B labeled TRP/Glue with the addition of ATP. As shown in Figure 2d, the fluorescence intensity at 580 nm increased along with the increased concentration of ATP. This result suggested that the adhesion between trypsin and molecular glue could be detached by the competitive binding of ATP. By fitting the dissociation profile to the competitive binding model, the association constant for molecular glue toward ATP was determined to be $1.2 \times 10^6\ M^{-1}$ (Figure 2e). Taken together, the molecular glues could act as an effective tool for ATP-responsive modulation of the activity of the enzyme, which provides a foundation for the construction of programmable protein delivery for cancer therapy.

To construct pH AND ATP logic controlled therapeutic protein delivery system, we further biomineralized the molecular glues adhered enzyme by adding sodium hydrogen phosphate into the solution containing TRP/Glue (ratio of trypsin and molecular glue as 1:2) and calcium ions. It is well known that the carboxyl residues of protein could provide active nucleation sites for CaP nucleation.[18] Therefore, TRP/Glue could be incorporated into calcium phosphate during the formation of nanoparticles to obtain the final product designed as

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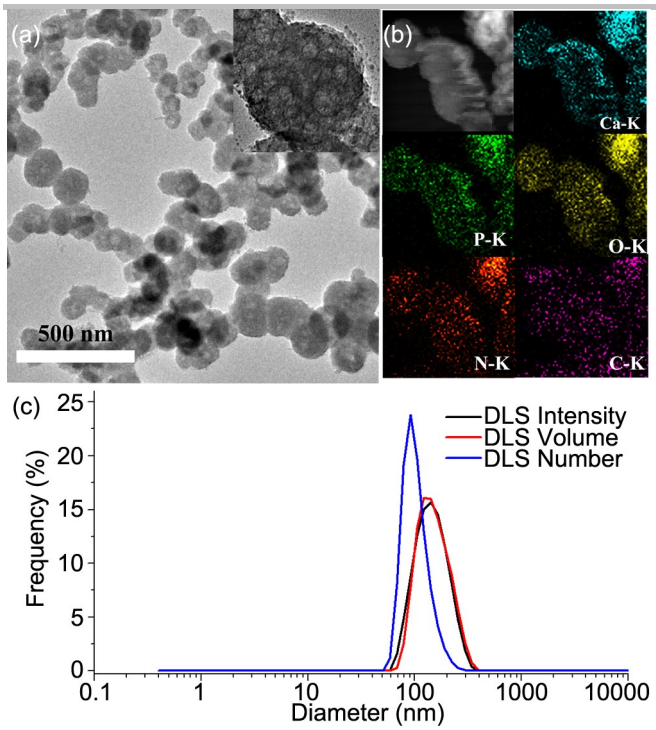

Figure 3. Characterizations of the TRP/Glue@CaP nanoparticles. (a) TEM image of the TRP/Glue@CaP. (Inset) the amplified TEM image of the TRP/Glue@CaP. (b) The energy dispersive X-ray elemental mapping images of Ca-K, P-K, O-K, N-K, and C-K. (c) The diameter distribution of the TRP/Glue@CaP measured by DLS.

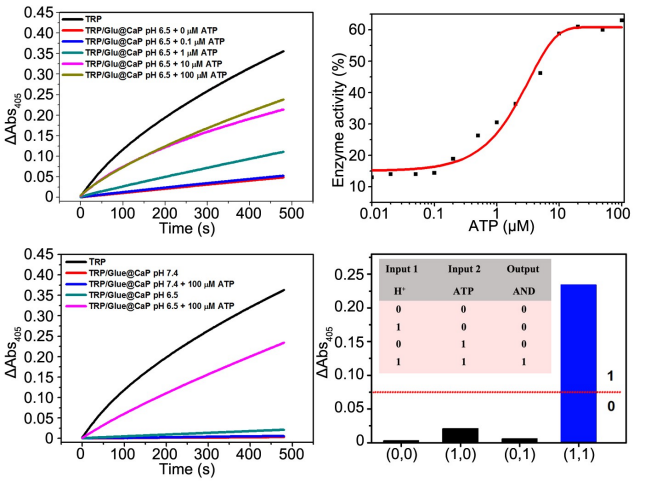

Figure 4. (a) Absorbance changes at 405 nm of TRP, and TRP/Glue@CaP at pH 6.5 in the presence of ATP in Tris-HCl buffer containing BAPNA. The enzymatic activity can be measured by TRP-catalyzed hydrolysis of BAPNA to p-nitroaniline, which has a significant absorbance at 405 nm. (b) Normalized enzymatic activity of TRP/Glue@CaP at pH 6.5 in the presence of ATP. (c) Absorbance changes at 405 nm of the TRP/Glue@CaP at pH 6.5 or 7.4 in the absence or presence of ATP in the Tris-HCl buffer containing BAPNA. (d) Absorbance intensity at 405 nm was recorded for the AND logic gate. (Inset) truth table for the AND gate.

TRP/Glue@CaP. Transmission electron microscopy (TEM) image revealed that the spherical and uniform morphology of Trp/Glue@CaP with an average diameter of 110 nm (Figure 3a). The superficial CaP inorganic materials phase facilitates direct observation without any staining treatment. Moreover, energy dispersive X-ray elemental (EDX) mapping images further demonstrated that Ca, P, O, C, N elements were distributed in the nanoparticles, which indicated that TRP/Glue was successfully coated by CaP layer (Figure 3b). The result of the diameter distribution of Trp/Glue@CaP with a polydispersity index of 0.104 measured by DLS was also in accordance with TEM images (Figure 3c). Compared with TRP/Glue, the size increase was evidently due to the precipitated mineral layer. Besides, the amount of trypsin in Trp/Glue@CaP was calculated as 4.2 μM in 1 mg/mL TRP/Glue@CaP (Figure S6). Considering CaP was a pH-sensitive mineral phase, which was stable in physiology condition while could be degraded at the acidic environment. Therefore, the above results indicated that TRP/Glue@CaP had the potential to be a protein delivery system for pH and ATP logic controlled activation of therapeutic effect.

We then examined the protein activity of TRP/Glue@CaP through a trypsin-catalyzed hydrolysis assay. N-α-benzoyl-DL-arginine 4-nitroanilide (BAPNA) could be hydrolyzed to p-nitroaniline by active trypsin, which resulted in the characteristic absorption at 405 nm increase (Figure S7). TRP/Glue@CaP at pH 7.4 showed nearly no enzymatic activity, which indicated CaP shell could greatly protect the protein active site from contacting its substrate (Figure S8). In addition, no enzymatic activity of TRP/Glue@CaP at pH 7.4 was observed even in the presence of ATP. As CaP shell was pH-sensitive, the enzymatic activity of TRP/Glue@CaP in the presence of ATP (100 μM) was dependent on pH (Figure S9). We assumed the process as that acidic environment could degrade the CaP shell and subsequently the presence of ATP could competitively bind with molecular glue to liberate the trypsin. We also proved that ATP has no influence on the hydrolytic activity of trypsin (Figure S10). Besides, TRP/Glue@CaP at pH 6.5 without the addition of ATP also showed nearly very low hydrolytic activity (Figure 4a). This result indicated that TRP/Glue exposed very few active sites to the substrate even CaP was degraded. However, the enzymatic activity of TRP/Glue@CaP at pH 6.5 gradually recovered with the addition of ATP. The correlation between the enzyme activity and concentration of ATP showed a distinct difference of activity at the ATP concentration of 0.1 μM and 100 μM (Figure 4b). Considering the concentration of extracellular ATP in tumor tissue is more than 100 μM while that in normal tissue is less than 0.1 μM, TRP/Glue@CaP can achieve tumor-specific targeting activation of the enzyme. Based on the above results, we designed an AND logic gate that employed acidic pH and ATP (100 μM) as inputs, and the enzymatic activity of TRP/Glue@CaP as the output.[19] The signal response after application of a different combination of inputs was shown in Figure 4c and 4d. In the absence of both or either of the inputs (0/0, 0/1, 1/0), enzymatic activity was very low and no increase absorption at 405 nm was observed. Therefore, TRP/Glue@CaP remained in an OFF state (output 0). However, in the presence of both inputs (1/1), the enzymatic activity was activated to a very high level, which was considered as an ON state (output 1). This AND gate could increase the sensing specificity towards tumor microenvironment by integrating two signals, each of which may be too general to identify the tumor tissue. Moreover, this platform was based on the multiple salt bridges between guanidinium and oxyanionic groups, which not only suitable for proteins but also for nucleic acids. Therefore, TRP/Glue@CaP can be extended to a broad range of applications such as sensing, imaging, delivery as well as therapy.

Finally, to demonstrate the potential of pH and ATP logic controlled therapeutic protein delivery as an approach for specific tumor therapy, the capability of TRP/Glue@CaP to selectively inhibit tumor cell proliferation was studied. It has

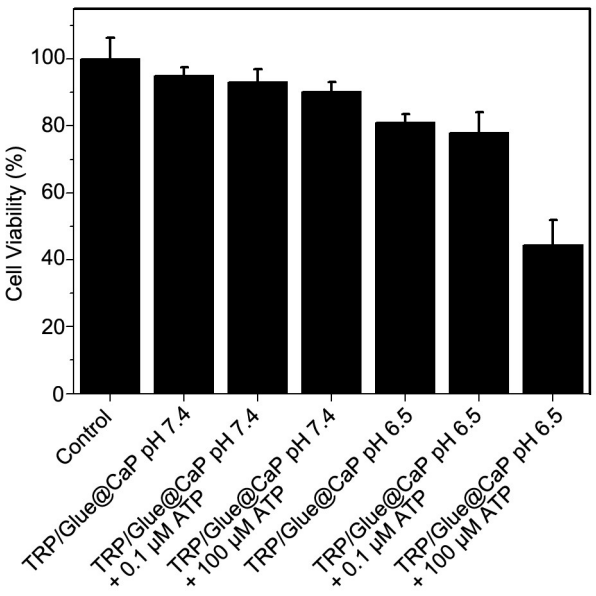

Figure 5. Cell viability of HeLa cells treated with TRP/Glue@CaP at pH 6.5 or 7.4 in the absence and presence of ATP.

proved that long-term exposure of trypsin makes cells weak and even to cell death through down-regulating superficial integrins to regulate the apoptotic ability of cells.[20] TRP/Glue@CaP showed no toxicity to HeLa cells at pH 7.4 in the absence of ATP even at the concentration of 1 mg/mL after 24 h treatment, which indicated that TRP/Glue@CaP has no function in normal tissues. The cancer cells decreased to 50% in viability only at pH 6.5 in the presence of ATP (100 μM), while either pH 7.4 or low concentration of ATP (0.1 μM) could keep the cell viability over 80%. These results suggest that the highly selective toxicity of TRP/Glue@CaP against cancer cells is attributed to the acidic pH and high concentration of ATP in tumor tissue, which can activate the therapeutic effect of protein than in normal tissues.

In conclusion, we have constructed a modular AND gate-controlled delivery platform for tumor microenvironment specific activation of protein activity based on molecular glue and biomineralization. The AND gate integrates the specific microenvironment of tumor tissues (acidic pH and a certain concentration of ATP) as inputs and activates the therapeutic activity of protein only when both inputs are active. Trypsin was applied as a proof of concept to prepare the TRP/Glue@CaP nanoparticles to show high specific toxicity for cancer cells in the tumor microenvironment. Besides, this modular AND gate is based on the multiple salt bridges between guanidinium and oxyanionic groups, which can be suitable for many therapeutic proteins and nucleic acids.[21] We hope this AND gate can be extended to a broad range of applications such as sensing, imaging, delivery as well as therapy.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: protein therapy • logic gate • molecular glue• biomineralization • drug delivery

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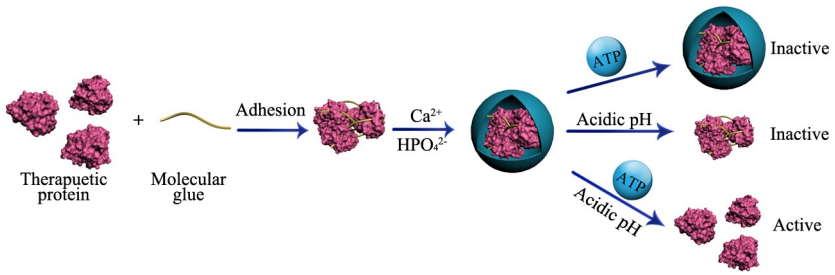

A modular AND gate-controlled delivery platform for tumor microenvironment specific activation of therapeutic protein activity was constructed based on biomineralization and molecular glue, which greatly increase the specificity and efficiency of protein therapy.

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