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Boronic Acid-Appended Molecular Glues for ATP-Responsive Activity Modulation of Enzymes

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Supporting Information

ABSTRACT: Water-soluble linear polymers Gu_mBA_n (m/n = 18/6, 12/12, and 6/18) with multiple guanidinium ion (Gu^+) and boronic acid (BA) pendants in their side chains were synthesized as ATP-responsive modulators for enzyme activity. Gu_mBA_n polymers strongly bind to the phosphate ion (PO_4^-) and 1,2-diol units of ATP via the Gu^+ and BA pendants, respectively. As only the Gu^+ pendants can be used for proteins, Gu_mBA_n is able to modulate the activity of enzymes in response to ATP. As a proof-of-concept study, we demonstrated that trypsin (Trp) can be deactivated by hybridization with Gu_mBA_n. However, upon addition of ATP, Trp was liberated to retrieve its hydrolytic activity due to a higher preference of Gu_mBA_n toward ATP than Trp. This event occurred in a much lower range of [ATP] than reported examples. Under cellular conditions, the hydrolytic activity of Trp was likewise modulated.

Chemicals that act site-selectively in tumor tissue are attractive for tumor chemotherapy and drug delivery. Tumor targeting is mostly based on a difference in pH between the tumor (pH 5.9−7.6) and normal tissue (pH 7.3−8.0).[1,2] Adenosine triphosphate (ATP; Figure 1b) represents a promising tumor indicator, as the concentration of extracellular ATP in tumor tissue ([ATP] > 100 μM)[3] is more than 4 orders of magnitude higher than that in normal tissue ([ATP] = 1−10 nM).[4] Previously, a few examples of ATP-responsive drug delivery carriers using DNA aptamers[5] and phenylboronic acid[6] have been reported. However, these systems were designed to distinguish extracellular and intracellular matrices and operate at [ATP] > 1 mM. Recently, we reported an ATP-responsive drug carrier, consisting of tubularly connected chaperon units, which can operate at [ATP] = 10−50 μM, albeit that the system requires esterase to cleave off a drug linker for the release of encapsulated drugs.[7] Here we report a series of water-soluble polymers, Gu_mBA_n, as ATP-responsive molecular glues that carry multiple guanidinium ion (Gu^+) and boronic acid (BA) pendants in their side chains (Figure 1a). Gu_mBA_n polymers can temporarily suppress the activity of an enzyme by hybridization (Figure 2). However, upon addition of ATP, the enzyme is liberated and can retrieve its intrinsic activity. Most importantly, this event occurs in a much lower range of [ATP] (1−10 μM) than previous examples including our chaperon nanotubes.[5−7]

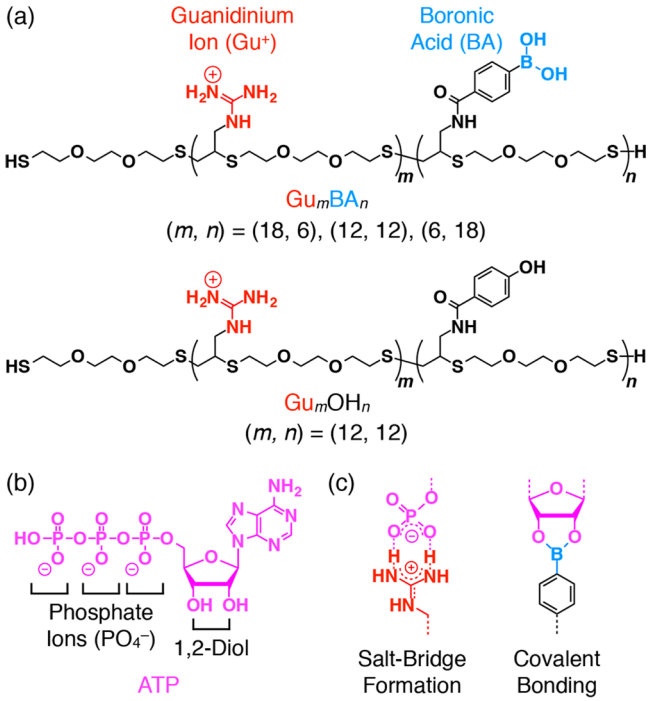

Figure 1. (a) Molecular glues Gu_mBA_n (m/n = 18/6, 12/12, and 6/18) with randomly incorporated guanidinium ion (Gu^+) and boronic acid (BA) moieties in their side chains, as well as of Gu_mOH_n (m/n = 12/12) without BA units; (b) adenosine triphosphate (ATP); (c) schematic illustration of (left) a salt-bridge between phosphate ion (PO_4^-) and Gu^+, and (right) covalent bonds between 1,2-diol and BA moieties.

Previously, we developed dendritic[8a−e] and linear[8f−i] molecular glues containing multiple Gu^+ units.[9] These molecular glues strongly adhere to proteins,[8a,c,e,g] nucleic acids,[8h,i] phospholipid membranes,[8d] and clay nanosheets[8b,f] via multiple salt bridges between Gu^+ and oxyanionic groups[9] located on the targets. Gu_mBA_n (Figure 1a) were designed as a new class of molecular glues containing not only Gu^+ but also BA units that can covalently bind to 1,2-diols (Figure 1c).[10] The cooperative effect of multiple Gu^+/PO_4^- salt bridges and covalently bound BA/1,2-diol moieties (Figure 1c) should result in a high affinity of Gu_mBA_n toward ATP (Figure 1b), which may be able to liberate proteins from their Gu_mBA_n conjugates (Figure 2). As a proof-of-

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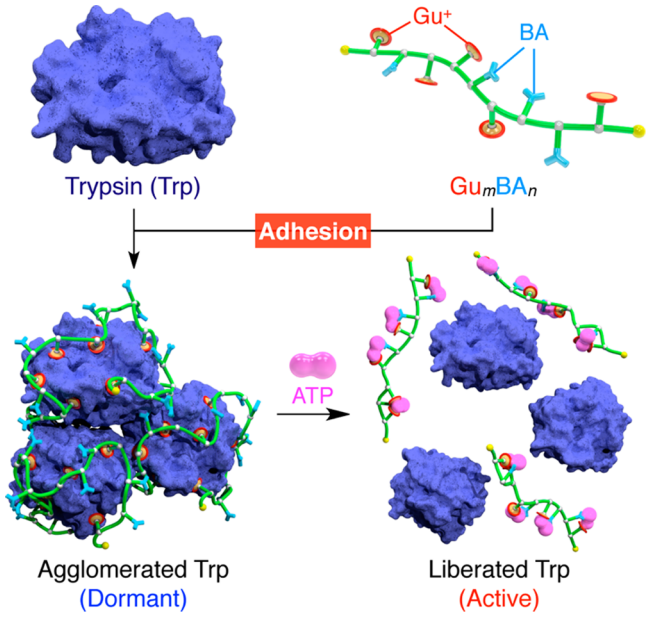
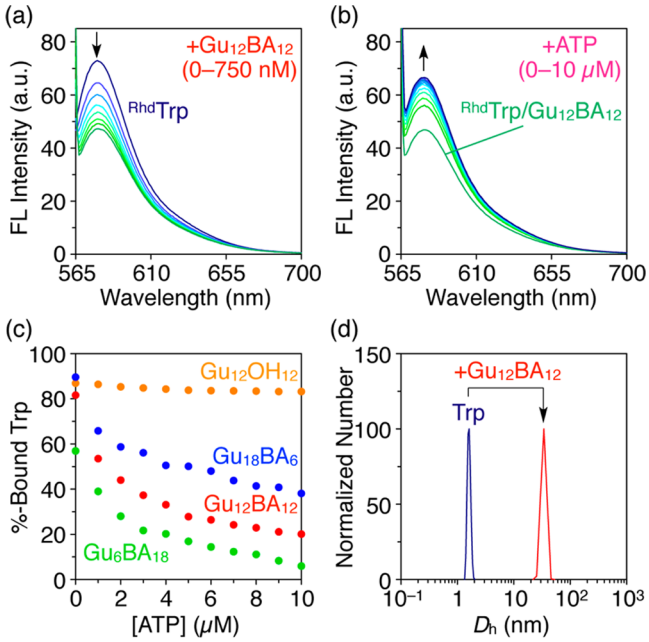


Figure 2. Schematic illustration of the modulation of the enzymatic activity of trypsin (Trp) by Gu_mBA_n and ATP. Gu_mBA_n adheres to the surface of Trp to form agglomerates and thus suppresses Trp enzymatic activity (dormant state). ATP competitively binds to Gu_mBA_n, which allows its dissociation from Trp, thus restoring the enzymatic activity.

concept study, we chose a hydrolytic enzyme trypsin (Trp) as a target protein. Trp is known to inhibit the proliferation and metastasis of cancer cells[11] and thus may possibly exhibit tumor-suppressing activity. However, Trp is not tissue selective and thus also deteriorates normal tissue.[11] Here we highlight that the hydrolytic activity of Trp, which is intrinsically independent of ATP, can be modulated via conjugation with Gu_mBA_n and that this conjugation is essentially reversible upon addition of ATP (Figure 2).

Gu_mBA_n polymers (Figure 1a) were synthesized using the "thiol−yne" reaction[12] between alkyne-appended monomers containing Gu^+ and BA units, and a triethylene glycol with thiol termini. With different molar ratios of two alkyne-appended monomers, $Gu_{18}BA_6$, $Gu_{12}BA_{12}$, and Gu_6BA_{18} were synthesized. The oxidation of $Gu_{12}BA_{12}$ with H_2O_2 furnished $Gu_{12}OH_{12}$ as a reference, which contains hydroxyl (OH) groups instead of BA units (Figure 1a). The average molecular weights of Gu_mBA_n and Gu_mOH_n were evaluated by quantification of the thiol termini (Table S1).[13] Subsequently, we labeled Trp with a fluorescence probe sulforhodamine B (^{Rhd}Trp, 0.5 μM) and conducted titration experiments with $Gu_{12}BA_{12}$ (0−750 nM) in Tris-HCl buffer (20 mM, pH 7.8) at 25 °C, as the adhesion of $Gu_{12}BA_{12}$ to ^{Rhd}Trp quenches the fluorescence emission at 578 nm (λ_{ext} = 560 nm; Figure 3a).[14] According to an established method,[15] we fitted the fluorescence intensity changes to a 1:1 binding model and obtained an association constant (K_{Trp}) of 1.3×10^7 M^{-1} for the binding between $Gu_{12}BA_{12}$ and Trp (Figure 3e). A similar fluorescence-quenching profile was observed for ^{Rhd}Trp with $Gu_{18}BA_6$ ($K_{Trp} = 2.4 \times 10^7$ M^{-1}; Figures 3e and S12a)[13] as well as $Gu_{12}OH_{12}$ ($K_{Trp} = 2.5 \times 10^7$ M^{-1}; Figures 3e and S12e).[13] In contrast, Gu_6BA_{18} barely quenched the fluorescence (Figure S12c),[13] and a much smaller K_{Trp} value (3.1×10^5 M^{-1}; Figure 3e) relative to that of $Gu_{12}BA_{12}$ was observed, indicating that the binding to Trp occurs predominantly via Gu^+. Dynamic light scattering (DLS) measurements revealed that $Gu_{12}BA_{12}$ (700 nM) adheres to Trp (0.5 μM; Figure 3d, dark blue) to yield



(e)

	K_{Trp} (M^{-1})	K_{ATP} (M^{-1})	K_{ATP}/K_{Trp}
$Gu_{12}BA_{12}$	1.3×10^7	3.8×10^6	0.29
Gu_6BA_{18}	3.1×10^5	3.5×10^6	11
$Gu_{18}BA_6$	2.4×10^7	2.7×10^6	0.11
$Gu_{12}OH_{12}$	2.5×10^7	5.6×10^4	0.0022

Figure 3. Fluorescence spectra (λ_{ext} = 560 nm) of sulforhodamine B-labeled Trp (^{Rhd}Trp, 0.5 μM) at 25 °C in Tris-HCl buffer (20 mM, pH 7.8) upon successive titration with (a) $Gu_{12}BA_{12}$ (0−750 nM) and (b) ATP (0−10 μM). (c) Dissociation profiles of $Gu_{12}B_{12}$ (red; 750 nM), $Gu_{18}BA_6$ (blue; 800 nM), Gu_6BA_{18} (green; 4.5 μM), and $Gu_{12}OH_{12}$ (orange; 700 nM) from ^{Rhd}Trp (0.5 μM) at 25 °C in Tris-HCl buffer. Fractions of bound Trp were calculated from the fluorescence intensity at 578 nm. (d) DLS histograms of Trp (0.5 μM) in the absence (dark blue) and presence (red) of $Gu_{12}BA_{12}$ (700 nM) at 25 °C in Tris-HCl buffer. (e) Association constants of $Gu_{12}B_{12}$, $Gu_{18}BA_6$, Gu_6BA_{18}, and $Gu_{12}OH_{12}$ toward Trp (K_{trp}) and ATP (K_{ATP}) determined according to the reported methods.[15,16]

agglomerates with an average diameter of ∼70 nm (Figure 3d, red).[8g]

Subsequently, Gu_mBA_n that adheres to Trp can be detached by addition of ATP. For example, when ATP (0−10 μM) was added to a conjugate of ^{Rhd}Trp (0.5 μM) and $Gu_{12}BA_{12}$ (750 nM; Figure 3a), the fluorescence emission at 578 nm increased as expected (Figure 3b). The fraction of $Gu_{12}BA_{12}$-bound ^{Rhd}Trp decreased nonlinearly as a function of [ATP] (Figure 3c, red), as evaluated from the change of the fluorescence profile using the extinction coefficient of $Gu_{12}BA_{12}$ for ^{Rhd}Trp.[15] For example, when 10 μM of ATP was added, the %-fraction of $Gu_{12}BA_{12}$-bound ^{Rhd}Trp dropped from 80% to 20% (Figure 3c, red). An association constant for $Gu_{12}BA_{12}$ toward ATP (K_{ATP}) of 3.8×10^6 M^{-1} (Figure 3e) was obtained by fitting the dissociation profile (Figure 3c, red) to the competitive binding model.[16] Likewise, K_{ATP} values for Gu_6BA_{18} (3.5×10^6 M^{-1}; Figure 3c, green) and $Gu_{18}BA_6$ (2.7×10^6 M^{-1}; Figure 3c, blue) were determined (Figure 3e). Although the K_{Trp} of $Gu_{12}OH_{12}$ is comparable to that of $Gu_{12}BA_{12}$, ATP was virtually unable to liberate Trp from $Gu_{12}OH_{12}$ (Figure 3c, orange). For $Gu_{12}OH_{12}$, a much smaller K_{ATP} value (5.6×10^4 M^{-1}; Figure 3e) relative to that of $Gu_{12}BA_{12}$ was observed, which highlights the crucial role

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of the BA units in the ATP-responsive nature of Gu_mBA_n. The binding of Gu_mBA_n to ATP strongly depends on multivalent Gu^+/PO_4^- salt-bridge interactions. In fact, toward AMP, an ATP analogue with only one PO_4^- unit, the association constant for $Gu_{12}BA_{12}$ (K_{AMP}: 8.7×10^2 M^{-1}; Figure S14)[13] was 4,400-fold smaller than the corresponding K_{ATP}. The relative affinity toward ATP and Trp (K_{ATP}/K_{Trp}; Figure 3e) can be tuned via the Gu^+/BA ratio. Among the examined Gu_mBA_n polymers, Gu_6BA_{18} exhibited the highest K_{ATP}/K_{Trp} ratio (11; Figure 3e) due to its small K_{Trp} value. Although $Gu_{12}BA_{12}$ exhibited $K_{ATP}/K_{Trp} < 1$, it was used for the following studies on account of its high K_{ATP} and K_{Trp} values (Figure 3e). In principle, BA/1,2-diol binding is pH-sensitive.[10] However, judging from the binding behavior to Alizarin Red S, a fluorescent diol (Figure S12),[13] the BA units of $Gu_{12}BA_{12}$ could certainly bind to the 1,2-diol unit of ATP also at a typical pH (6.8) of tumor tissue.

We then examined the ATP-responsive enzymatic activity of Trp. Upon mixing *N*-α-benzoyl-DL-arginine 4-nitroanilide (BAPNA, 1 mM) with Trp (0.5 μM) at 37 °C in Tris-HCl buffer (50 mM, pH 7.8) containing $CaCl_2$ (10 mM), the characteristic absorption at 405 nm increased (Figure 4b, dark

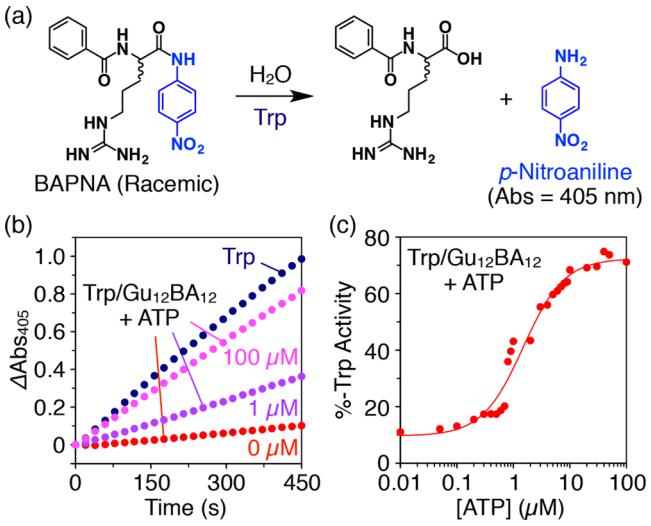

Figure 4. (a) Schematic representation of Trp-catalyzed hydrolysis of BAPNA to *p*-nitroaniline. (b) Absorption changes at 405 nm of Trp (0.5 μM, dark blue), as well as of a mixture of Trp, $Gu_{12}BA_{12}$ (10 μM), and ATP (0, 1, and 100 μM; red, purple, and pink, respectively) in Tris-HCl buffer (50 mM, pH 7.8) containing BAPNA (1 mM) and $CaCl_2$ (10 mM) at 37 °C. (c) Normalized enzymatic activity of Trp (0.5 μM) in the presence of $Gu_{12}BA_{12}$ (10 μM) and ATP (0.01−100 μM).

blue), indicating that BAPNA was hydrolyzed to *p*-nitroaniline (Figure 4a).[17] Conversely, BAPNA was hydrolyzed only very sluggishly (Figure 4b, red) when $Gu_{12}BA_{12}$ (10 μM) was used under otherwise identical conditions. This result might be ascribed to the agglomeration of Trp (Figure 3d), which would lower the accessibility of the active site of Trp. However, the hydrolysis, which was suppressed by $Gu_{12}BA_{12}$, could be accelerated by addition of ATP (1 and 100 μM; Figure 4b, purple and pink, respectively). When Gu_mBA_n was absent, the hydrolytic activity of Trp remained intact to the addition of ATP (Figure S17).[13] The hydrolytic activity of Trp in the presence of $Gu_{12}BA_{12}$ and ATP was evaluated by using pseudo-first order reaction kinetics and normalized relative to that of untreated Trp. A sigmoidal correlation was observed between the %-activity of Trp and [ATP] (Figure 4c), which resulted in an abrupt increase in hydrolytic activity of Trp upon increasing [ATP] from 1 to 10 μM. Considering the concentrations of ATP in tumor and normal tissues (*vide supra*), this threshold is favorable for tumor targeting.

Then, we attempted to extend this strategy to cellular systems. Trp can be used to detach cells from a culture substrate by degrading cell surface components involved in the adhesion to the substrate. Therefore, we immersed human hepatocellular carcinoma Hep3B cells, fluorescently labeled with CellBrite Orange,[13] in a Hank's balanced salt solution (HBSS, pH 7.8) containing Trp (5 μM), before we subjected the sample to confocal laser scanning microscopy (λ_{ext} = 552 nm). As shown in Figure 5a, the cells were detached from the substrate.

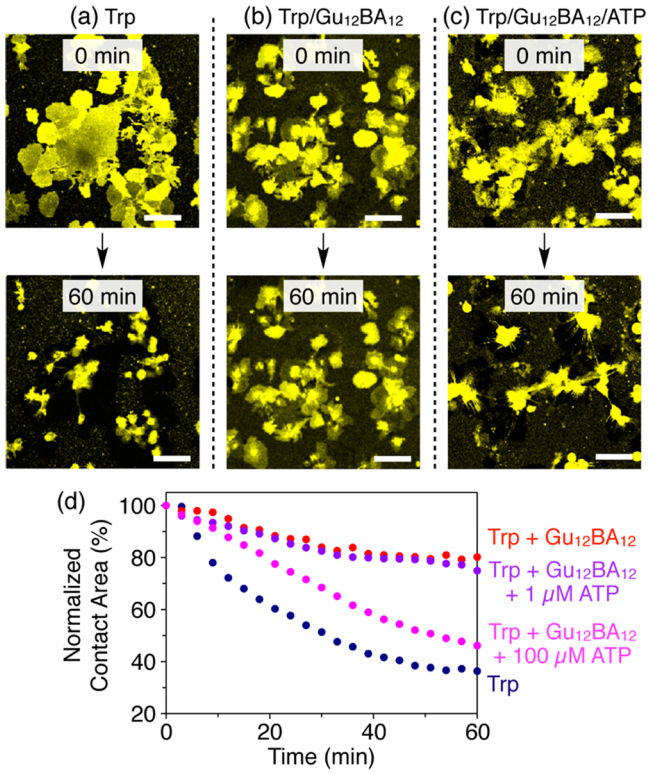

Figure 5. Confocal laser scanning micrographs (λ_{ext} = 552 nm; 0−60 min) of Hep3B cells (5.0×10^3 cells/chamber) fluorescently labeled with CellBrite Orange in HBSS (pH 7.8) containing Trp (5 μM) at 25 °C. Micrographs recorded in the absence (a) and presence of (b) $Gu_{12}BA_{12}$ (10 μM) or (c) both $Gu_{12}BA_{12}$ (10 μM) and ATP (100 μM). Scale bars = 100 μm. (d) Normalized contact areas between Hep3B cells and the substrate in HBSS containing Trp (5 μM) at 25 °C in the absence (dark blue) and presence of $Gu_{12}BA_{12}$ (10 μM, red), as well as in the presence of both $Gu_{12}BA_{12}$ and ATP (1 or 100 μM, purple and pink, respectively); calculated from the micrographs using the ImageJ software.

Accordingly, the cell/substrate contact area, calculated from the fluorescence micrographs by using the ImageJ software, decreased over a period of 60 min (Figure 5d, dark blue).[18] In sharp contrast, Hep3B cells barely detached (Figures 5b,d, red) when they were treated with a mixture of Trp (5 μM) and $Gu_{12}BA_{12}$ (10 μM) under otherwise identical conditions, thus indicating the suppression of enzymatic activity of Trp. Subsequently, we added 100 μM of ATP to the system, whereupon the cells started to detach efficiently, even in the presence of $Gu_{12}BA_{12}$ (Figures 5c,d, pink). It is noteworthy that 1 μM of ATP hardly affected the cell detachment rate (Figure 5d,

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purple). These results suggest that $Gu_{12}BA_{12}$ remains bound to Trp in an ordinary cellular environment but liberates Trp in ATP-rich areas such as tumor tissue.

In conclusion, we developed novel molecular glues, Gu_mBA_n, carrying multiple guanidinium ion (Gu^+) and boronic acid (BA) pendants, as ATP-responsive modulators for the activities of enzymes. Gu_mBA_n binds tightly to ATP as well as to proteins. The enzymatic activity of trypsin (Trp) was effectively modulated *in vitro* and in cellular systems using Gu_mBA_n and ATP. As Trp/Gu_mBA_n conjugates may potentially be sensitive to ATP-rich (>100 μM) tumor tissue, *in vivo* pharmacological studies may furnish interesting results.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b02664.

Synthesis of Gu_mBA_n and Gu_mOH_n; NMR, MALDI-TOF mass spectrometry, fluorescence spectroscopy, and related experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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