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Electronic Supporting Information

Controllable Multivalent LYTACs Enhance Targeted Protein Degradation

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Materials

All DNA oligonucleotides were purchased from Sangon Biotech and purified by high-performance liquid chromatography (HPLC). Detailed sequences and modifications of DNA oligonucleotides can be found in Table S1. HepG2 cell line was purchased from ATCC.

Synthesis of 1-, 3-, and 9-valent GalNAc-Biotin LYTACs.

Synthesis of b*a_n*. 100-μL reactions were prepared in 1×PBS with final concentrations of 10 mM MgSO4, 800 U/mL Bst LF polymerase (NEB, M0275S), 600 μM each of dATP, dCTP, and dTTP (NEB, N0446S), 100 nM of Clean.G, 0.15 μM Hairpin and water to 90 μL. After Clean.G incubation for 15 min at 37 °C, 10 μL of 10 μM Primer: b*a* was added to obtain 1 μM final concentration, and then the reaction was incubated for another 1.5 h at 37 °C followed by 20 min at 80 °C to heat inactivate the polymerase. The synthesis of concatemer b*a_n* was verified by agarose gel electrophoresis. Among them, the Tm of "a*" was 25.6 °C.[1]

Synthesis of Multivalent GalNAc-Biotin LYTACs. b*a_n* reacted with Tri-GalNac-b and Biotin-aa at a molar ratio of 1:1:1, 1:1:3, or 1:1:9 at 80 °C for 5 min. Then, the samples were cooled down to 4 °C at a rate of -0.5 °C/min. The synthesis of 1-, 3-, and 9-valent LYTACs were verified by agarose gel electrophoresis.

Synthesis of 1-, 3-, and 9-valent GalNAc-Apt Met LYTACs.

Synthesis of b*a_n*. 100-μL reactions were prepared in 1×PBS with final concentrations of 10 mM $MgSO_4$, 800 U/mL Bst LF polymerase, 600 μM each of dATP, dCTP, and dTTP, 100 nM of Clean.G, 0.15 μM Hairpin and water to 90 μL. After Clean.G incubation for 15 min at 37 °C, 10 μL of 10 μM Primer: b*a* was added to obtain 1 μM final concentration, and then the reaction was incubated for another 1.5 h at 37 °C followed by 20 min at 80 °C to heat inactivate the polymerase. The synthesis of concatemer b*a_n* was verified by agarose gel electrophoresis. Among them, the Tm of "a*" was 25.6 °C.

Synthesis of Multivalent GalNAc-Apt Met LYTACs. b*a_n* reacted with Tri-GalNac-b and Apt Met-aa at a molar ratio of 1:1:1, 1:1:3, or 1:1:9 at 80 °C for 5 min. Then, the samples were cooled down to 4 °C at a rate of -0.5 °C/min. The synthesis of 1-, 3-, and 9-valent LYTACs were verified by agarose gel electrophoresis and HPLC.

Synthesis of 1-, 3-, and 9-valent Apt IGFIIR-Apt Met LYTACs.

100-μL reactions were prepared in 1×PBS with final concentrations of 10 mM $MgSO_4$, 800 U/mL Bst LF polymerase, 600 μM each of dATP, dCTP, and dTTP, 100 nM of Clean.G, 0.15 μM Hairpin and water to 90 μL. After Clean.G incubation for 15 min at 37 °C, 10 μL of 10 μM Primer: Apt IGFIIR-a* was added to obtain 1 μM final concentration, and then the reaction was incubated for another 1.5 h at 37 °C followed by 20 min at 80 °C to heat inactivate the polymerase. The synthesis of Apt IGFIIR-a_n* was verified by agarose gel electrophoresis. Among them, the Tm of "a*" was 25.6 °C.

Apt IGFIIR-a_n* reacted with Apt Met-aa at a molar ratio of 1:1, 1:3, or 1:9 at 80 °C for 5 min. Then, the samples were cooled down to 4 °C at a rate of -0.5 °C/min. The synthesis of 1-, 3-, and 9-valent LYTACs were verified by agarose gel electrophoresis.

Synthesis of 3-valent GalNAc-Apt Met LYTACs with different ligand distance.

Synthesis of b*a_n*. 100-μL reactions were prepared in 1×PBS with final concentrations of 10 mM $MgSO_4$, 800 U/mL Bst LF polymerase, 600 μM each of dATP, dCTP, and dTTP,

100 nM of Clean.G, 0.15 μM Hairpin and water to 90 μL. After Clean.G incubation for 15 min at 37 °C, 10 μL of 10 μM Primer: b*a* was added to obtain 1 μM final concentration, and then the reaction was incubated for another 1/1.5/2/2.5/3 h at 37 °C followed by 20 min at 80 °C to heat inactivate the polymerase. The synthesis of concatemer b*a_n* was verified by agarose gel electrophoresis. Among them, the Tm of "a*" was 25.6 °C.

Synthesis of Multivalent GalNAc-Apt Met LYTACs. b*a_n* reacted with Tri-GalNac-b and Apt Met-aa at a molar ratio of 1:1:3 at 80 °C for 5 min. Then, the samples were cooled down to 4 °C at a rate of -0.5 °C/min. The synthesis of 3-valent LYTACs with different ligand distance were verified by agarose gel electrophoresis.

Analysis and purification of DNA products by HPLC-SEC.

A Waters 1260 HPLC system (Waters Corporation, USA), equipped with a size-exclusion chromatography (SEC) column, was used to purify and analyze DNA products. The column had a pore size of 300 Å and a particle size of 2.7 μm, optimized for size-based separation. The mobile phase consisted of TE buffer (20mM Tris-HCl, pH 7.4, 100mM NaCl, 1mM EDTA) at a flow rate of 0.3 mL/min. DNA samples (1μM ,100 μL) were injected at a column temperature of 25°C, with UV detection performed using a Waters 2489 UV detector at 260 nm, 495 nm, and 649 nm. A Waters Fraction Collector III was used for collection of eluted fractions. Data processing was conducted using Waters Empower 3 software, enabling purity and yield assessment based on peak area and retention time.

Streptavidin binding analysis by flow cytometry.

HepG2 cells were incubated with 100 nM of 1-, 3-, or 9-valent LYTACs on ice for 30 minutes, and then washed with 1×PBS three times at 150 g for 5 minutes. Cells were incubated with 100 nM of SA-AF647 (Yeasen biotechnology, 35104ES60) on ice for 30 minutes, and then washed with 1×PBS three times at 150 g for 5 minutes. Then, cells were resuspended in 200 μL DAPI diluted in 1×PBS. Flow cytometry was performed on BD FACSCanto, and FlowJo software was used to gate on single cells and live cells for analysis.

Streptavidin endocytosis analysis by confocal microscopy.

HepG2 cells were plated (100,000 cells in a 20 mm confocal dish) 1 d before the experiment. Cells were incubated with 10 nM 1-, 3-, or 9-valent LYTACs and 90 nM SA-AF647 for 1 h at 37°C. After three times washing with fresh DMEM, LysoTracker (Beyotime, C1047S) and Hoechst (Beyotime, C1027) were performed according to the manufacturer's protocol. Cells were imaged with Zeiss LSM 880 confocal laser scanning microscopy using a 63× oil immersion objective lens.

Serum stability test.

500 nM of 1-, 3-, or 9-valent LYTACs were incubated with 10% fetal bovine serum for different time (0-24 h). 10% native polyacrylamide gel in 1×TBE was run at 90 V for 1 hour and then stained with Gel Red staining solution.

Multivalent LYTACs binding affinities analysis by flow cytometry.

HepG2 cells were incubated with a series of different concentrations of 1-, 3-, or 9-valent LYTACs on ice for 30 minutes. The cells were washed twice with 1×PBS, centrifuged at 150 g for 5 minutes, and then resuspended in 200 μL DAPI diluted in 1×PBS. Flow cytometry was performed on BD FACSCanto, and FlowJo software was used to gate on

single cells and live cells for analysis. Median fluorescence intensity (MFI) of each cell population was calculated from at least 50,000 live cells. The dissociation constants (Kd) of chimeras were obtained by fitting the dependence of fluorescence intensity (Y) and the concentrations of chimeras (X) into the onesite saturation equation Y = Bmax X/(Kd + X), using GraphPad Prism.

Cell surface Met degradation analysis by flow cytometry.

HepG2 cells were plated (80,000 cells per well in a 24-well plate) 1 d before the experiment. Cells were incubated with 250 μL complete DMEM with 100 nM chimeras or controls for 24 h, and then washed with 1×PBS three times, trypsinized for 4 min and transferred to centrifuge tubes. Cells were washed three times with FACS buffer (0.5% BSA+5 mM EDTA in PBS), centrifuged at 150 g for 5 minutes. Then, cells were incubated with primary antibody for 30 min on ice, washed three times with FACS buffer and incubated with secondary antibody for 30 min on ice. After washing three times with FACS buffer, cells were suspended in diluted DAPI buffer. Flow cytometry was performed on BD FACSCanto, and FlowJo software was used to gate on single cells and live cells for analysis. Median fluorescence intensity (MFI) of each cell population was calculated from at least 50,000 live cells.

Cell surface Met degradation analysis by western blot.

HepG2 cells were plated (80,000 cells per well in a 24-well plate) 1 d before the experiment. Cells were incubated with 250 μL complete DMEM with 100 nM chimeras or controls for 24 h. Cells were washed three times with PBS and lysed with RIPA buffer supplemented with 1 mM PMSF on ice for 20 min. The cells were scraped, transferred to tubes and lysed for another 10 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected and the protein concentration was determined by BCA assay (Beyotime, P0010S). 20 μg proteins were separated by SDS-PAGE. The gel was then transferred onto a nitrocellulose membrane (Beyotime, FFN02) and blocked with Blocking Buffer (Beyotime, P0252) for 1 h at room temperature. The membrane was incubated with primary antibody overnight at 4 °C and washed three times with TBSTw (0.1% Tween-20 in TBS) (Beyotime, ST673). Subsequently, the membrane was incubated with secondary antibody for 1 h at room temperature and washed three times with TBSTw for visualization with a Tanon 2500 automatic digital gel image analysis system. ImageJ software was used to quantify band intensities.

Cell surface Met degradation analysis by confocal microscopy.

HepG2 cells were plated (30,000 cells per well in an 8-well ibidi plate) 1 d before the experiment. Cells were incubated with 200 μL complete DMEM with 100 nM chimeras or controls for 24 h. Cells were then washed three times with 1×PBS, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, washed three times and blocked in 2% BSA in PBS for 30 min at room temperature and incubated with primary antibody for 1 h at room temperature. Cells were then washed with 1×PBS and incubated with secondary antibody for 1 h at room temperature. After washing with 1×PBS, DID and DAPI were performed according to the manufacturer's protocol. Cells were imaged with Zeiss LSM 880 confocal laser scanning microscopy using a 63× oil immersion objective lens.

Statistical analysis

Data were represented as the mean of three independent experiments ± s.e.m. *p<0.05, **p<0.01, ***p<0.001.

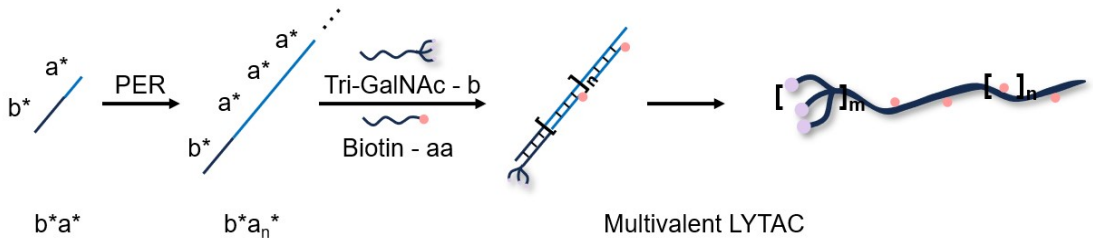


Fig. S1. Flowchart of multivalent LYTACs (GalNAc-Biotin) synthesis via DNA self-assembly.

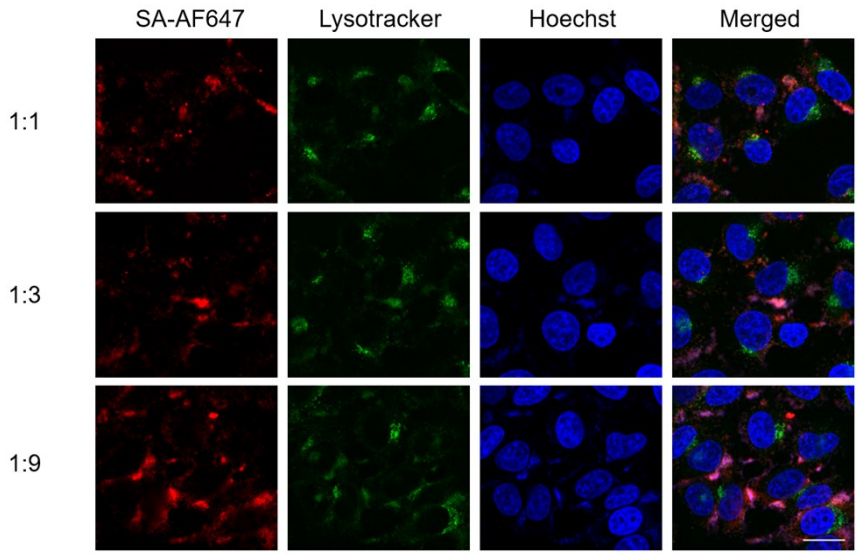


Fig. S2. Characterization of streptavidin endocytosis ability in HepG2 cells using fluorescence imaging with 1-, 3-, or 9-valent LYTACs under optimal imaging conditions. AF647 in the 1:1, 1:3, and 1:9 groups colocalized with Lysotracker, with correlation coefficients of 0.39, 0.32, and 0.44, respectively. Scale bar=20 μm.

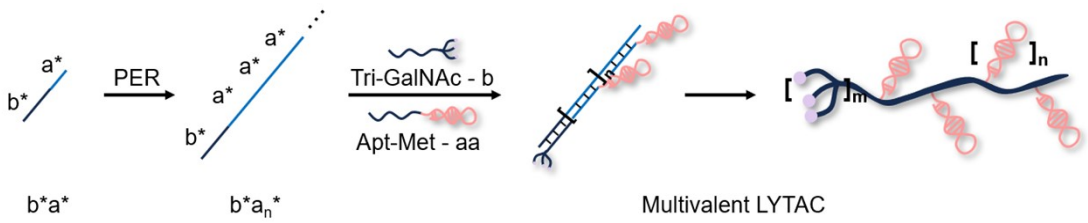
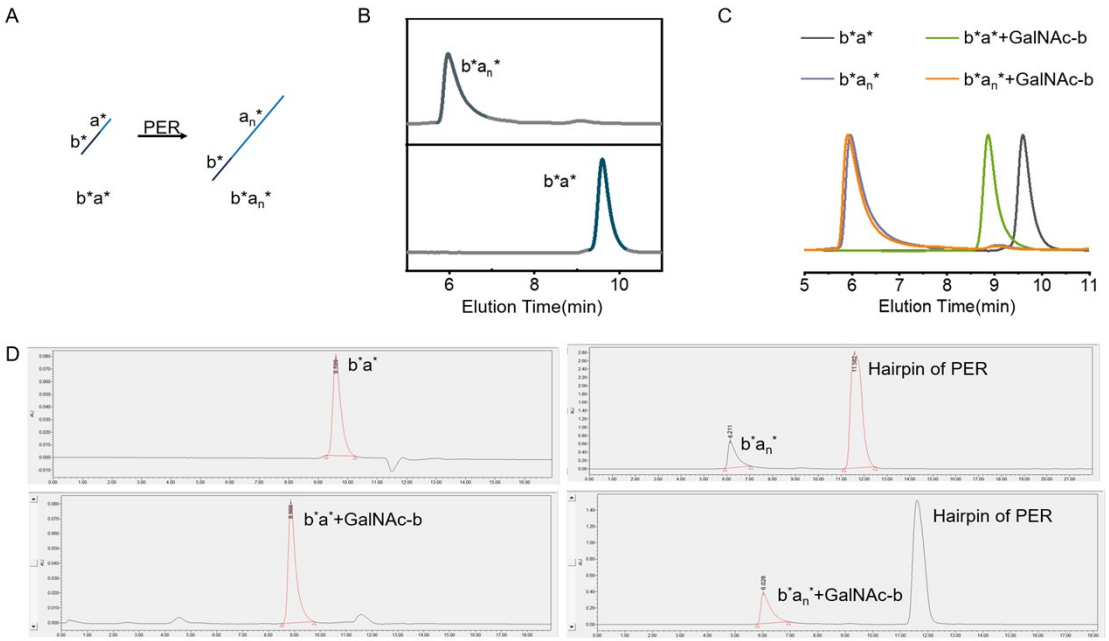
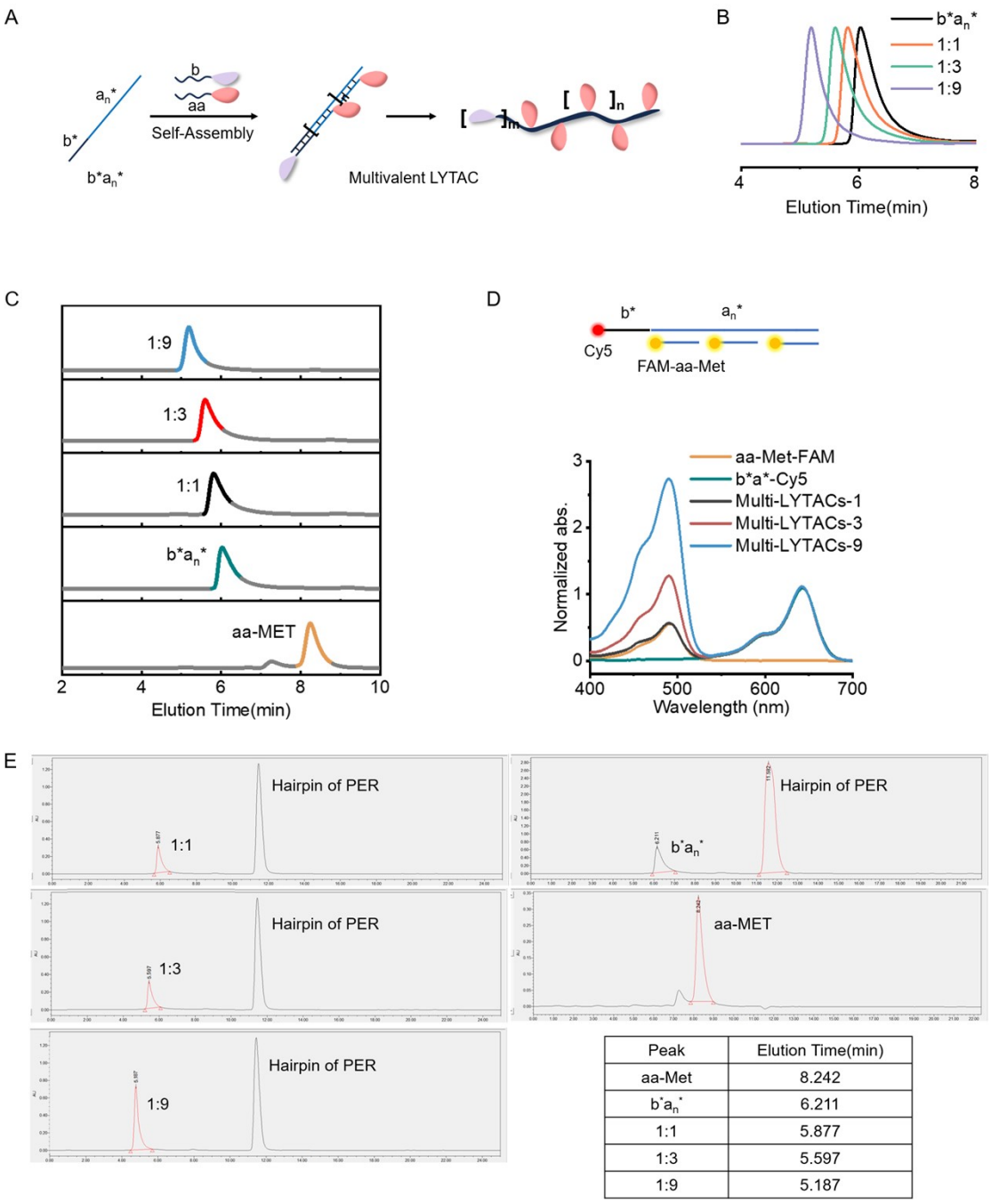


Fig. S3. Flowchart of multivalent LYTACs (GalNAc-Apt Met) synthesis via DNA self-assembly.



Peak	Elution Time(min)
b*a*	9.599
b*a*-GalNAc	8.866
b*a_n*	6.211
b*a_n*-GalNAc	6.028

Fig. S4. (A) Flowchart illustrating the synthesis of b*a_n* through PER. (B) HPLC characterization diagram before and after PER. The left shift of the peak in the upper figure indicated the successful synthesis of b*a_n*. (C) HPLC characterization diagram of b*a* and b*a_n* before and after complementation with GalNAc-b. The left shift of the peak indicated their successful complementation. (D) The unprocessed analytical HPLC data of (B) and (C).



Peak	Elution Time(min)
aa-Met	8.242
$b^*a_n^*$	6.211
1:1	5.877
1:3	5.597
1:9	5.187

Fig. S5. (A) Flowchart illustrating the synthesis of multivalent LYTACs through DNA self-assembly. (B) HPLC characterization of different valent chimeras. The gradual leftward shift of the peaks indicated the formation of different valent chimeras. (C) HPLC purification of chimeras. Color in the peaks represented the collection position. (D) Quantification of chimeras after purification. Cy5 was introduced into the main chain "b*a_n*" and FAM was introduced into the POI ligand. The concentration of chimeras was quantified according to the absorbance at 495 nm and the valence of chimeras was qualitatively determined according to the absorbance at 649 nm. (E) The unprocessed analytical HPLC data of (B) and (C).

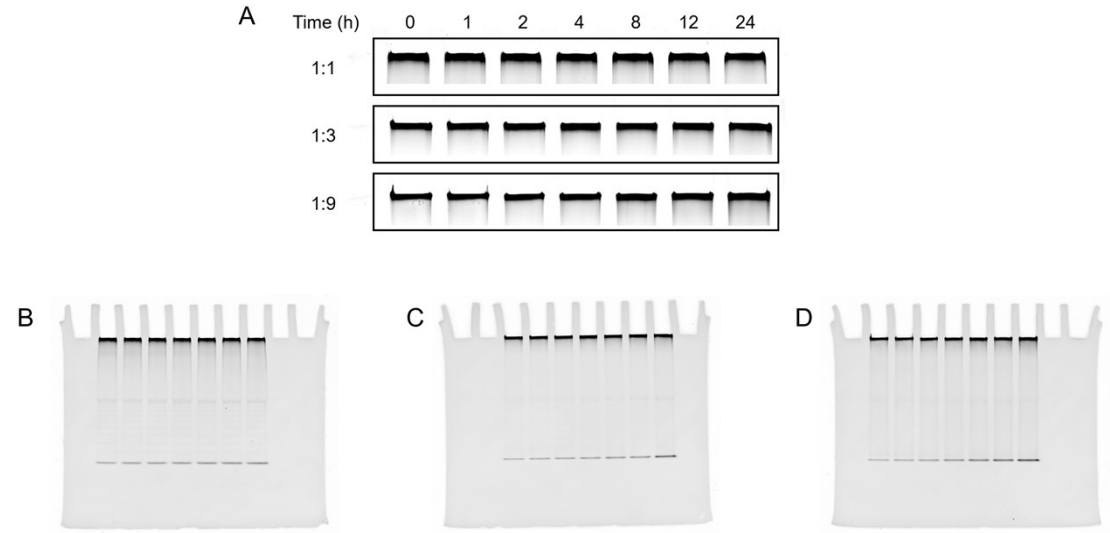


Fig. S6. (A) Native polyacrylamide gel analysis of the stability of 1-, 3-, and 9-valent LYTACs (1:1, 1:3, 1:9) in 10% FBS for 0, 1, 2, 4, 8, 12, 24 hours. The raw images of 1:1 (B), 1:3 (C), and 1:9 (D).

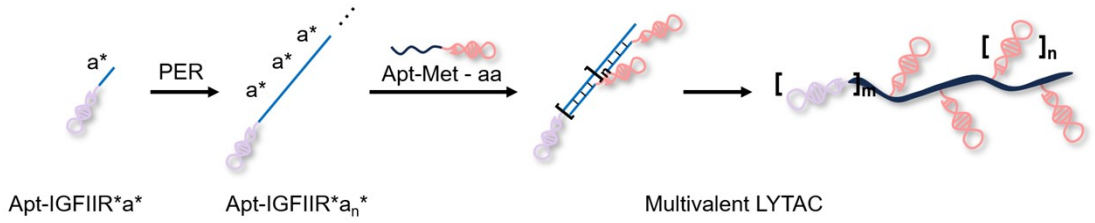


Fig. S7. Flowchart of multivalent LYTACs (Apt IGFIIR-Apt Met) synthesis via DNA self-assembly.

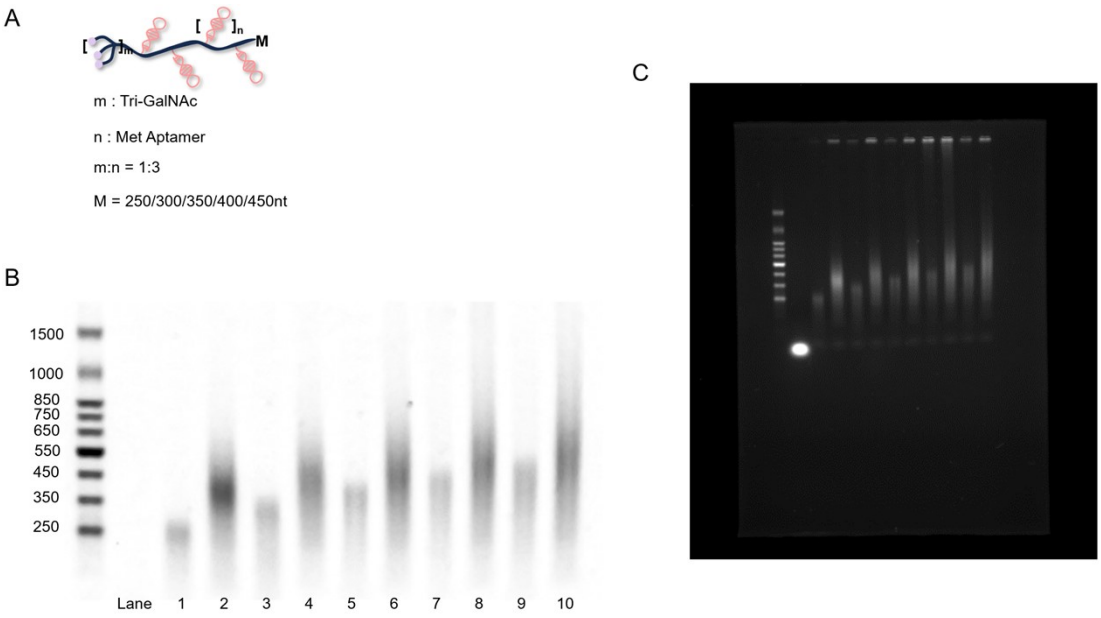


Fig. S8. (A) Structure of 3-valent LYTACs (GalNAc-Apt Met) with different ligand distances. (B) Synthesis of 3-valent LYTACs with different ligand distances visualized through agarose gel electrophoresis. Lane 1, 3, 5, 7, 9: b*a_n* with about 250, 300, 350, 400, 450 nucleotides. Lane 2, 4, 6, 8, 10: 3-valent LYTACs with different length. (C) The raw image of (B).

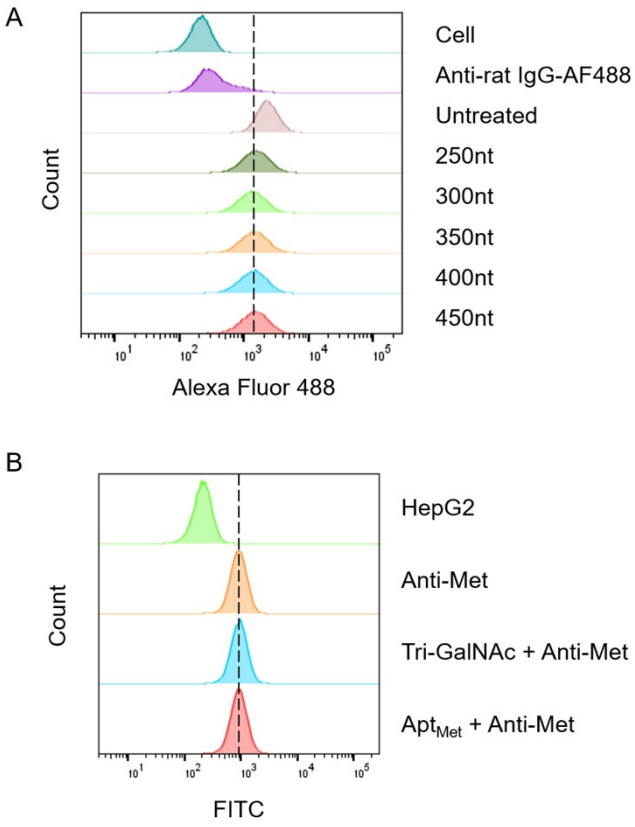


Fig. S9. (A) Flow cytometry analysis of total Met levels in HepG2 cells after treatment with 100 nM 3-valent LYTACs with different ligand distances for 24 h. (B) Flow cytometry analysis of total Met levels in HepG2 cells after treatment with Tri-GalNAc only or Apt-Met only. It can be seen that Tri-GalNAc and Apt-Met do not affect anti-Met antibody staining.

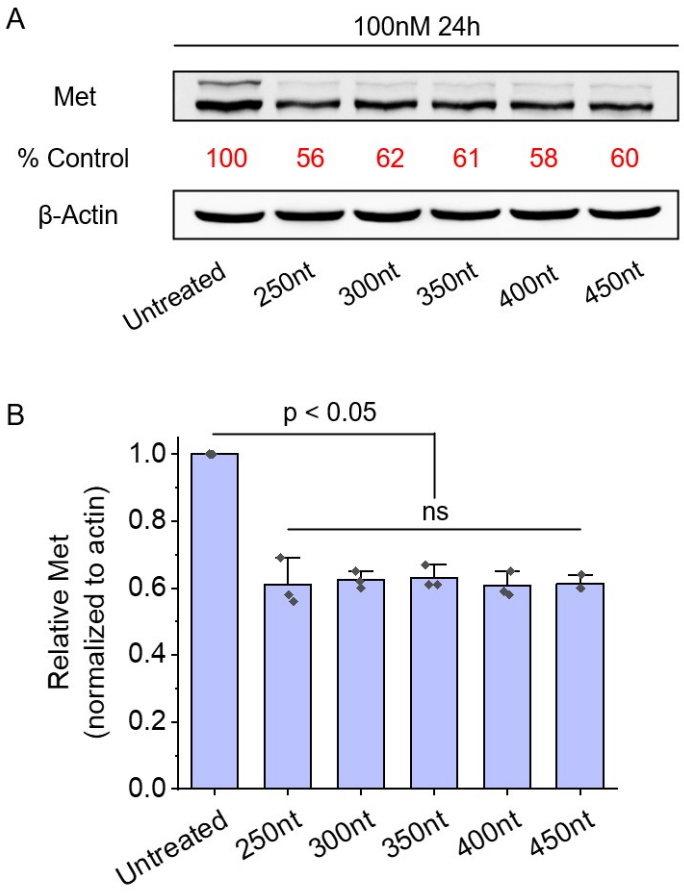


Fig. S10. Western blot analysis of total Met levels in HepG2 cells after treatment with 100 nM 3-valent LYTACs with different ligand distances for 24 h. Data are represented as the mean of three independent experiments ± s.e.m.

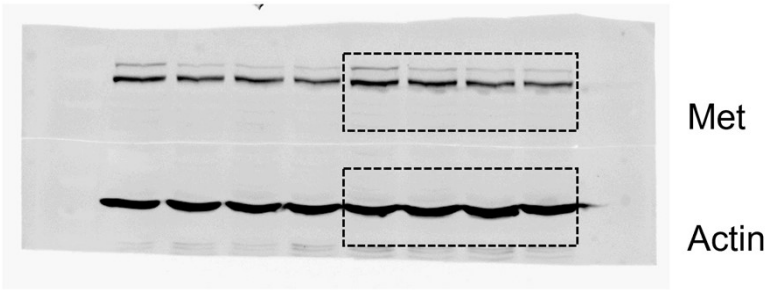


Fig. S11. Uncropped blot corresponding to Fig. 3C.

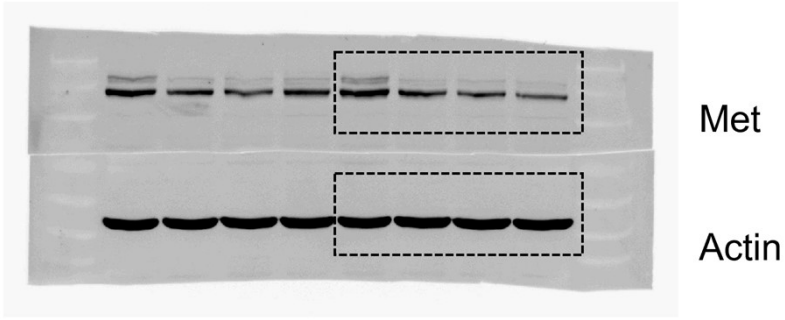


Fig. S12. Uncropped blot corresponding to Fig. 4C.

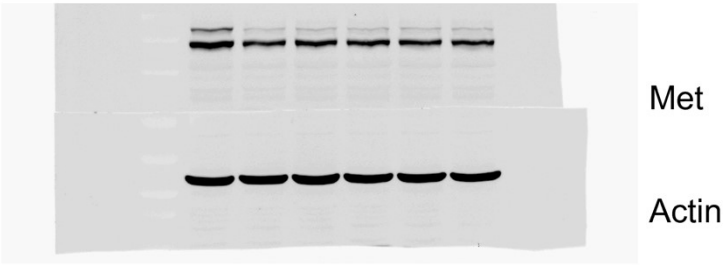


Fig. S13. Uncropped blot corresponding to Fig. S10A.

Table S1. Oligonucleotide sequences.

LYTACs	Name	Sequence (5’ to 3’)
GalNAc-biotin	b*a*	GGACGCTAAATATAGGAAACTTCCAATAATA
	GalNAc-b	GTTTCCTATATTTAGCGTCC-GalNAc
	Biotin-aa	Biotin-TTTATTATTGGTTATTATTGGT
GalNAc-Apt Met	b*a*	GGACGCTAAATATAGGAAACTTCCAATAATA
	GalNAc-b	GTTTCCTATATTTAGCGTCC-GalNAc
	Met-aa	ATCAGGCTGGATGGTAGCTCGGTCGGGGTGGGTGGGTTGGCAAGTCTGATTTTATTATTGGTTATTATTGGT
Apt IGFIIR-Apt Met	IGFIIR-a*	GGGCGCGTAGATGACGAGCAGTCCTAACATCGTTTAGGACCCAATAATA
	Met-aa	ATCAGGCTGGATGGTAGCTCGGTCGGGGTGGGTGGGTTGGCAAGTCTGATTTTATTATTGGTTATTATTGGT
	Hairpin	ACCAATAATAGGGCCTTTTGGCCCTATTATTGGTTATTATTGG-Inverted dT
	Clean.G	CCCCGAAAGTGGCCTCGGGCCTTTTGGCCCGAGGCCACTTTCG

Table S2. The binding affinity of Met aptamer (Apt-Met), IGFIIR aptamer (Apt-IGFIIR)[2] and GalNAc.[3]

Name	Kd [nM]
Apt-Met	45
GalNAc	10-26
Apt-IGFIIR	34

References

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3 X. Huang, J. Leroux and B. Castagner, *Bioconjug. Chem.*, 2017, **28**, 283-295.