Supporting Information

Defining the Design Parameters for *in Vivo* Enzyme Delivery Through Protein Spherical Nucleic Acids

Caroline D. Kusmierz†‡, Katherine E. Bujold†‡, Cassandra E. Callmann†‡, and Chad A. Mirkin*†‡

1Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States
2International Institute for Nanotechnology, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States.

Table of Contents

Safety Statement ................................................................................................................................................. 2
S1. Synthesis and Characterization of β-Galactosidase Protein Spherical Nucleic Acids .............. 2
  1.1 Oligonucleotide synthesis ....................................................................................................................... 2
  1.2 Synthesis of β-Galactosidase (β-Gal) Protein Spherical Nucleic Acids (ProSNA) [2] .... 3
  1.3 Characterization of GQ Sequences and ProSNAs by Circular Dichroism ................................. 9
S2. *in Vitro* Investigations on ProSNA Variants ....................................................................................... 12
  2.1 Cellular Uptake in HeLa Cells ............................................................................................................... 12
  2.2 Cellular Uptake in C166 Cells .............................................................................................................. 15
  2.3 Kinetic Analysis of β-Galactosidase Activity ................................................................................... 15
S3. *in Vivo* Investigations Using Model ProSNA Variants ................................................................. 19
  3.1 Blood Circulation Time ....................................................................................................................... 19
  3.2 *Ex Vivo* Near-Infrared Fluorescence (NIRF) Imaging ..................................................................... 19
  3.3 Enzymatic Colorimetric Activity Assay of Main Organs [6] .......................................................... 22
S4. References .................................................................................................................................................. 25
Safety Statement

No unexpected or unusually high safety hazards were encountered in this work.

S1. Synthesis and Characterization of β-Galactosidase Protein Spherical Nucleic Acids

1.1 Oligonucleotide synthesis

Oligonucleotides were synthesized using standard phosphoramidite chemistry protocols on an ABI 392 using 1000 Å universal CPG solid supports (ChemGenes) and phosphoramidites, as well as coupling reagents purchased from Glen Research. The DBCO-dT-CE phosphoramidite was hand coupled in a glove box using a mild oxidizing agent ((1S)-(+)-(10-Camphorsulfonyl)-oxaziridine, CSO) to increase coupling efficiency and preserve the integrity of the strained alkyne in a water- and air-free environment. Synthesized strands were cleaved using 30% aqueous ammonia (Sigma Aldrich) for 16 h at room temperature and purified by HPLC using a reverse-phase C4 column (Shimadzu) running a gradient from 0 to 100% acetonitrile in triethylammonium acetate buffer (Sigma Aldrich) over 45 min. After HPLC purification, the final dimethoxytrityl group was removed in 20% acetic acid (Sigma Aldrich) for 2 h and extracted in ethyl acetate (Sigma Aldrich). The masses of the oligonucleotides were confirmed using matrix-assisted laser desorption ionization-time of flight (MALDI-MS) mass spectroscopy on a Bruker AutoFlex-III using 2’,6’-dihydroxyacetophenone (Sigma Aldrich) as the matrix. Molecular weights and extinction coefficients were calculated based on estimates using the IDT OligoAnalyzer tool [1] (Table S1.1).

Table S1.1 Oligonucleotide sequences, molecular weights, and extinction coefficients.

| Name     | Sequence (5’ to 3’) | MW Expected [Da] | MW Observed [Da] | ε_{260} [M^{-1}cm^{-1}] |
|----------|---------------------|------------------|------------------|------------------------|
| T₄       | DBCO-dT T₃₄          | 11054            | 10991            | 276000                 |
| T₈       | DBCO-dT T₃₈          | 12271            | 12237            | 308400                 |
| T₁₂      | DBCO-dT T₄₂          | 13488            | 13423            | 340800                 |
| (sp18)₂  | DBCO-dT (sp18)₂ T₃₀ | 10526            | 10524            | 243600                 |
| (sp18)₄  | DBCO-dT (sp18)₄ T₃₀ | 11215            | 11206            | 243600                 |
Table S1.2 Molecular weights and extinctions for chemical modifications onto β-Gal.

| Name                        | MW [Da] | $\varepsilon_{\text{max}}$ [M$^{-1}$cm$^{-1}$] |
|-----------------------------|---------|-----------------------------------------------|
| β-Galactosidase             | 464,000 | 1,142,000 (280nm) 596,268 (260nm)               |
| Alexa Fluor® 647 dye        | 1250    | 265,000 (650nm)                                  |
| NHS-PEG$_4$-azide           | 388     | N/A                                            |

Figure S1. Synthesis scheme for ProSNAs. Cysteine residues are first modified with an Alexa Fluor 647 tag, then NHS-PEG$_4$-azide linkers are conjugated to surface accessible lysine residues. Using copper-free click chemistry, DBCO-terminated DNA strands can react with the azide-modified protein resulting in Protein Spherical Nucleic Acids (ProSNA). All reactions are held at pH 7.4 in phosphate containing buffers at either 4°C or ambient temperatures.

1.2 Synthesis of β-Galactosidase (β-Gal) Protein Spherical Nucleic Acids (ProSNA) [2]

Molecular weights and extinction coefficients pertaining to synthesis and characterization of β-Gal ProSNAs are detailed in Table S1.2.
Reaction of Surface-Accessible Cysteines with Alexa Fluor 647 (AF647). β-Gal from an E. coli overproducer was purchased from Roche. The lyophilized protein was first dissolved in 1X phosphate-buffered saline (1X PBS; Thermo Fisher Scientific) and washed via 3 rounds of centrifugation using a 100 kDa centrifugal filter (Millipore), resuspending the protein in 1X PBS after each wash. Protein concentration was determined from its 280 nm absorbance peak collected on a Cary-5000 UV-Vis spectrophotometer in a 1 cm pathlength cuvette. Then, 10 equivalents of Alexa Fluor 647-C₂-maleimide (Thermo Fisher Scientific), dissolved in DMSO, were added to ~19 μM β-Gal in 1500 μL 1X PBS and the reaction was shaken (900 rpm) overnight at 4 °C. Unconjugated Alexa Fluor 647 was removed by repeated rounds of centrifugation using a 100 kDa filter until the filtrate did not have a detectable absorbance at 650 nm by UV-Vis. The number of Alexa Fluor 647 modifications per protein was calculated based on UV-Vis spectroscopy (Figure S2).

Figure S2. Representative UV-Vis spectrum of fluorophore modified β-Galactosidase. UV-Vis spectrum and calculation of the number of AF647 fluorophores on β-Gal in 1X PBS. Spectra were collected at ambient temperature with a 1 cm pathlength cuvette on a Cary5000 spectrophotometer. Inset calculations detail the steps used to calculate the number of fluorophores per protein.
Reaction of Surface-Accessible Lysines with NHS-PEG₄-Azide. 200 equivalents of NHS-PEG₄-azide crosslinker (Thermo Fisher Scientific), dissolved in anhydrous DMSO at a concentration of 100 mM, were added to ~ 45 μM β-Gal-AF647 in 550 μL 1X PBS. The reaction was shaken (900 rpm) overnight at 25°C. Unconjugated linker was removed by 10 rounds of centrifugation using a 100 kDa filter. The number of azide modifications was assessed by MALDI-MS using sinapinic acid (Thermo Fisher Scientific) as a matrix in a Bruker AutoFlex-III (Figure S3).

\[
\Delta m/z_{\text{monomer}} = \beta\text{GalAF647Azide} - \beta\text{GalAF647} = 120349 - 116744 = 3605
\]

\[
\Delta m/z_{\text{tetramer}} = \Delta m/z_{\text{monomer}} \times 4 = 3605 \times 4 = 14420
\]

\[
\Delta m/z_{\text{tetramer}} + MW_{\text{NHSEG₄azide}} = \frac{14420}{388} = 37 \text{ azide per protein}
\]

Figure S3. MALDI-MS spectra of azide modified and unmodified β-Gal-AF647. To calculate the number of azides per monomer, the mass difference between an unmodified and azide modified protein can be determined using MALDI-MS. The mass shown is of the monomer; therefore, to determine a mass change of the entire protein—a tetramer—this difference should be multiplied by 4.
Functionalization with DBCO-dT Terminated T-Rich Oligonucleotides. 350 equivalents of DBCO-dT terminated DNA strands were first lyophilized, then 6.5 μM β-Gal-AF647-azide in 450 μL 1X PBS was added to rehydrate the DNA. This solution was allowed to incubate for 72 h at 25 °C with shaking (900 rpm). Unreacted DNA strands were removed by successive rounds of centrifugation in a 100 kDa filter until the filtrate did not have a detectable absorbance at 260 nm. Typically, complete removal of DNA required 30-40 washing steps. The number of DNA strands per protein was calculated based on UV-Vis spectroscopy (Figure S4). Loading densities from ProSNAs described in this work appear in Table S1.3 unless otherwise specified.

![Protein Spherical Nucleic Acid](image)

Figure S4. Representative UV-Vis spectrum of a protein spherical nucleic acid. UV-Vis spectrum and DNA loading calculations for a T₄T₃₀ ProSNA in 1X PBS. Spectra were collected at ambient temperature with a 1 cm pathlength cuvette on a Cary5000 spectrophotometer. Inset calculations detail the steps used to calculate the number of T₄T₃₀ DNA per protein.
Functionalization with DBCO-dT Terminated GQ Oligonucleotides. β-Gal-AF647-azide was first buffer exchanged in lithium phosphate buffer (10 mM LiH$_2$PO$_4$, Sigma-Aldrich, pH 7.4) using five successive rounds of centrifugation in a 100 kDa filter. 350 equivalents of DBCO-dT terminated GQ DNA were lyophilized, then 6.5 μM β-Gal-AF647-azide in 450 μL LiPO$_4$ buffer were added to rehydrate the DNA. This solution was allowed to incubate for 72 h at 25 °C with shaking (900 rpm). Unreacted DNA strands were removed by successive rounds of centrifugation in a 100 kDa filter using the LiPO$_4$ buffer as the washing buffer until the filtrate did not have a detectable absorbance at 260 nm. Typically, complete removal of DNA required 30-40 washing steps. The number of DNA strands per protein was calculated based on UV-Vis spectroscopy (Figure S4). Loading densities from ProSNAs described in this work, unless otherwise specified, appear in Table S1.3. After complete removal of unconjugated DNA strands, ProSNAs were buffer exchanged back into 1X PBS buffer using five successive rounds of centrifugation in a 100 kDa filter.

Figure S5. UV-Vis spectra of protein spherical nucleic acids. UV-Vis spectra of ProSNAs with different linker identities demonstrating their equal DNA loading. Spectra were collected at ambient temperature with a 1 cm pathlength cuvette on a Cary5000 spectrophotometer.
Table S1.3 Oligonucleotide loading of ProSNA constructs (unless otherwise stated)

| Name            | Sequence (5' to 3')                  | DNA per β-Gal |
|-----------------|---------------------------------------|---------------|
| T₄              | DBCO-dT T₃₄                           | 31            |
| T₈              | DBCO-dT T₃₈                           | 30            |
| T₁₂             | DBCO-dT T₄₂                           | 30            |
| (sp₁₈)₂         | DBCO-dT (sp₁₈)₂ T₃₀                  | 32            |
| (sp₁₈)₄         | DBCO-dT (sp₁₈)₄ T₃₀                  | 32            |
| (sp₁₈)₆         | DBCO-dT (sp₁₈)₆ T₃₀                  | 33            |
| T₃₀ (sp₁₈)₆     | DBCO-dT T₃₀ (sp₁₈)₆                 | 31            |
| T₄(GGT)₁₀       | DBCO-dT T₄(TGG)₁₀                   | 32            |
| 2KF7[³]        | DBCO-dT T₄(G₃T₂A)₃G₃T               | 35            |
| 1KF1[⁴]        | DBCO-dT T₄AG₃(T₂AG₃)₃               | 35            |
| 2JPZ[⁵]        | DBCO-dT T₄(T₂AG₃)₄T₂                  | 37            |
| 148D[⁵]        | DBCO-dT T₄G₂T₂G₂TGTG₂T₂G₂            | 37            |

*SDS-PAGE Analysis of ProSNAs.* Protein samples (2 pmol) were incubated at 80 °C for 5 min in 1X Laemmlie Sample Buffer (Bio-Rad) and 355 mM 2-mercaptoethanol (Sigma-Aldrich) before loading. Samples were run on a 4-15% TGX mini-Protean pre-cast gradient gel (Bio-Rad) for 90 min at 100 V in 1X Tris/Glycine/SDS running buffer (Bio-Rad). Gels were stained using SimplyBlue SafeStain (Invitrogen) before imaging on a ChemiDoc™ MP Imaging System (Figure S6-8).

*Figure S6. 4-15% SDS PAGE gel of native β-gal and ProSNA variants with different linker identity.* Gel was run for 90 min at 100 V in 1X Tris/Glycine/SDS running buffer. Lower mobility bands are indicative of higher molecular weights for ProSNA variants and confirm the successful conjugation of DNA strands onto the protein.
1.3 Characterization of GQ Sequences and ProSNAs by Circular Dichroism

CD spectra of G-Quadruplex DNA strands in 1X PBS buffer. Circular dichroism (CD) spectra were recorded in a 10 mm pathlength cuvette at 25°C with a Jasco J-1700 spectropolarimeter equipped with a temperature controller at a concentration of 7.5 μM DNA in 1X PBS buffer (pH 7.4). The instrument parameters to record the CD spectra were: 190-350 nm measurement range, 0.1 nm data pitch, 1 nm bandwidth, 50 nm/min scanning speed, and 5 accumulations. CD spectra were normalized to molar ellipticity ([θ], deg cm² dmol⁻¹) using $[\theta] = \frac{\theta \times 10}{c \times l}$ where
θ is the CD ellipticity in millidegrees, c is the sample concentration in mol/L, and l is the pathlength in cm (Figure S9). Expected and observed peaks and troughs are tabulated in Table S1.4.

Figure S9 Circular dichroism (CD) spectra of free GQ DNA. CD spectra of 7.5 μM free DBCO-dT terminated strands with a T₄ DNA linker in 1X PBS. Sequences are detailed in Table S1.3. Spectra match the corresponding topology of (a) parallel⁵, (b) antiparallel basket⁴, (c) parallel⁴, (d) mixed⁵, and (e) antiparallel chair⁵.
Table S1.4 G-Quadruplex Circular Dichroism Peak and Trough Signatures

| Name             | Sequence (5’ to 3’)                  | Expected Peak or Trough | Observed Peak or Trough |
|------------------|--------------------------------------|-------------------------|-------------------------|
| T₄(GGT)₁₀[⁽²⁾]   | DBCO-dT T₄(TGG)₁₀                 | 245 265                 | 210 245 270             |
| 2KF7[⁽³⁾]        | DBCO-dT T₄(G₃T₂A)₃G₃T              | 235 250 260 290          | 215 240 250 260 290     |
| 1KF1[⁽⁴⁾]        | DBCO-dT T₄AG₃(T₃AG₃)₃              | 215 240 290             | 215 245 280             |
| 2JPZ[⁽⁵⁾]        | DBCO-dT T₄(T₃AG)₃T₂                | 240 270 290             | 215 250 280             |
| 148D[⁽⁵⁾]        | DBCO-dT T₄G₂T₂G₂TGTG₂T₂G₂          | 230 250 270 290         | 210 230 250 265 290     |

Calculated CD spectra of Conjugated DNA strands on ProSNAs. Circular dichroism (CD) spectra were recorded in a 1 mm pathlength cuvette at 22 °C with a Jasco J-1700 spectropolarimeter equipped with a temperature controller at a concentration of 300 nM β-Gal in 1X PBS buffer (pH 7.4) or 10 mM LiH₂PO₄ (pH 7.4). The instrument parameters to record the CD spectra were: 200-400 nm measurement range, 0.1 nm data pitch, 1 nm bandwidth, 200 nm/min scanning speed, and 10 accumulations. CD spectra were normalized to molar ellipticity ([θ], deg cm² dmol⁻¹) using \[\theta = \frac{\theta \times 10}{c \times l}\] where \(\theta\) is the CD ellipticity in millidegrees, \(c\) is the sample concentration in mol/L, and \(l\) is the pathlength in cm. Calculated spectra of the DNA conjugated to the protein were obtained by subtracting the spectra of β-Gal-AF647 from the spectra of the ProSNAs in their respective buffers (Figure S10).

\[[\theta]_{DNA \text{ conjugated to protein}} = [\theta]_{ProSNA} - [\theta]_{\beta-Gal-AF647}\]

![T₃₄ DNA Shell on Protein](image1.png) ![T₄(GGT)₁₀ DNA Shell on Protein](image2.png)

Figure S10. Calculated circular dichroism (CD) spectra of DNA conjugated to protein. Calculated spectra of T-rich or GQ DNA on protein in two different buffered conditions: KPO₄ (solid) and LiPO₄ (dashed). A strong shift in spectra for T₄(GGT)₁₀ DNA strands on the ProSNA indicates the loss of GQ secondary structure in the presence of a lithium based buffer.
S2. *in Vitro* Investigations on ProSNA Variants

2.1 Cellular Uptake in HeLa Cells

HeLa cells (ATCC) were cultured in a 96-well plate in DMEM (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Gibco). Once 80% confluent, cells were treated with either Alexa Fluor 647-modified native β-Gal or ProSNA variants for 2-4 h at 5-10 nM β-Gal (specific treatments are specified in figure captions). “Untreated” samples refer to cells treated with the same volume of 1XPBS. Post-treatment, cells were washed with 1X PBS, trypsinized (Gibco), and fixed in 4% paraformaldehyde (Thermo Fisher Scientific) and subsequently analyzed by flow cytometry using a BD LSRFortessa measuring the fluorescence (excitation 640 nm, emission 655-685 nm) of at least 5000 single-cell events per sample. Raw FCS files were gated based on forward and side scatter intensities and analyzed on FlowJo (Figure S11-14).

Figure S11. Representative histograms depicting cellular uptake of fluorophore modified ProSNA variants and native protein in HeLa cells. Flow cytometry was used to measure the uptake of ProSNA variants or native protein in HeLa cells (n = 3) after 4 h treatments with 5 nM β-Gal in serum-containing media. Representative histogram of n = 1 per treatment, which shows the fluorescence intensity versus the number of events detected.
Figure S12. Median Alexa Fluor 647 fluorescence intensity from HeLa cells using flow cytometry. HeLa cells were treated with 5 nM β-Gal for 4 h in serum-containing media and subsequently analyzed by flow cytometry. The median fluorescence intensity of n = 3 wells was determined in FlowJo. The linker’s deleterious uptake properties were further supported by switching the location of Spacer18 on the protein: cellular uptake recovers, confirming that placing Spacer18 near the protein surface is detrimental to internalization (orange bars).
Figure S13. Representative histograms depicting cellular uptake of fluorophore modified ProSNA variants and native protein in HeLa cells. HeLa cells \((n = 3)\) were treated with 10 nM β-Gal for 2 h in serum-containing media and subsequently analyzed by flow cytometry. Representative histogram of \(n = 1\) per treatment, which shows the fluorescence intensity versus the number of events detected. Median MFI is plotted in Figure 2B.

Figure S14. Representative histograms depicting cellular uptake of fluorophore modified ProSNA variants and native protein in HeLa cells. HeLa cells \((n = 5)\) were treated with 10 nM β-Gal or ProSNA for 2 h in serum-containing media and subsequently analyzed by flow cytometry. Representative histogram of \(n = 1\) per treatment, which shows the fluorescence intensity versus the number of events detected. Median MFI is plotted in Figure 2C.
2.2 Cellular Uptake in C166 Cells

C166 cells (ATCC) were cultured in a 96-well plate in DMEM (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Gibco). Once 80% confluent, cells were treated with either Alexa Fluor 647-modified native β-Gal or ProSNA variants for 4 h at 10 nM β-Gal. Post-treatment, cells were washed with 1X PBS, trypsinized (Gibco), and fixed in 4% paraformaldehyde (Thermo Fisher Scientific) and subsequently analyzed by flow cytometry using a BD LSRFortessa measuring the fluorescence (excitation 640 nm, emission 655-685 nm) of at least 5000 single-cell events per sample. Raw FCS files were gated based on forward and side scatter intensities and analyzed on FlowJo (Figure S15).

Figure S15. Median Alexa Fluor 647 fluorescence intensity from C166 cells using flow cytometry. C166 cells were treated with 10 nM β-Gal for 4 h in serum-containing media and subsequently analyzed by flow cytometry. The median fluorescence intensity of n = 3 wells was determined in FlowJo. The study demonstrates the generalizability of the linker’s deleterious effect on a ProSNA’s uptake in two different cell lines.

2.3 Kinetic Analysis of β-Galactosidase Activity

o-Nitrophenyl β-D-galactopyranoside (ONPG) was purchased from Thermo Fisher Scientific. Sodium phosphate, magnesium chloride, and 2-mercaptoethanol were obtained from Sigma
Aldrich. All reactions were run in an assay buffer consisting of 100 mM sodium phosphate (pH 7.0), 1 mM MgCl₂, and 50 mM 2-mercaptoethanol. To determine Michaelis-Menten values, a dilution series of ONPG ranging from 4 mM to 0.0625 mM was made using the assay buffer as the diluent in a black 96-well clear bottom plate (Corning). Reactions were initiated by the addition of either native β-Galactosidase or ProSNA variants, to a final [β-Gal] of 0.5-1 nM, using a multichannel pipette (specified concentration in the figure caption). Absorbance readings were taken immediately at 420 nm every minute for a total of 45 min at ambient temperature with shaking between each reading in a BioTek Cytation 5 plate reader. To determine Michaelis-Menten values, the absorbance reading was converted to the concentration of o-nitrophenol using its extinction (ε₄₂₀ = 4500 M⁻¹cm⁻¹) and a calculated plate pathlength of 0.23 cm. Excel was used to calculate the slope of the linear part of the line (R² ≥ 0.99) in a graph of [o-nitrophenol] v. time (Figure S16). Finally using GraphPad Prism nonlinear regression software, data were fit to a curve using the following model (Figure S17):

\[ y = \frac{E_t \ k_{cat} \ x}{K_m + x} \]

The average k_{cat} of three separate trials is graphed in Figure S18-19.
Figure S16. Representative kinetic reaction of the T₃₄ ProSNA at various ONPG concentrations. The rate of ONPG turnover to o-nitrophenol in the presence of 0.5 nM T₃₄ ProSNA at indicated ONPG concentrations. The kinetic reactions were run in a 96 well plate and the absorbance at 420 nm was taken every 30 s using a BioTek Cytation5. In Excel, the absorbance readings were converted to concentrations of product and the slope in the linear portion of the curve ($R^2 \geq 0.99$) was calculated to be the initial rate, $V_o$.

Figure S17. Representative graph of the effect of substrate concentration on the enzyme’s initial rate. The initial rate of the T₃₄ ProSNA was first determined kinetically (see Fig. S15) and plotted against the ONPG substrate concentration. GraphPad Prism nonlinear regression software was then used to fit the data to a curve and thus determine the $k_{cat}$ of the ProSNA variants.
Figure S18. Average $k_{cat}$ ($n = 3$) of the ProSNA variants and native protein. DNA modification of the protein does not significantly affect its enzymatic activity; however, with the addition of longer linker lengths, the activity of the enzyme decreases. This effect is more dramatic for the Spacer18 linkers (pink v. purple bars). In addition, there is a more than 50% decrease in activity after conjugating only Spacer18 (light orange) on the protein surface. This loss can be partially reversed by introducing a $T_{30}$ oligonucleotide strand between the Spacer18 and the protein (dark orange). [$\beta$-Gal] = 0.5 nM

Figure S19. Average $k_{cat}$ ($n = 3$) of the ProSNA variants and native protein. DNA modification of the protein with a T-rich sequence does not significantly affect its enzymatic activity; however, by introducing a GQ DNA shell, the activity of the enzyme decreases. The $k_{cat}$ was determined by an ONPG assay of ProSNAs (both 20 DNA/ $\beta$-Gal) and the native protein. Relative activity is shown in Figure 2D of the main text. [$\beta$-Gal] = 1 nM
S3. *in Vivo* Investigations Using Model ProSNA Variants

Female CD-1 mice (strain code: 022) were procured from Charles River Laboratory at 8 weeks of age and maintained in conventional housing. All animal experiments were carried out in accordance with the approved IACUC protocol issued by Northwestern University.

3.1 Blood Circulation Time

Pharmacokinetic studies were performed in female CD1 mice (28-30g). Alexa Fluor 647 modified native β-Galactosidase or ProSNA variants were administered in a single injection via the tail vein at a dose of 4 mg β-Gal / kg body weight. Blood (n = 3 per time point) was collected in a heparinized tube via retro-orbital blood draw and stored on ice. Blood was centrifuged at 1200 rpm for 7 min, and the fluorescence of the resulting supernatant, or plasma, was measured in a black 384-well plate using the BioTek Cytation 5 plate reader (excitation 640 nm, emission 680 nm).

3.2 *Ex Vivo* Near-Infrared Fluorescence (NIRF) Imaging

NIRF imaging studies were performed using a Caliper Life Sciences In Vivo Imaging System (IVIS) to determine the extent of protein accumulation in main organs at different time points post-injection. Female CD1 mice (28-30 g) were administered a single injection via tail vein at a dose of 4 mg β-Gal / kg body weight. At different time points post-injection, as specified in the figure, mice (n = 3 per time point) were humanely euthanized by cardiac perfusion while anesthetized. Tissues were harvested post-cardiac perfusion with 1X PBS and fixed in 4% paraformaldehyde for 1 h at 4 °C. Post-fixation, organs were washed three times with 1X PBS and stored in 1X PBS at 4 °C. Finally, organs were imaged using an IVIS system using 650 nm/700 nm excitation/emission filters and data was quantified by measuring fluorescence counts with the Living Image software. **Figure S20-S21** displays the IVIS images from each main organ post the indicated treatment time. **Figure S22** displays the IVIS image from each main organ post a 1 h injection.
Figure S20. Temporal distribution of fluorophore modified ProSNAs and native protein in four main organs. The distribution of two ProSNA variants—with and without Spacer18 linker—and native protein were studied by measuring the fluorescence using IVIS (excitation 650 nm, emission 700 nm) in four main organs: a) liver, b) lung, c) kidney, and d) spleen. Post the indicated treatment (see key; e), mice were sacrificed, perfused, and their organs dissected and fixed. T$_{30}$ ProSNA demonstrates enhanced distribution and retention in imaged organs in comparison to the (sp18)$_6$T$_{30}$ ProSNA and native protein. Using the Living Image software, a region of interest was drawn around each organ and fluorescence counts were quantified (see Figure 3b).
Figure S21. Temporal distribution of fluorophore modified ProSNAs and native protein in four main organs. The distribution of two ProSNA variants—with and without Spacer18 linker—and native protein were studied by measuring the fluorescence using IVIS (exicitation 650 nm, emission 700 nm) and reported versus time in four main organs: a) liver, b) lung, c) kidney, and d) spleen. Post the indicated treatment (x-axis), mice were sacrificed, perfused, and their organs dissected and fixed. T\textsubscript{30} ProSNA demonstrates enhanced distribution and retention in imaged organs in comparison to the (sp18)\textsubscript{6}T\textsubscript{30} ProSNA and native protein.
Female CD1 mice (29-35 g) were administered a single injection via tail vein at a dose of 6.5 mg β-Gal / kg body weight. One-hour post-injection, mice (n = 3) were humanely euthanized by cardiac perfusion while anesthetized. Each mouse was first perfused with 20 mL of 1X PBS, then 20 mL of 4% paraformaldehyde (PFA; Thermo Fisher Scientific), and main organs were dissected and stored in 4% PFA with shaking at 4 °C for 2 h. Organs were rinsed in many changes of 1X PBS and equilibrated overnight in 15% sucrose (Sigma Aldrich) in 1X PBS solution at 4 °C. The organs were subsequently transferred to a 30% sucrose in 1X PBS solution and stored at 4 °C until the organs sunk to the bottom of their respective container. Each organ was then embedded in optimal cutting temperature compound and cryo-sectioned at 10 μm (kidney, liver, spleen) or 20 μm (lung) thickness. The slides were stored at -80 °C until staining.

3.3 Enzymatic Colorimetric Activity Assay of Main Organs \cite{6}

Female CD1 mice (29-35 g) were administered a single injection via tail vein at a dose of 6.5 mg β-Gal / kg body weight. One-hour post-injection, mice (n = 3) were humanely euthanized by cardiac perfusion while anesthetized. Each mouse was first perfused with 20 mL of 1X PBS, then 20 mL of 4% paraformaldehyde (PFA; Thermo Fisher Scientific), and main organs were dissected and stored in 4% PFA with shaking at 4 °C for 2 h. Organs were rinsed in many changes of 1X PBS and equilibrated overnight in 15% sucrose (Sigma Aldrich) in 1X PBS solution at 4 °C. The organs were subsequently transferred to a 30% sucrose in 1X PBS solution and stored at 4 °C until the organs sunk to the bottom of their respective container. Each organ was then embedded in optimal cutting temperature compound and cryo-sectioned at 10 μm (kidney, liver, spleen) or 20 μm (lung) thickness. The slides were stored at -80 °C until staining.

Figure S22. Temporal distribution of fluorophore modified ProSNAs and native protein in four main organs. The distribution of two ProSNA variants—T-rich and GQ sequence—and native protein were studied by measuring the fluorescence using IVIS (excitation 650 nm, emission 700 nm) in five main organs: brain, liver, lung, kidney, and spleen. Post 1 h treatment, mice were sacrificed, perfused, and their organs dissected and fixed. Using the Living Image software, a region of interest was drawn around each organ and fluorescence counts were quantified (see Figure 3c).
EGTA, sodium deoxycholate, potassium ferrocyanide, potassium ferricyanide, glutaraldehyde, xylene, Nuclear Fast Red, and aluminum sulfate were obtained from Sigma Aldrich. NP-40 Alternative was from Santa Cruz Biotechnology. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), Permount, and ethanol were purchased from Thermo Fisher Scientific. Table S3.1 provides the details of solutions used during the X-Gal histology staining protocol. Slides were first fixed in the Fixative solution for 10 min at room temperature. Subsequently, slides were washed in a Wash Buffer for 5 min three times. The slides were then stained at 37 °C for 6 h with shaking at 40 rpm in a Reaction Buffer. Slides were washed three times for 5 min in PBS-Mg$^{2+}$ then once more in Milli-Q filtered (MQ) water. To counterstain, slides were stained for 5 min in a Histology Counterstain and quickly washed three times in MQ water. Slides were then dehydrated through ethanol and xylene baths as follows: 70% ethanol (EtOH) (1 min), 95% EtOH (2.5 min), 100% EtOH (3.5 min), xylene (twice, 3 min). A coverslip was then mounted to each slide using Permount. A BioTek Cytation 5 Imaging Multi-Mode Reader was used to image each slide at a 10X magnification. Figure S23 is a microscope image of the entire organ section.

Table S3.1 X-Gal Staining Reagents

| Solution Name         | Component            | [Component] | Diluent          |
|-----------------------|----------------------|-------------|------------------|
| Fixative              | Glutaraldehyde       | 0.2%        | 1X PBS           |
|                       | EGTA                 | 5 mM        |                  |
|                       | MgCl$_2$             | 100 mM      |                  |
| Wash Buffer           | Sodium deoxycholate  | 0.01%       | 1X PBS           |
|                       | NP-40                | 0.02%       |                  |
|                       | MgCl$_2$             | 2 mM        |                  |
| Reaction Buffer       | Potassium Ferrocyanide | 5 mM   | Wash Buffer      |
|                       | Potassium Ferricyanide | 5 mM   |                  |
|                       | X-Gal                | 1 mg/mL     |                  |
| PBS-Mg$^{2+}$         | MgCl$_2$             | 2 mM        | 1X PBS           |
| Histology Counterstain| Nuclear Fast Red     | 0.1%        | Milli-Q Water    |
|                       | Aluminum Sulfate     | 5%          |                  |

S23
S4. References

1. PrimerQuest® program, IDT, Coralville, Iowa, USA.
   https://www.idtdna.com/SciTools.

2. Brodin, J. D.; Sprangers, A. J.; McMillan, J. R.; Mirkin, C. A. DNA-Mediated Cellular Delivery of Functional Enzymes. *J. Am. Chem. Soc.* **2015**, *137*, 47, 14838-14841.

3. Lim, K. W.; Amrane, S.; Bouaziz, S; Xu, W.; Mu, Y.; Patel, D. J.; Luu, K. N.; Phan, A. T. Structure of the Human Telomere in K⁺ Solution: A Stable Basket-Type G-Quadruplex with Only Two G-Tetrad Layers. *J. Am. Chem. Soc.* **2009**, *131*, 12, 4301-4309.

4. Marchand, A.; Gabelica, V. Native Electrospray Mass Spectroscopy of DNA G-Quadruplexes in Potassium Solution. *J. Am. Soc. Mass Spectrom.* **2014**, *25*, 7, 1146-1154.

5. Villar-Guerra, R. D.; Trent, J. O.; Chaires, J. B. G-Quadruplex Secondary Structure Obtained from Circular Dichroism Spectroscopy. *Angew. Chem. Int. Ed.* **2018**, *57*, 7171-7175.

Figure S23. *In vivo* catalytic activity and tissue distribution of native β-Gal and ProSNAs. Representative light micrographs of histology slides after incubation with the β-Gal substrate, X-Gal. The blue color apparent in tissue dissected from mice (n = 3) 1 hour post intravenous injection of 6.5 mf enzyme/kg mouse results from the hydrolysis of X-Gal and the formation of an insoluble blue product. Scale = 2000 μm.
6. Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. In vivo protein transduction: delivery of biologically active protein into the mouse. Science. 1999, 285, 5433, 1569-1572.