A biomimetic approach to promote cellular uptake and enhance photoacoustic properties of tumor-seeking dyes

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ABSTRACT: The attachment of glucose to drugs and imaging agents enables cancer cell targeting via interactions with GLUT1 overexpressed on the cell surface. While an added benefit of this modification is the remarkable solubilizing effect of carbohydrates, in the context of imaging agents, aqueous solubility does not guarantee decreased π-stacking or aggregation. The resulting broadening of the absorbance spectrum is a detriment to photoacoustic (PA) imaging since the signal intensity, accuracy, and image quality all rely on reliable spectral unmixing. To address this major limitation and further enhance the tumor-targeting ability of imaging agents, we have taken a biomimetic approach to design a multivalent glucose moiety (mvGlul). We showcase the utility of this new group by developing aza-BODIPY-based contrast agents boasting a significant PA signal enhancement greater than 11-fold after spectral unmixing. Moreover, when applied to targeting cancer cells, effective staining could be achieved with ultra-low dye concentrations (50 nM) and compared to a non-targeted analog, the signal intensity was >1000-fold higher. Lastly, we employed the mvGlul technology to develop a logic-gated acoustogenic probe to detect intratumoral Cu(I), which is an emerging cancer biomarker, in a murine model of breast tumor. This was exciting application not possible using other acoustogenic probes previously developed for copper sensing.

INTRODUCTION

The targeted delivery of drugs and diagnostic imaging agents to a tumor site can limit off-target toxicities and non-specific tissue distribution, respectively.1 A common strategy to achieve this is by appending a targeting ligand that is preferentially taken up by cancer cells relative to surrounding tissue.2-3 For instance, rapidly dividing cancer cells will upregulate certain surface proteins such as the folate receptor, which binds to and internalizes folate utilized to synthesize DNA, RNA, as well as other key building blocks for cell division. A notable example that leverages this interaction is CYTALUX™, an FDA approved folate-cyanine conjugate used for imaging-guided ovarian cancer surgery.4 Likewise, the overexpression of the glucose transporter 1 (GLUT1) is essential in providing cancer cells with fuel via aerobic glycolysis.5-7 The reliance on this process for energy is known as the Warburg effect and has been exploited to deliver a variety of cargo.8 Of note, the attachment of glucose to a molecule imparts additional key benefits such as improved aqueous solubility. This property is of particular interest to the imaging community because dyes suitable for in vivo applications are often large, hydrophobic, and exhibit poor solubility.9-10

Our group and others have contributed to the development of activity-based sensing (ABS) probes designed to monitor cancer biomarkers such as hypoxia,11-14 reactive oxygen and nitrogen species,15-21 enzymes,22-29 and thiols.30-34 via photoacoustic (PA) imaging.32-33 This modality is best described as a “light-in, sound-out” approach in which absorbed light is converted to ultrasound via the PA effect.34-35 Because sound can readily propagate through tissue, it is possible to obtain high resolution images in deep regions of the body. However, the quality (and accuracy) of a PA image relies on a well-resolved in vivo PA spectrum of the imaging agent to deconvolute its signal from that of background. Unfortunately, the spectra of aggregation-prone dyes, which encompass many PA imaging agents, are typically broad, spectrally shifted, and poorly defined. Recently, we attempted to address this by installing 2 glucose residues onto an aza-BODIPY (AB) dye (infra vide, Figure 2C). While we were able to enhance aqueous solubility, only moderate improvements to the PA spectra were observed, presumably because the dyes are still able to π-stack. This is consistent with reports that have demonstrated that the

Figure 1. Schematic showing the targeted delivery of cargo (i.e., dye) is enhanced when a multivalent glucose targeting ligand is employed.
inclusion of strategically positioned functional groups such as oligoglycerol dendrons, quaternary ammonium centers or PEG shielding arms are required to block dye aggregation.

In the present study, we developed a new multivalent glucose targeting moiety (herein named mvGlu) by mimicking Nature’s solution to weak binding interactions, which is to display numerous copies of a ligand to maximize receptor contact. mvGlu was designed to: 1) solubilize and 2) prevent π-stacking of the appended dye; and 3) enhance cancer targeting relative to a single glucose residue (Figure 1).

Using this new aggregation blocking and tumor-seeking motif, we prepared a panel of aza-BODIPY-based contrast agents for in vivo PA cancer imaging. Moreover, we developed a logic-gated acoustogenic probe by integrating this technology to detect elevated labile copper (Cu) in a murine model of breast cancer.

**RESULTS AND DISCUSSION**

**Design of mvGlu-based Contrast Agents**

We hypothesized the attachment of two glucose units to a glycerol scaffold through the primary alcohols would afford a branched configuration that can effectively attenuate π-stacking interactions, while simultaneously maximizing interactions with GLUT1. Of note, we chose to connect glucose through the anomeric center (as opposed to the C6 position) because this would lock the sugar into its pyranose form to increase the number of alcohols available for hydrogen bonding. Furthermore, modification of the C1 position has been shown to tolerate large cargo without decreasing GLUT1 interaction. We opted to install an azido group to facilitate attachment to alkynylated dyes (or other cargo) via click chemistry. We prepared a panel of three mvGlu-based aza-BODIPY dyes to determine the minimum number of mvGlu units required to inhibit aggregate formation, as well as to evaluate the impact of mvGlu placement (bottom versus top hemispheres of the dye). The chemical structures of the non-glycosylated control AB (AB-OMe), mvGlu-AB1, mvGlu-AB2, and mvGlu-AB3 are shown in Figure 2a. Synthetic details, including full characterization can be found in the SI.

*In vitro* Characterization of mvGlu-dye Conjugates

With the mvGlu-AB series in hand, we began by determining the experimental octanol-water partition coefficients at pH 7.4 (logDv). The measured logDv values in ascending order (most to least water soluble) are: mvGlu-AB2 (-1.12 ± 0.16), mvGlu-AB3 (-0.71 ± 0.11), mvGlu-AB1 (0.75 ± 0.02), and AB-OMe (2.51 ± 0.92) (Figure 2b). These results indicate the installation of a single mvGlu unit already has a dramatic effect on aqueous solubility and the inclusion of a second mvGlu group can further shift dye distribution from octanol to favor water. Next, we obtained the absorbance spectrum of each dye (3:7 v/v acetonitrile:PBS). The peak appearance of a dye is an excellent indicator of its aggregation status (Figure 2c). For instance, owing to the broad spectrum of AB-OMe, it is likely to be aggregated and thus, we would anticipate spectral unmixing to be sub-optimal and the corresponding PA signal to be weak. However, when two mvGlu groups are present (regardless of the position), the peak shape is sharp, well defined, and resembles that of AB-OMe in a pure organic solvent such as DMSO. In addition to improving spectral unmixing, a sharp peak is also associated with a larger extinction coefficient (ε) value. According to PA Brightness Factor (PABF) calculations, a large ε is the most influential property that determines the strength of a PA signal. Because the ε for mvGlu-AB2 (2.6 ×

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**Figure 2.** a) Chemical structures of AB-OMe2, mvGlu-AB1, mvGlu-AB2, and mvGlu-AB3. b) Table summarizing photophysical properties including ωmax, λem, ε, Φ, PABF, and logDv. c) Absorbance spectra of mvGlu-AB2, AB-OMe1, and AB-OMe2. Spectrally unmixed images of d) AB-OMe2 and e) mvGlu-AB2 (f) quantified data from d) and e). n = 3. Error bars = SEM. Statistical analysis was performed using a two-tailed t-test (α = 0.05, *** P < 0.001).

**Figure 3.** a) Representative fluorescent images of A549 human lung cancer cells treated with AB-OMe2, mvGlu-AB2, mvGlu-AB2 + glucose, or L-mvGlu-AB2. b) Quantified data from a). n = 4. Error bars = SEM. Statistical analysis was performed using a two-tailed t-test (α = 0.05, ** P < 0.01, *** P < 0.001).
was difference in signal intensity between the mvGlu

First, is cause relative to man lung cancer advanced to cellular imaging in Live 15, 46 (~4 same dye concentration corresponding during endogenous PA such high concentrations was a) two-tailed t-test (α = 0.05, *P < 0.01, **P < 0.001***, P < 0.0001****).

Figure 4. a) Representative fluorescent image of mouse bearing 4T1 orthotopic breast cancer prior to injection (left) and following injection of mvGlu-AB2 at timepoint = 16 hr (right). b) Quantified data from a) and timepoints 6, 8, 12, and 48 hrs (shown in SI) c) Representative PA image of tumor in cross-sectional view at timepoint = 6 hr. d) Lateral view of tumor at timepoint = 6 hr. e) Quantified data from initial PA signal of tumor and e) Error bars = SEM. Statistical analysis was performed using a two-tailed t-test (α = 0.05, *P < 0.01, **P < 0.001***, P < 0.0001****).

10⁴ M⁻¹cm⁻¹) is 8.7-fold higher than AB-OMe₂ (0.3 × 10⁴ M⁻¹cm⁻¹), we anticipate this will translate into a substantially stronger PA signal. To test this property experimentally, we cast a tissue-mimicking phantom using agar and milk. Samples of AB-OMe₂ or mvGlu-AB2 in water were then dosed with hemoglobin (10 mg/mL), embedded in the phantom, and imaged using a multispectral optoacoustic tomography (MSOT) imaging system (Figure 2d and 2e). Hemoglobin at such high concentrations was added because it is the major endogenous PA-active pigment that causes interference during in vivo imaging. The PA signal of AB-OMe₂ was barely above background after unmixing; however, the corresponding PA intensity of mvGlu-AB2 was 11-fold higher at the same dye concentration (Figure 2f). Compared to representative strategies we have devised previously including conformational restriction (~3-4-fold) and steric relaxation (~4-fold) mvGlu is the most successful approach to date.15, 46

Evaluating the Cancer Targeting Ability of mvGlu-AB2 in Live Cells

Encouraged by these promising improvements, we advanced to cellular imaging to evaluate the targeting ability of mvGlu. For these experiments, we cultured A549 human lung cancer and 4T1 murine breast cancer cells because relative to their non-cancerous counterparts, GLUT1 is reported to be overexpressed by these solid tumors.47-48 First, A549 cells were stained with either AB-OMe₂ or mvGlu-AB2 and imaged after 20 minutes (Figure 3a). The difference in signal intensity between the two conditions was dramatic, with the mvGlu-AB2-treated cells being >1000-fold brighter (Figure 3b). Additionally, a clear fluorescent signature was apparent even at a low dye concentration of 50 nM, indicating excellent sensitivity (Figure S1). Next, we performed two complimentary experiments to assess the involvement of GLUT1. First, A549 cells were pre-incubated with glucose (15 mM), before the addition of mvGlu-AB2 (2 μM) (Figure 3a). The concentration of glucose was maintained throughout the duration of the experiment. We hypothesized if uptake of the dye involved GLUT1, the presence of excess glucose in the media would compete for uptake, resulting in a decrease of cellular fluorescence. This was confirmed as the signal intensity was 2-fold higher when glucose was absent (Figure 3b). To further corroborate these results, we synthesized L-mvGlu-AB2 using L-glucose instead of D-glucose (shown in SI). Because L-glucose is not recognized by GLUT1, we anticipate that there will also be no interaction with the L-mvGlu isomer which will allow us to distinguish between nonspecific staining and GLUT1-mediated uptake. Analysis of the imaging results revealed cells treated with mvGlu prepared from the natural sugar were 3-fold brighter (Figure 3a and 3b). This implicates the enhancement of water solubility is likely increasing the effective dye concentration in the media (c.f. results from AB-OMe₂ experiment) but ultimately, it is GLUT1 that is mediating significant dye uptake. Additional controls comparing cell permeability between A549s modified with AB-Glu1 and an oligoglycerol dendron are shown in Figure S2. Moreover, similar results were obtained when these experiments were performed in 4T1 breast cancer cells (Figure S3).

Application of mvGlu-AB2 to Image Tumors In Vivo

One of the most exciting envisioned applications of PA imaging agents augmented with robust tumor-targeting capabilities is diagnostic cancer imaging. For a dye to be suitable for this purpose it must be minimally cytotoxic. This property was assessed using a standard MTT assay where we found no loss in cell viability up to a dye concentration of 15 μM (6-hour incubation) (Figure S4-5). In addition, after systemic administration, the dye must preferentially localize to the tumor site and the signal enhancement in the
lesion should be at least 2-fold. A large dynamic range is important because it will decrease the likelihood of an incorrect assessment. To monitor biodistribution in orthotopic breast tumor-bearing BALB/c mice, we employed fluorescence imaging and found that relative to the initial background scan, the signal enhancement of the tumor site had reached ∼2-fold after 8 and 12 hours for mvGlu-AB2 (Figure 4a and 4b) and mvGlu-AB3 (Figure S6), respectively. We corroborated these findings by performing PA imaging with spectral unmixing (Figure 4c). Interestingly, the PA signal appears to span the entire tumor (Figure 4d), and the difference between the tumor prior to and after injection of mvGlu-AB2 was substantial (4.1-fold) (Figure 4e). The ability to specifically detect a tumor mass within deep tissue is a testament to the impressive PA properties of our new mvGlu-based dyes. Results obtained for mvGlu-AB3 were comparable and are summarized in the SI (Figures S6-7).

**Design, synthesis, and in vitro testing of mvPCu**

In addition to developing powerful contrast agents, we wanted to extend the use of mvGlu to augment our analyte sensing capabilities. Recently, we developed an acoustogenic ABS probe for Cu(I) (named PACu-1), which we applied to visualize pathological levels of hepatic copper in a Wilson’s disease model. PACu-1 is equipped with a tris(2-pyridyl)methyl]amine (TPA) trigger that can be appended to an AB via an ether linkage. Upon coordination to Cu(I), an oxidative cleavage event can then take place which releases the dye. Of note, we selected the TPA trigger in this design because it has been used to develop a variety of sensors for other modalities and can effectively compete against glutathione to access intracellular Cu(I). However, when PACu-1 was employed in an attempt to image breast tumors, most of the probe localized to the liver owing to its hydrophobic properties. This result was disappointing because elevated copper is linked to aggressive breast malignancies and its detection could serve as a diagnostic marker. Moreover, this finding highlights the unpredictable nature of many molecules not equipped with a targeting group. The design of the second-generation copper probe to overcome this challenge is based on the mvGlu-AB3 architecture where one of the anisole rings was replaced with a 2,6-dichlorophenol moiety to provide a site for trigger installation. Synthesis of the proposed probe, multivalent probe for Cu(I) (mvPCu), began with the preparation of 1 and 2, which were accessed from the corresponding chalcone precursors via aldimine condensation and Henry reactions (Scheme S4 and S5). The aza-BODIPY dye scaffold was synthesized via Paal-Knorr cyclization of the two precursors, followed by metalation using boron trifluoride etherate in 22% yield over 2-steps. Next, copper-mediated click chemistry with the acetyl protected mvGlu azide (Scheme S7 and S8), yielded the glycoside incorporated aza-BODIPY 5. A Tsuji-Trost deallylation reaction was employed to remove the allyl protecting group on the 2,6-dichlorophenol moiety in 81% yield. The TPA trigger was then installed via nucleophilic substitution. Global deprotection of the acetyl groups with potassium methoxide generated in situ simultaneously exchanged the fluoro substituents with methoxy groups (Scheme 1).

After synthesis, we obtained the absorbance spectra for mvPCu and the turned over product (t-mvPCu) (Figure 5a). In both instances, the peaks were well defined which satisfied the criteria for in vivo spectral unmixing. Moreover,
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Figure
5a
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product
or
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b) Dose-dependent
ratiometric
turn-on
of
mvpCu
following
1
hour
incubation
with
0,25,50,
and
100 µM
Cu(I)
in
1:1
v/v
DMF:HEPES
(2
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GSH,
pH=7.4).
c) Ratiometric
turn-on
following
incubation
of
mvpCu
(5
µM)
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d) PA
tissue-mimicking
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images
of
mvpCu
and
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(670
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was
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a
two-tailed
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test
(*,
α
=0.05;**,
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P
<0.001).

We
observed
a
3.25-fold
turn-on
response
relative
to
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indicating
successful
detection
of
intratumoral
Cu(I)
(Figure
6c).
As
mentioned
above,
because
the
TPA
trigger
used
in
this
design
is
only
able
to
detect
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labile
Cu
pool
(defined
as
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associated
with
glutathione),
we
also
performed
ICP-MS
measurements
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whether
the
total
Cu
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also
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the
tumors.
Although
not
statistically
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our
results
appear
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(breast
tumors
= 3.88 ± 0.12
ppm
vs.
healthy
beast
tissue
= 3.19 ± 0.81
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S14).
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the
performance
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PACu-1
was
compared
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mvpCu,
it
failed
to
detect
intratumoral
Cu(I)
(Figure
6d).
This
finding
demonstrates
the
powerful
tumor
targeting
ability
of
our
multivalent
approach.

Conclusion

Cellular
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their
environment,
and
in
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of
immune
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and
mount
an
appropriate
response.
For
instance,
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cells
feature
a
dense
network
of
carbohydrates
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glycocalyx.
Depending
on
the
type
of
sugars
present
and
their
connectivity,
these
carbohydrates
can
serve
as
ligands
for
a
specific
binding
partner
(e.g.,
cell
surface
receptor).
However,
the
binding
affinity
of
a
single
sugar
residue
is
typically
weak,
but
Nature
has
evolved
to
overcome
this
through
a
robust
multivalent
approach,
where
numerous
copies
of
a
sugar
are
linked
and
presented
in
a
manner
to
increase
binding
interactions.
This
incredibly
simple
solution
to
an
otherwise
challenging
problem,
inspired
us
to
develop
mvGlu
featured
in
the
present
work.

Application
of
mvpCu
to
detect
intratumoral
Cu(I)
via
PA
imaging

The
ability
to
employ
mvpCu
to
image
intracellular
Cu(I)
within
tumors
can
be
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for
diagnostic
purposes
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high
Cu(I)
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To
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end,
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mg/kg,
100
µL)
for
PA
imaging.
Because
the
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about
product
is
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distinct
from
mvpCu,
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were
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perform
ratiometric
imaging
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irradiating
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670
and
765
nm,
respectively.
Experimental Details

3-(4-(Allyloxy)-3,5-dichlorophenyl)-5,5-difluoro-7-(4-methoxyphenyl)-1,9-bis(4-(prop-2-yn-1-yloxy)phenyl)-5H-p14,614-dipyrrrolo[1,2-c:2',1'-f][1,3,5,2]triazaborinine (4). To a round-bottom flask, 1 (693 mg, 1.5 mmol, 1 equiv) and 2 (1.1 g, 3.1 mmol, 2 equiv.) were suspended in n-butanol (9.3 mL). The solution was heated to 130 °C for 45 minutes in an oil bath containing rice bran oil. Then, ammonium acetate (1.8 g, 23 mmol, 15 equiv.) was added, and the reaction was allowed to heat at 130 °C overnight. The reaction was cooled to room temperature, diluted with water, and extracted with DCM (3×). The organic layers were combined, dried with sodium sulfate, and concentrated via rotary evaporation. The excess n-butanol was removed azeotropically with toluene. The resulting shiny blue solid was used without further purification. The solid was dissolved in DCM (150 mL) and cooled to 0 °C. DIPEA (2.7 mL, 15 mmol, 10 equiv.) and BF3·OEt2 (2.7 mL, 22 mmol, 14 equiv.) were added sequentially. The reaction was warmed to room temperature and was allowed to stir overnight. The reaction was quenched with aqueous sodium bicarbonate and extracted with DCM (3×). The combined organic layers were dried with sodium sulfate and concentrated via rotary evaporation. The resulting solid was purified via column chromatography (SiO2, toluene) to afford the desired compound as a blue solid (0.26 mg, 0.34 mmol, 22%). 1H NMR (500 MHz, CDCl3) δ 8.07 (d, J = 8.9 Hz, 2H), 7.99 (d, J = 8.8 Hz, 2H), 7.95 (d, J = 8.9 Hz, 2H), 7.88 (s, 2H), 7.04 – 6.94 (m, 6H), 6.74 (s, 1H), 6.11 (dd, J = 16.5, 10.4, 5.9 Hz, 1H), 5.41 (dd, J = 17.2, 1.6 Hz, 1H), 5.25 (dd, J = 10.3, 1.4 Hz, 1H), 4.72 (dd, J = 5.0, 2.4 Hz, 4H), 4.57 (dd, J = 6.0, 1.3 Hz, 2H), 3.84 (s, 3H), 2.53 (dt, J = 3.8, 2.4 Hz, 2H). 13C NMR (125 MHz, CDCl3) δ 163.02, 159.30, 158.71, 133.17, 132.38, 131.25, 130.86, 129.81, 125.79, 123.58, 119.09, 115.32, 115.27, 114.71, 78.47, 76.29, 76.18, 74.78, 56.06, 55.77.

Compound 5. To a round bottom flask, 4 (489 mg, 0.64 mmol, 1 equiv.) and CuSO4·H2O (1.6 g, 6.4 mmol, 10 equiv.) were dissolved in THF (32 mL). Sodium ascorbate (650 mg, 3.3 mmol, 5.1 equiv.) was dissolved in water (6.5 mL) and was added to the reaction suspension dropwise. The reaction was allowed to stir at room temperature overnight. The reaction was diluted with water and extracted with EtOAc (3×). The combined organic layers were dried with sodium sulfate and concentrated via rotary evaporation. The resulting blue oil was purified via column chromatography (SiO2, 4:1 v/v EtOAc:Hexane then 1:19 v/v MeOH:DCM) to afford the desired compound as a blue solid (1.0 g, 0.44 mmol, 68%). 1H NMR (500 MHz, Chloroform-d) δ 8.17 – 7.90 (m, 6H), 7.82 (s, 2H), 7.71 (d, J = 13.1 Hz, 2H), 7.18 (d, J = 7.8 Hz, 2H), 7.11 (s, 2H), 7.07 – 6.79 (m, 4H), 6.10 (dd, J = 17.0, 10.9, 5.7 Hz, 1H), 5.62 – 5.52 (m, 1H), 5.42 (dd, J = 28.3, 16.9 Hz, 1H), 5.32 – 5.25 (m, 2H), 5.16 (q, J = 9.8, 9.3 Hz, 4H), 5.04 (d, J = 7.8 Hz, 4H), 5.01 – 4.89 (m, 4H), 4.85 (d, J = 9.7 Hz, 2H), 4.74 (dd, J = 12.6, 6.8 Hz, 2H), 4.61 (d, J = 6.1 Hz, 4H), 4.47 (qd, J = 14.6, 10.2, 7.5 Hz, 2H), 4.28 – 4.18 (m, 4H), 4.17 – 4.06 (m, 7H), 3.86 (d, J = 16.5 Hz, 4H), 3.68 (t, J = 8.0 Hz, 2H), 3.34 (d, J = 6.7 Hz, 2H), 2.12 – 1.86 (m, 48H). 13C NMR (125 MHz, CDCl3) δ 170.60, 170.09, 169.59, 169.42, 169.29, 157.88, 155.37, 133.15, 132.91, 132.34, 132.19, 131.31, 131.09, 130.69, 130.31, 129.64, 129.56, 128.77, 128.64, 121.12, 119.49, 118.83, 115.35, 114.48, 100.90, 100.80, 100.51, 96.98, 79.58, 74.67, 74.55, 73.29, 72.40, 72.37, 72.06, 71.95, 70.95, 69.96, 68.22, 68.13, 67.95, 67.25, 62.97, 61.70, 61.63, 55.55, 41.49, 38.43, 20.77, 20.74, 20.63, 20.56, 20.50.

Compound 6. To a round bottom flask, 5 (324 mg, 0.14 mmol, 1 equiv.) and 1,3-dimethylbarbituric acid (35.0 mg, 0.22 mmol, 1.6 equiv.) were dissolved in DMF (16 mL). The reaction was degassed by bubbling N2 for 5 minutes. Then, palladium tetrakis (48.5 mg, 0.042 mmol, 0.3 equiv.) was added, and the reaction stirred at room temperature for 2 hours. The solvent was concentrated via rotary evaporation and directly purified via column chromatography (SiO2, 4
Then, the solvent was removed via rotary evaporation. 68.80, 68.40, 68.28, 68.24, 67.98, 67.01, 66.64, 63.13, 62.22, 71.04, 70.99, 70.95, 70.80, 123.57, 123.32, 123.23, 122.44, 121.16, 120.64, 115.01, 69.93, 68.27, 63.02, 61.68, 56.97, 55.05, 42.82, 29.45, 27.57, 19.91, 19.84, 19.69.

**Compound 7.** To a round bottom flask, TPA-Cl (32.8 mg, 0.097 mmol, 1.1 equiv.) and sodium iodide (73.3 mg, 0.44 mmol, 5 equiv.) were dissolved in acetone (8.8 mL) and heated to reflux for 1 hour. The reaction was cooled. Then, 6 (200.2 mg, 0.088 mmol, 1 equiv.) and triethylamine (0.037 mL, 0.27 mmol, 3 equiv.) were added, and the reaction was heated to 40 °C overnight. The reaction was cooled, diluted with brine, and extracted with DCM (3×). The combined organic layers were dried with sodium sulfate and concentrated by rotary evaporation. The crude residue was purified via flash chromatography on neutral alumina (Al2O3, DCM then 1:19 v/v MeOH:DCM) to afford the product as a blue-green solid (49.9 mg, 0.019 mmol, 22%). 1H NMR (500 MHz, 1:1 v/v CD3Cl2:CD3OD) δ 8.33 (d, J = 5.1 Hz, 2H), 7.83 (s, 2H), 7.74 – 7.61 (m, 4H), 7.56 – 7.48 (m, 12H), 7.43 (d, J = 5.3 Hz, 6H), 7.24 (s, 2H), 7.21 – 7.09 (m, 2H), 6.89 (d, J = 8.1 Hz, 2H), 5.30 – 5.23 (m, 4H), 5.18 – 5.08 (m, 7H), 5.05 (s, 4H), 4.96 (t, J = 9.9 Hz, 5H), 4.92 – 4.69 (m, 6H), 4.64 – 4.42 (m, 4H), 4.27 – 3.87 (m, 18H), 3.71 (d, J = 11.2 Hz, 4H), 2.02 – 1.86 (m, 48H). 13C NMR (125 MHz, 1:1 v/v CD3Cl2:CD3OD) δ 194.46, 171.17, 171.06, 170.98, 170.52, 170.50, 170.30, 170.06, 170.00, 169.83, 169.79, 169.67, 169.50, 163.24, 158.91, 158.72, 157.82, 155.18, 154.93, 148.34, 143.38, 137.66, 137.21, 133.74, 133.65, 133.57, 132.75, 132.73, 132.40, 132.38, 131.89, 131.81, 131.77, 130.94, 129.98, 128.88, 128.80, 128.75, 128.71, 128.61, 128.15, 123.64, 123.57, 123.32, 123.23, 122.44, 121.16, 120.64, 115.01, 100.78, 100.42, 89.71, 79.65, 75.30, 72.98, 72.74, 72.50, 72.43, 71.81, 71.74, 71.59, 71.40, 71.04, 70.99, 70.95, 70.80, 68.80, 68.40, 68.28, 68.24, 67.98, 67.01, 66.64, 63.13, 62.22, 61.75, 61.69, 61.52, 60.54, 59.91, 59.81, 41.50, 38.58, 36.26, 35.46, 30.97, 30.18, 29.51, 27.71, 27.19, 27.09, 22.53, 20.20, 20.15, 20.12, 20.09, 20.05, 20.03, 20.00.

**mvpCu (B).** To a round bottom flask, (42.2 mg, 0.016 mmol, 1 equiv.) and potassium carbonate (42.5 mg, 0.31 mmol, 18.8 equiv.) were suspended in methanol (1.6 mL). The reaction was stirred at room temperature for 3 hours. Then, the solvent was removed via rotary evaporation and purified via reverse-phase chromatography (C18, 100% H2O → 50% Acetonitrile/H2O) to afford the product as a blue-green solid (24.6 mg, 0.013 mmol, 78%). 1H NMR (500 MHz, 1:1 v/v CD3CN:CH2OAcetonitrile-d3) δ 8.29 (s, 2H), 7.77 (s, 2H), 7.65 (d, J = 25.1 Hz, 4H), 7.44 (q, J = 17.4, 15.5 Hz, 11H), 7.13 (s, 7H), 6.85 (s, 4H), 5.07 (s, 11H), 4.32 – 4.17 (m, 3H), 3.96 (s, 2H), 3.87 (s, 4H), 3.73 (s, 12H), 3.65 (d, J = 18.5 Hz, 15H), 3.55 (s, 18H), 3.42 (s, 4H), 3.37 – 3.02 (m, 5H).

**PA Imaging Unmixing Experiment in Tissue-Mimicking Phantoms.** Tissue mimicking phantoms were prepared as reported in the SI. mvGlu-AB2 or AB-OMe2 (30 µM) were prepared in water containing hemo-globin from bovine blood (10 mg/mL). The solutions were pipetted in a plastic tube and sealed with hot glue. PA measurements were taken at 5 nm intervals (660 – 850 nm). The subsequent images were spectrally un-mixed using the iThera Invision software with spectra of hemoglobin and the PA spectra of mvGlu-AB2 and AB-OMe2. The resulting values at the λPA are reported as the mean PA signals (Mean Pixel Intensity, MSOT a.u.).

**Fluorescence Imaging with mvGlu-AB2 and mvGlu-AB3.** 4T1 cells were trypsinized, neutralized with growth media, and counted on the Countess II FL Cell Counter (Invitrogen, Thermo Fisher Scientific). Cells were spun down, media was aspirated, and a solution was made in 1:1 serum-free RPMI media to VitroGel® hydrogel. Mice were injected in the mammary fat pad with 300,000 cells (50 µL) of the cell solution. Tumors grew for around 1.5-2 weeks and the mice were imaged once tumors reached 100 mm3. Dyes (1.2 mg/kg) were dissolved in 0.9% saline (5% DMSO) and injected retroorbitally. Mice were imaged using the CRI Maestro In-Vivo Fluorescence Imaging System at timepoints = 0, 6, 8, 12, 16, and 48 hours. Excitation occurred at 680 nm, and light was collected from 690-850 nm.

**PA Imaging with mvGlu-AB2.** 4T1 cells were trypsinized, neutralized with growth media, and counted on the Countess II FL Cell Counter (Invitrogen, Thermo Fisher Scientific). Cells were spun down, media was aspirated, and a solution was made in 1:1 serum-free RPMI media to Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix. Mice were injected in the mammary fat pad with 250,000 cells (50 µL) of the cell solution. Tumors grew for around 1.5-2 weeks and the mice were imaged once tumors reached 100 mm3. mvGlu-AB2 was reconstituted in 0.9% saline (5% DMSO) and injected retroorbitally. PA images were taken at timepoints = 0 and 6 hours in 5 nm increments from 660-700 nm. Images were spectrally unmixed from hemoglobin, deoxyhemoglobin, and melanin.

**PA Imaging with mvpCu.** 4T1 cells were trypsinized, neutralized with growth media, and counted on the Countess II FL Cell Counter (Invitrogen, Thermo Fisher Scientific).
Cells were spun down, media was aspirated, and a solution was made in 1:1 serum-free RPMI media to Gel-trex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix. Mice were injected in the mammary fat pad with 500,000 cells (50 μl) of the cell solution. Tumors grew for around 1.5-2 weeks and the mice were imaged once tumors reached 100 mm³.

ASSOCIATED CONTENT
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ABBREVIATIONS
DNA, deoxyribonucleic acid; FDA, Food and Drug Administration; GSH, glutathione; Kₐ, dissociation constant; MSOT, multispectral optoacoustic tomography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; RNA, ribonucleic acid; SEM, standard error of the mean, SWIR, short wave infrared.

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standard glucose targeting

multivalent glucose targeting

dye signal

enhanced tumor uptake

absorbance

prevents aggregation

improved imaging quality