Supplemental Information

for the

Two-Step Synthesis Galactosylated Human Serum Albumin for use as targeted optical imaging agent for peritoneal carcinomatosis

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Table of Contents

I. Synthesis of Galactosylated (or Glycosylated GSA) via initial Lysine blocking before amidation.

Reductive amination of glyceraldehyde to HSA before amidation. To a 100 mg of HSA in 5 mL 0.2 M sodium phosphate at pH ~7, add 2 x 21 mg glyceraldehyde and 3 x 51 g sodium cynanoborohydride. The reaction mixture was then placed in a shaking water bath at 37ºC for 11 d. The amination mixture was then exhaustively dialyzed in 0.1 MES pH ~5.25 and concentrated to 100 mL to obtain a propylene glycol-HSA product. Galactosamine (540 mg) and EDC (307 mg) were then added sequentially to the propylene glycol-HSA and then placed in a shaking water bath at 37ºC for 12 hr. The amidation reaction was then quenched with 20 mL 1.0 M sodium acetate pH 4.5 and then exhaustively dialyzed against 10 mM sodium acetate pH ~7 using a Tangential Flow Filtration system equipped with a Pellicon XL 50 cm^2 Biomax 30 cassette. Total protein concentration for this product **ggGSA** was determined using the Lowry method.

NOTE: Anlaogs using the bovine serum albumin as the protein base were also synthesized to compare that the reactivity is not lost by changing from bovine serum albumin (also made by Sigma-Aldrich which was previously used) to human serum albumin.

II. Characterization and SE-HPLC chromatograms

High Performance Liquid Chromatography. Size exclusion HPLC (SE-HPLC) was performed using a Beckman System Gold (Fullerton, CA) equipped with Model 126 solvent delivery module, a Model 168 UV detector (λ 254 and 280 nm), and a JASCO fluorescence detector (excitation 502 nm and emission at 532 nm) controlled by 32 Karat software. Size exclusion chromatography was performed on a Superose™ 12 10/300GL

column (GE Amersham) and TSKgel G2000SWxl eluted for 45 minutes using phosphate buffered saline (1X PBS) solution as the eluent at 0.5 mL/min.

Reversed phase HPLC was performed on a Beckman System Gold (Fullerton, CA) equipped with Model 126 solvent delivery module, a Model 168 UV detector (λ 254 and 280 nm) controlled by 32 Karat software using a Vydac C4 ODS column eluted on a linear gradient (30%B to 100%B in 25 min; A, 0.1% HOAc; B, 0.1% HOAc in MeCN) at 1.0 mL/min.

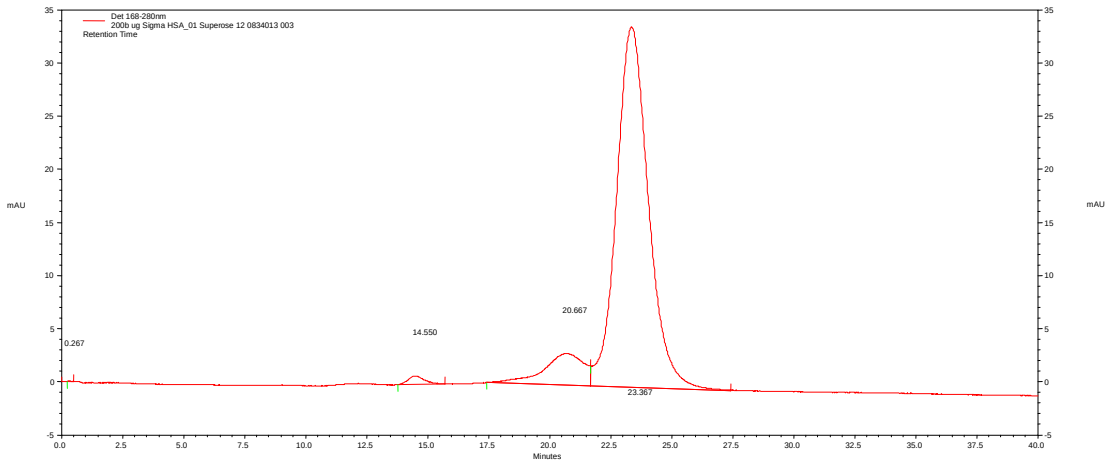

Figure S1. SE-HPLC chromatogram of the starting human serum albumin (Sigma) on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (280 nm). Major peak is at 23.4 min (89%) with some aggregates showing.

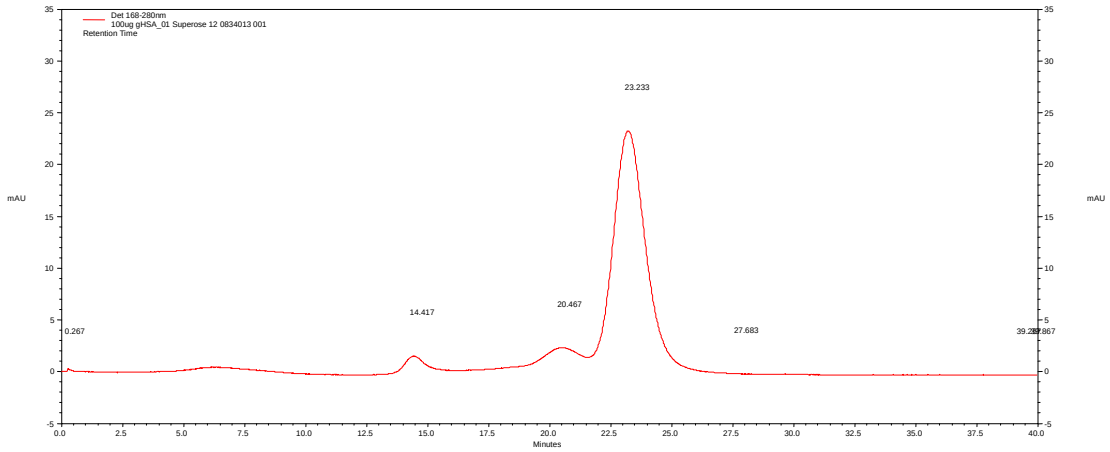

Figure S2. SE-HPLC chromatogram of the gHSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (280 nm). Major peak is at 23.2 min (88.9%).

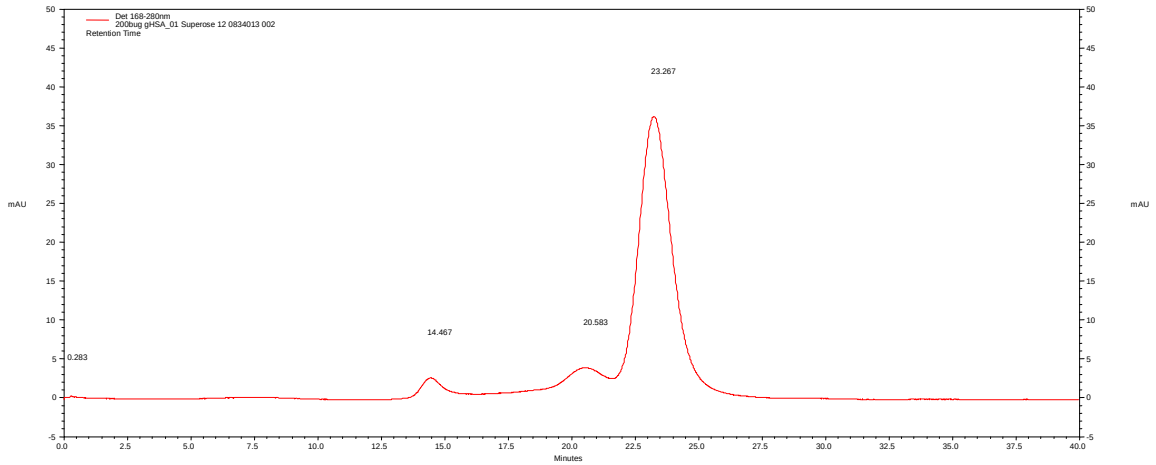

Figure S3. SE-HPLC chromatogram of the ggHSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (280 nm). Major peak is at 23.3 min (87.2%)

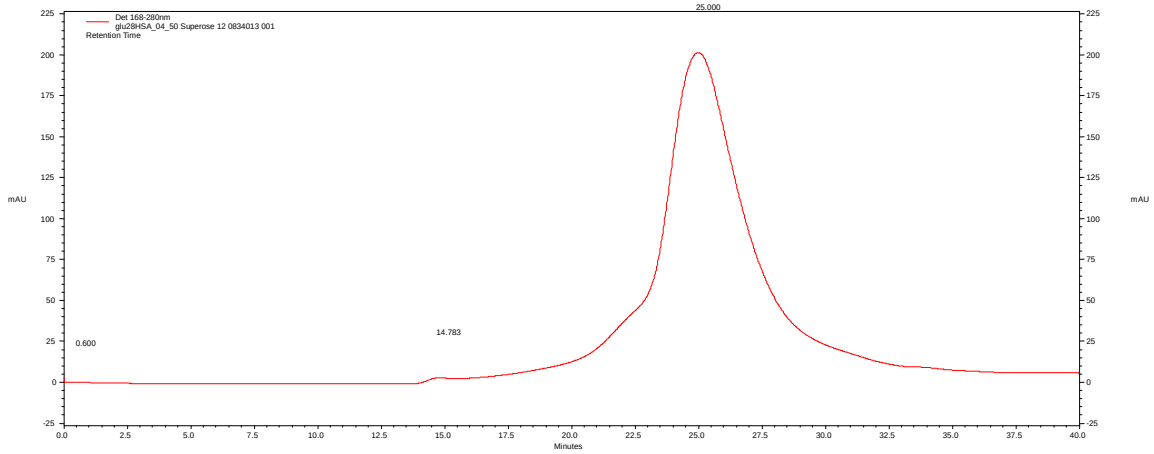

Figure S4. SE-HPLC chromatogram of the $(glu)_{28}$GSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (280 nm). Major peak at 25.0 min (99.2%).

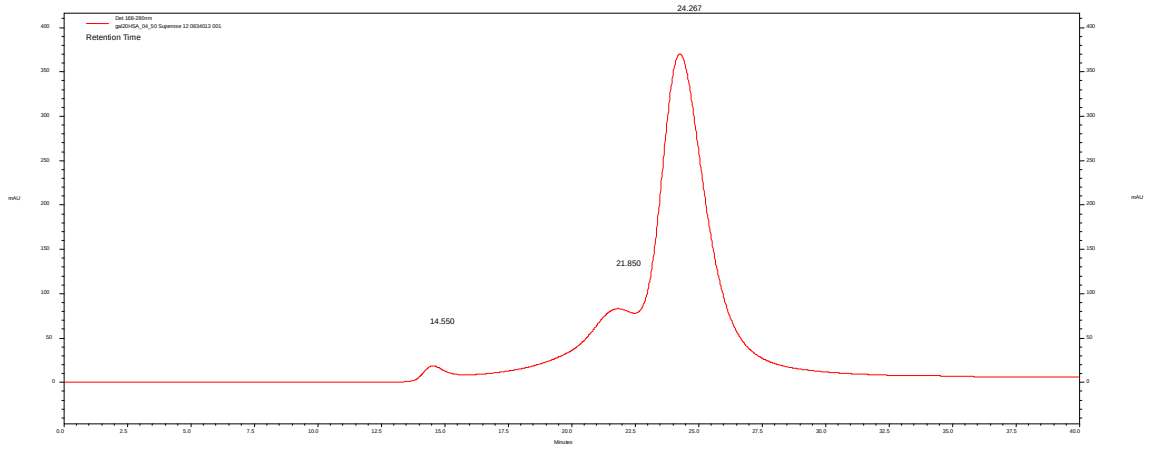

Figure S5. SE-HPLC chromatogram of the $(gal)_{20}$GSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (280 nm). Major peak at 23.5 min (88.4%).

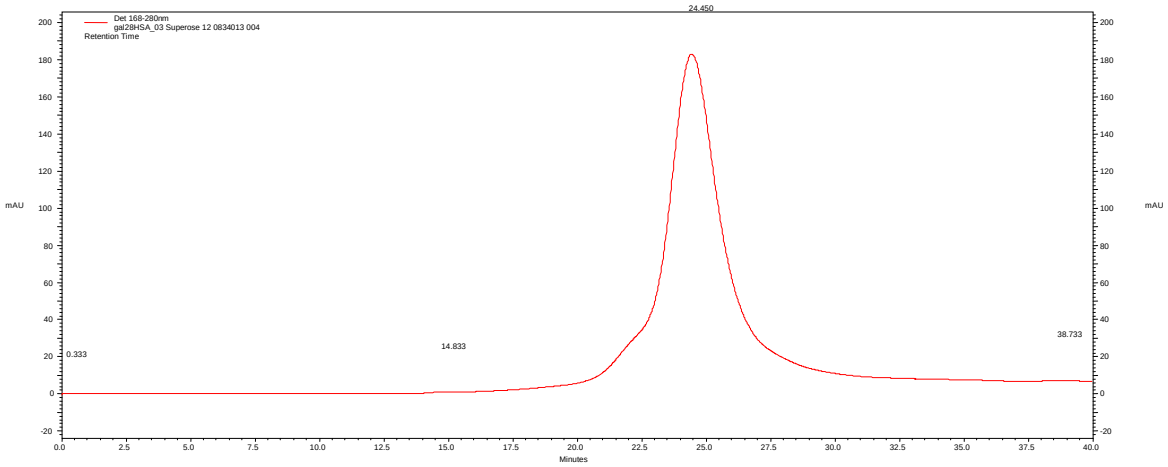

Figure S6. SE-HPLC chromatogram of the $(gal)_{28}$GSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (280 nm). Major peak at 24.5 min (99.6%).

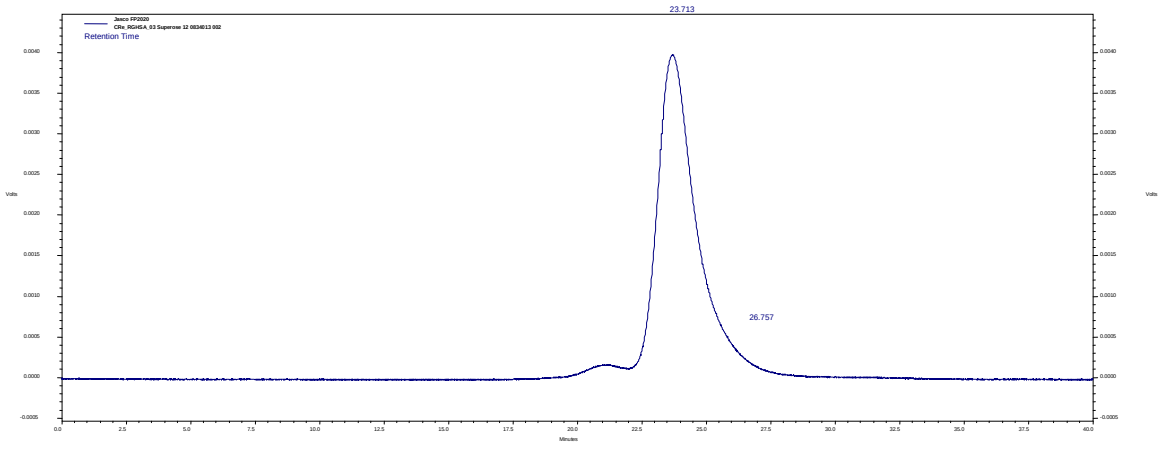

Figure S7. Fluorescence SE-HPLC chromatogram of the RG-HSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (excitation: 502 nm; emission 532 nm). Major peak 23.7 min (98.9%).

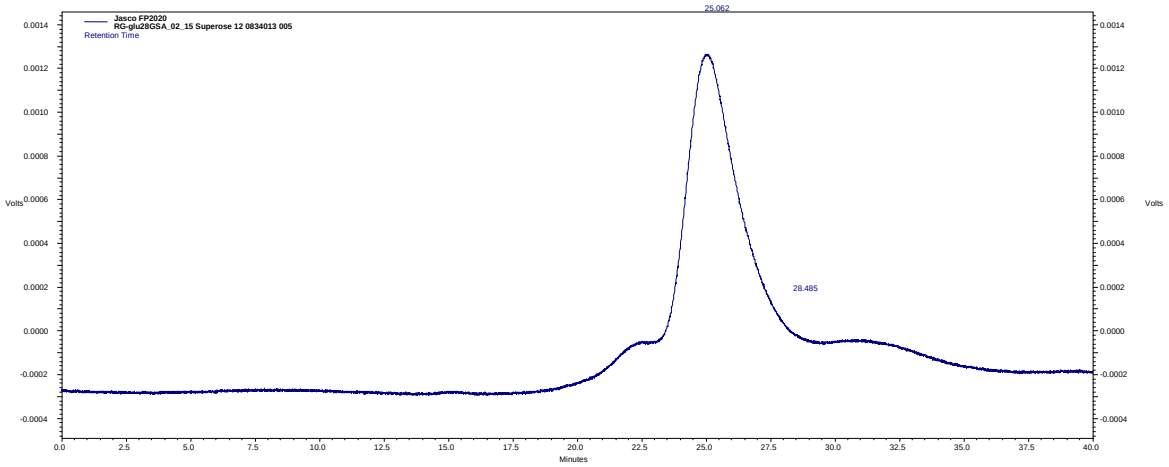

Figure S8. Fluorescence SE-HPLC chromatogram of the RG-$(glu)_{28}$GSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (excitation: 502 nm; emission 532 nm). Major peak is at 25.1 min (97.7%).

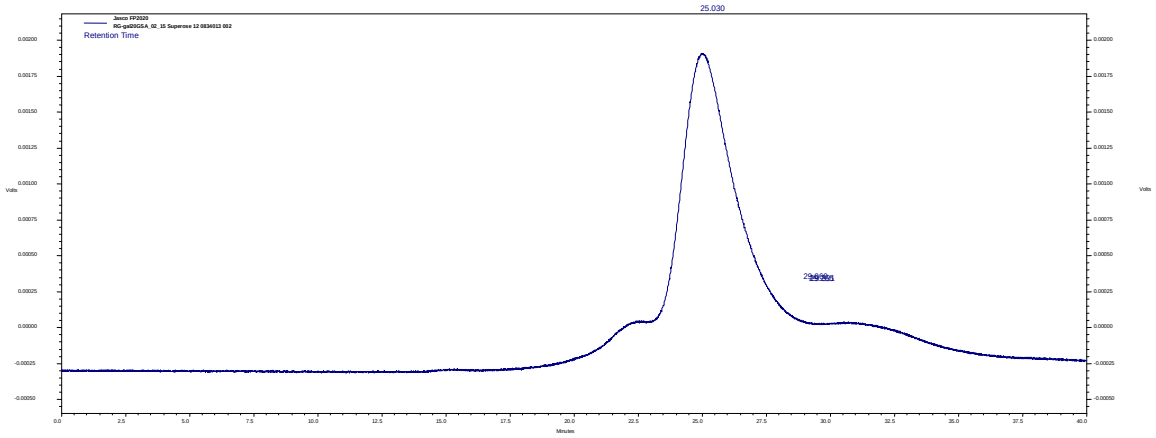

Figure S9. Fluorescence SE-HPLC chromatogram of the RG-$(gal)_{20}$GSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (excitation: 502 nm; emission 532 nm). Major peak is at 25.1 min (96.9%).

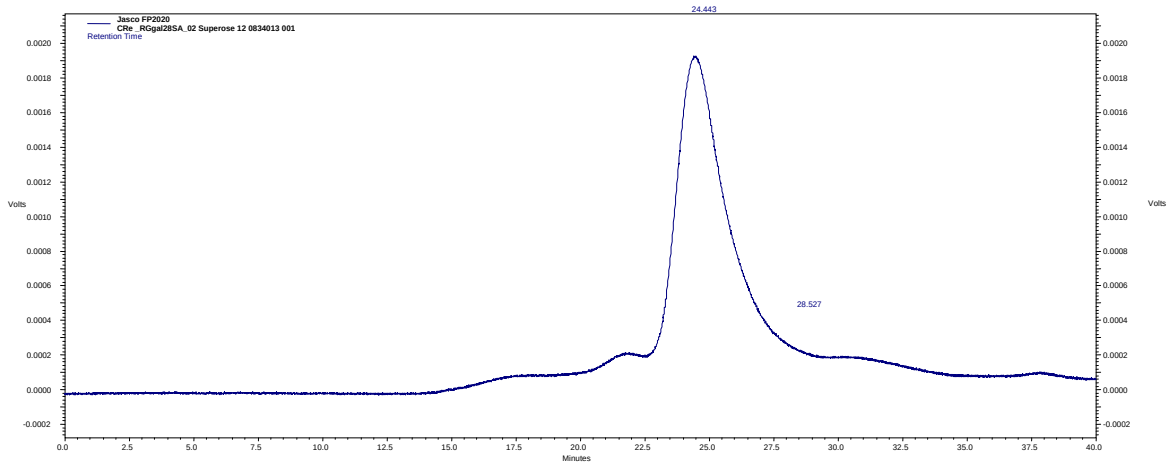

Figure S10. Fluorescence SE-HPLC chromatogram of the RG-$(gal)_{28}$GSA on a Superose 12 10/300GL eluted at 0.5 mL/min with 1x PBS (excitation: 502 nm; emission 532 nm). Major peak is at 24.4 min (98.5%).

Gel Electrophoresis

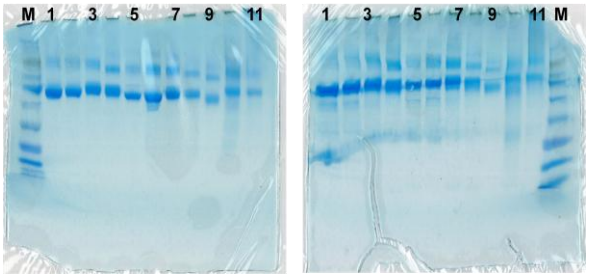

Figure S11. Non-denatured (left) and denatured (right) gel electrophoresis of the different forms of modified human serum albumin in 4-20% TRIS-gel. **M**, See-Blue Protein MW Marker; **1**, Sigma human serum albumin; **2**, glyceraldehydes-HSA; **3**, ggHSA; **4**, $(gal)_{28}$GSA; **5**, Sigma bovine serum albumin; **6**, glyceraldehydes-BSA; **7**, ggBSA; **8**, $(gal)_{28}$BSA; **9**, $(gal)_{20}$GSA; **10**, Sigma glucosylated BSA; and **11**, Sigma galactosyalted BSA.

III. Additional In Vitro Studies

Flow Cytometry Studies

Table 1. Summary of the flow cytometry studies using RG-$(gal)_{28}$GSA, RG-$(gal)_{20}$GSA, RG-$(glu)_{28}$GSA, and RG-HSA with different cell lines.

Cell Line	GSA type	% Gated	MFI
Ovarian			
OVCAR3	*RG-$(gal)_{28}$GSA*	99.21	2222.77
	RG-$(gal)_{20}$GSA	98.23	226.16
	RG-$(glu)_{28}$GSA	99.97	179.51
	RG-HSA	80.73	20.05
OVG-1	*RG-$(gal)_{28}$GSA*	99.87	2299.58
	RG-$(gal)_{20}$GSA	99.78	127.22
	RG-$(glu)_{28}$GSA	100	177.21
	RG-HSA	79.37	16.64
SKOV3	*RG-$(gal)_{28}$GSA*	99.75	1773.28
	RG-$(gal)_{20}$GSA	97.50	265.23
	RG-$(glu)_{28}$GSA	99.93	276.94
	RG-HSA	98.77	22.48
SHIN3	*RG-$(gal)_{28}$GSA*	99.06	613.83

	RG-(gal)$_{20}$GSA	96.81	66.60
	RG-(glu)$_{28}$GSA	97.12	62.43
	RG-HSA	5.68	10.52
Prostate			
PC3	*RG-(gal)$_{28}$GSA*	99.86	768.61
	RG-(gal)$_{20}$GSA	98.28	65.28
	RG-(glu)$_{28}$GSA	99.45	79.60
	RG-HSA	58.59	8.68
Pancreatic			
SHAW	*RG-(gal)$_{28}$GSA*	99.48	765.64
	RG-(gal)$_{20}$GSA	99.90	162.67
	RG-(glu)$_{28}$GSA	99.72	104.27
	RG-HSA	64.22	15.95
Colorectal			
LS 174T	*RG-(gal)$_{28}$GSA*	99.24	423.83
	RG-(gal)$_{20}$GSA	95.08	25.38
	RG-(glu)$_{28}$GSA	98.69	29.62
	RG-HSA	28.77	7.60
Breast			
MCF7	*RG-(gal)$_{28}$GSA*	99.92	697.59
	RG-(gal)$_{20}$GSA	99.89	92.30
	RG-(glu)$_{28}$GSA	99.97	93.17
	RG-HSA	18.60	5.78

Melanoma			
A431	*RG-(gal)$_{28}$GSA*	99.92	1133.58
	RG-(gal)$_{20}$GSA	98.36	62.95
	RG-(glu)$_{28}$GSA	99.30	51.27
	RG-HSA	92.28	20.49

Blocking Study

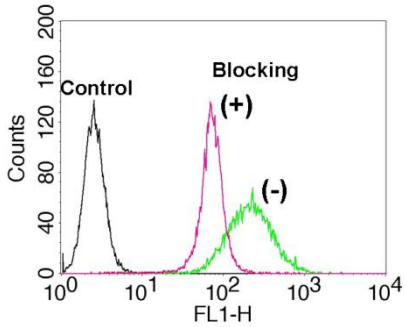

Figure S12. In vitro blocking study using flow cytometry using SHIN3 cell line after incubation with 3 μg/mL of RG-(gal)$_{28}$GSA (green), RG-(gal)$_{20}$GSA (blue), RG-(glu)$_{28}$GSA (pink), RG-HSA (orange), and buffer (black). The percentage fluorescently-gated cells and mean fluorescence intensity is highest with RG-(gal)$_{28}$GSA incubation for all cell lines tested.

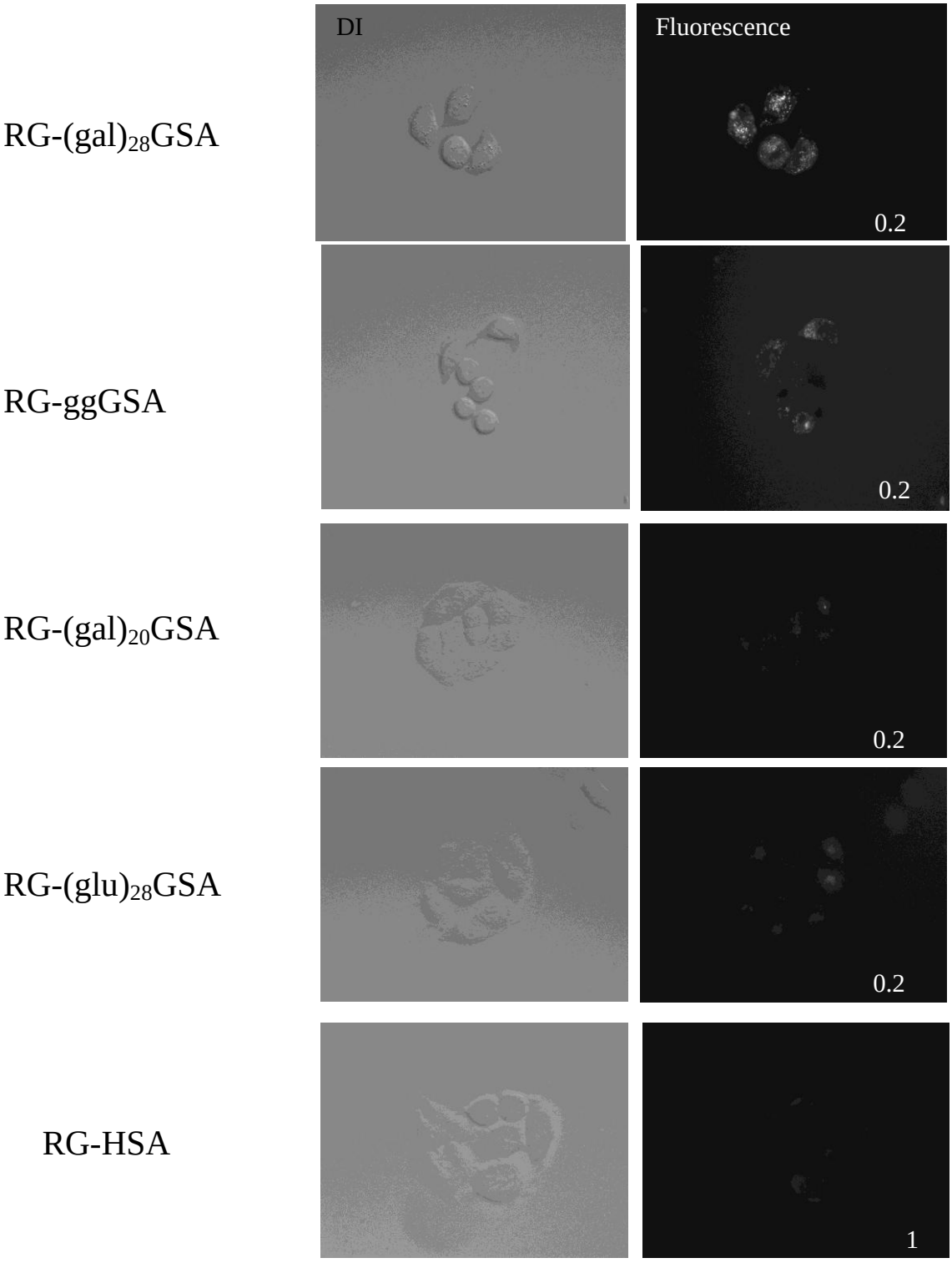

Figure S13. Fluorescence microscopy images (right panel) and differential interference contrast imaging (left panel) of SHIN3 cells 6 hr after incubation with 3 μg/mL of the Rhodamine dye conjugates of the glycosylated GSA and non-glycosylated HSA. Cells

incubated with the glycosylated GSA demonstrated internalization of the agent with the $(gal)_{28}$GSA showing the largest number of fluorescent dots within the cytoplasm under the same exposure time (200 ms) and with RG-HSA showing no fluorescence within the SHIN3 cells even at longer exposure time (1 s).

IV. Additional In vivo studies – gg series vs $(gal)_{28}$GSA

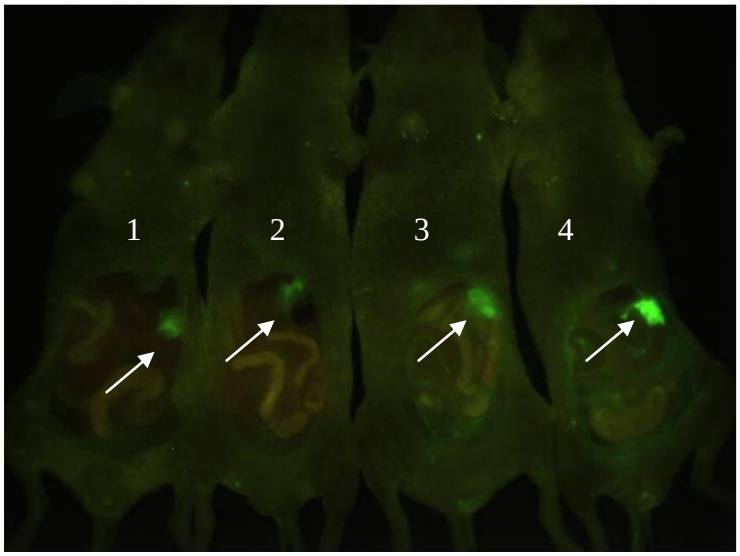

Figure S14. Spectral fluorescence imaging of the peritoneal cavities of SHIN3-xenografted mice after 4 hr intraperitoneal injection of 20 μg of the **1**:RG-ggBSA, **2**:RG-ggHSA, **3**:RG-$(gal)_{28}$BSA, and **4**:RG-$(gal)_{28}$GSA. Spectral composite Rhodamine Green fluorescence images are shown. Aggregated large tumor foci are pointed by the arrows.

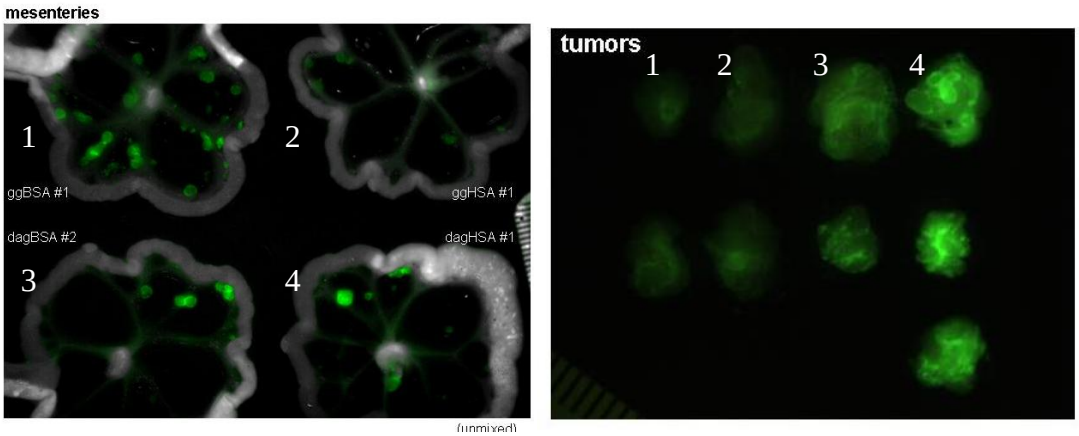

Figure S15. Spectral unmixed fluorescence imaging showing sub-millimeter SHIN3-implants in the peritoneal membranes (of mice from Figure S4) after 4 hr intraperitoneal injection of 20 µg of the **1**:RG-ggBSA, **2**:RG-ggHSA, **3**:RG-$(gal)_{28}$BSA, and **4**:RG-$(gal)_{28}$GSA (left panel). Spectral Rhodamine Green fluorescence images of the *ex vivo* aggregated large tumors from the SHIN3-xenografted mice (from Figure S4) after 4 hr intraperitoneal injection of 20 µg of the **1**:RG-ggBSA, **2**:RG-ggHSA, **3**:RG-$(gal)_{28}$BSA, and **4**:RG-$(gal)_{28}$GSA (right panel).