Supporting Information

Fluorescent Liposomes for Differential Interactions with Glycosaminoglycans

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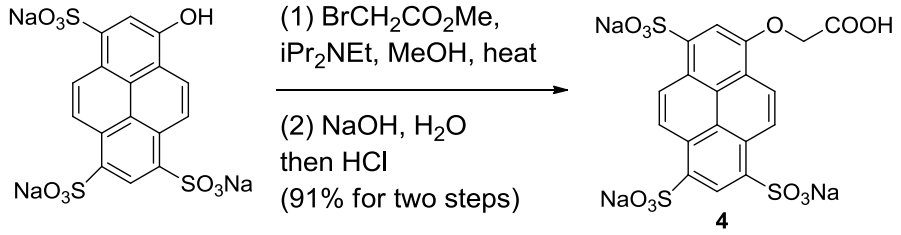
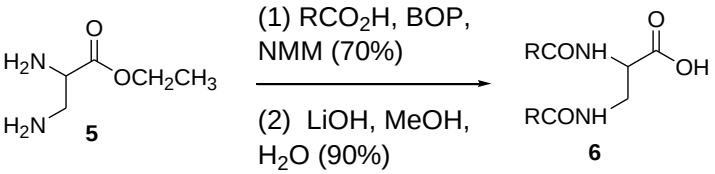
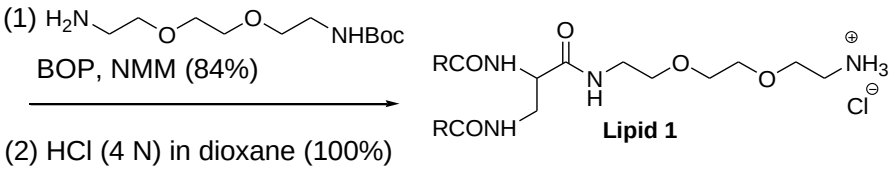
Index of contents:

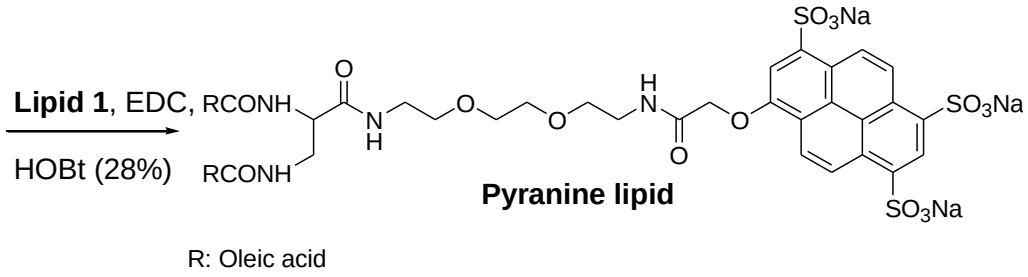
1. Synthesis of the fluorophore-containing lipids:

1.1 Pyranine lipid synthesis









R: Oleic acid

MW = 1329.61 g/mol
λ_{ex} = 390 nm; λ_{em} = 400-600 nm

Scheme S1. Synthesis of the pyranine lipid.

Cascade Blue (**4**): Methyl ester derivative of cascade blue (1.28 g, 2.14 mmol) was dissolved in water (20 mL) and stirred with NaOH (0.086 g, 2.15 mmol) for 30 min at 60^0 C. After cooling the reaction mixture, 100 µL of HCl was added. To this clear solution isopropanol (60 mL) was added to effect precipitation. The mixture was stirred for 20 minutes, filtered and dried to afford free flowing yellow powder (1.131 g, 91%). ^{1}H NMR (400 MHz, CD_3OD): δ 5.07 (s, 2H), 8.33(s, 1H), 8.76 (d, 1H, J = 10 Hz,), 9.11 (d, 1H, J = 10 Hz), 9.19-9.24 (overlapping doublet, 2H, J = 10.8 Hz and 10 Hz), 9.378 (s, 1H). ^{13}C NMR (100

MHz,D_2O):δ 66.64, 109.92, 120.87, 121.67, 123.26, 124.39, 124.69, 124.73, 125.63, 125.71, 127.41, 129.64, 130.28, 134.84, 138.88, 152.43, 174.10.

Compound 6: To a stirred mixture of diamino propanoic acid.2PTSA ethyl ester (5.12 g, 10 mmol) and oleic acid (5.64 g, 20 mmol) in $CHCl_3$ (150 mL) and DMF (50 mL), BOP (8.85 g, 20 mmol) was added. The reaction mixture was stirred for 5 minutes and NMM (6.5 mL, 60 mmol) was added drop wise. Overnight stirring at room temperature resulted in a clear reaction mixture. The reaction mixture was then diluted with 250 mL additional $CHCl_3$, quenched with brine, and the organic phase was washed successively with brine, 5% citric acid and 5% $NaHCO_3$ solution. Organic layer was dried on sodium sulfate and solvent evaporated under reduced pressure. Oily residue was purified by SiO_2 column chromatography employing 3:1 to 3:2 hexane ethyl acetate. (R_f = 0.3 in 1:1 ethyl acetate/hexane) to obtain the pure product as a colorless oil(4.6 g, 70%) ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.83-0.87 (t, 6H, J = 6.8 Hz), 1.18-1.32 (m, 43H), 1.56-1.61 (m, 4H), 1.94-2.01 (m, 8H), 2.11-2.3 (m, 4H), 3.58-3.62 (m, 2H), 4.15-4.21 (m, 2H), 4.53-4.58 (m, 1H), 5.26-5.33 (m, 4H), 6.15-6.18 (m, 1H), 6.71 (d, 1H, J = 7.2 Hz).

Bis oloeyl diaminopropanoic acid ethyl ester (3.6 g, 5.44 mmol) in CH_2Cl_2/MeOH (2:1, 45 mL) was treated with $LiOH.H_2O$ (0.458 g, 10.89 mmol) overnight at room temperature. After complete consumption of starting material the reaction was stopped and acidified to pH 2 with dilute HCl and solvent was evaporated under reduced pressure. The residue was taken into CH_2Cl_2 and was washed with water to remove LiCl. Drying of organic phase over sodium sulfate and solvent evaporation afforded clear liquid which slowly became waxy solid. This was used in the next step without further purification. Compound **6** was obtained in 90% yield (3.1 g). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78-0.82 (distorted triplet, 6H, J = 7.2 and 6.8 Hz), 1.17-1.25 (m, 40H), 1.47-1.54 (m, 4H), 1.89-1.94 (m, 8H), 2.06-2.25 (m, 4H), 3.28-3.34 (m, 1H), 3.51-3.55 (dd, 1H, J = 4 Hz and J = 9.6 Hz), 4.12-4.14 (q, 1H), 5.23-5.27 (m, 4H).

Lipid 1: Following a similar protocol as above **6** (3.6 g, 5.696 mmol) was conjugated with the mono BOC protected linker (1.447 g, 5.98 mmol) by employing BOP (2.646 g, 5.98 mmol) and NMM (1.876 mL, 17.08 mmol) in chloroform (100 mL). After work up and solvent evaporation it was purified by chromatography with CH_2Cl_2/MeOH (R_f = 0.3 in 5% methanol in CH_2Cl_2, iodine active). Pure product obtained 4.1 g (yield: 84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.83-0.87 (distorted triplet, 6H, J = 7.2 and 6.8 Hz), 1.2-1.38 (m, 40H), 1.41 (s, 9H), 1.54-1.62 (m, 4H), 1.95-1.99 (m, 8H), 2.14-2.22 (m, 4H), 3.32-3.36 (m, 2H), 3.42-3.58 (m, 12H), 4.44 (br s, 1H), 5.29-5.33 (m, 4H), 6.46 (br s, 1H).

Thus obtained Boc-protected compound (2.5 g, 2.90 mmol) was dissolved in minimum quantity of CH_2Cl_2 (5 mL) and stirred with 4 N HCl in dioxane (10 mL) for 3 hours. Evaporation of solvent afforded the compound **7** as HCl in quantitative yield. ^{1}H NMR (500 MHz, $CDCl_3$/2 drops CD_3OD): δ 0.88-0.90 (dd, 6H, J = 5.2 and 5.6 Hz), 1.28-1.32 (m, 40H), 1.58-1.61 (m, 4H), 2.00-2.02 (m, 8H), 2.21-2.28 (m, 4H), 3.15-3.3 (br s, 2H), 3.37-3.47 (m, 1H), 3.63-3.72 (m, 9H), 3.85-3.86 (m, 2H), 4.6-4.7 (m, 1H), 5.32-5.37 (m, 4H), 7.24 (br s, 1H), 7.73 (d, 1H, J = 5.2Hz), 8.20 (br s, 1H), 8.36 (br s, 3H).

Pyranine lipid: To a DMF (10 mL) solution of compound **7** (0.2 g, 0.25 mmol), cascade blue (0.161 g, 0.27 mmol) was added and stirred for 10 minutes to make a homogeneous solution. HOBt (0.036 g, 0.27

mmol) followed by EDC (0.052 g, 0.27 mmol) were added to the reaction mixture and stirred at room temperature for 36 hours. Solvent was evaporated under reduced pressure and little water was added to the residue followed by large excess of isopropyl alcohol. The resulting precipitate was filtered, dried and was subjected to chromatographic purification using 2:1 dichloromethane methanol mixture (R_f = 0.3) to afforded the pure product as a yellow waxy solid (92 mg, 28%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80-0.84 (distorted triplet, 6H, J = 7.2 and 6.4 Hz), 1.13-1.31 (m, 40H), 1.40-1.44 (m, 4H), 1.93-2.01 (m, 10H), 2.05-2.09 (t, 2H, J = 7.6 Hz), 3.14-3.51 (m, 14H), 4.26-4.29 (m, 1H), 4.85 (s, 2H), 5.26-5.31 (m, 4H), 7.68-7.73 (m,2H), 7.84-7.86 (m, 1H), 8.11 (s, 1H), 8.25-8.28 (m,1H), 8.51 (d, 1H, J = 9.6 Hz), 8.96 (d, 1H, J = 10 Hz), 9.02-9.04 (m, 2H), 9.15 (d, 1H, J = 9.6 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.95, 22.74, 25.41, 25.76, 25.89, 27.23, 27.30, 29.25, 29.33, 29.49, 29.63, 29.75, 29.83, 31.94, 36.02, 39.03, 69.50, 69.58, 70.23, 85.59, 109.78, 120.43, 121.37, 121.67, 124.51, 125.17, 125.69, 126.09, 126.68, 127.01, 128.29, 128.69, 130.26, 130.53, 134.25, 140.29, 140.41, 143.79, 151.28, 168.18, 170.71, 172.92, 173.43. MH^+ calcd. for $C_{63}H_{89}N_4Na_3O_{16}S$: 1324.51; found: 1324.57.

1.2 Lissamine Rhodamine B lipid: This lipid is commercially available from Avanti Polar Lipids, Alabaster, AL. MW = 1267.68 g/mol; λ_{ex} = 557 nm; λ_{em} = 567-700 nm

1.3 Dansyl lipid: This lipid is commercially available from Avanti Polar Lipids, Alabaster, AL. MW = 994.35 g/mol; λ_{ex} = 336 nm; λ_{em} = 400-650 nm

2. Fluorescence emission intensity ratios of the liposomes in presence of glycosaminoglycans:

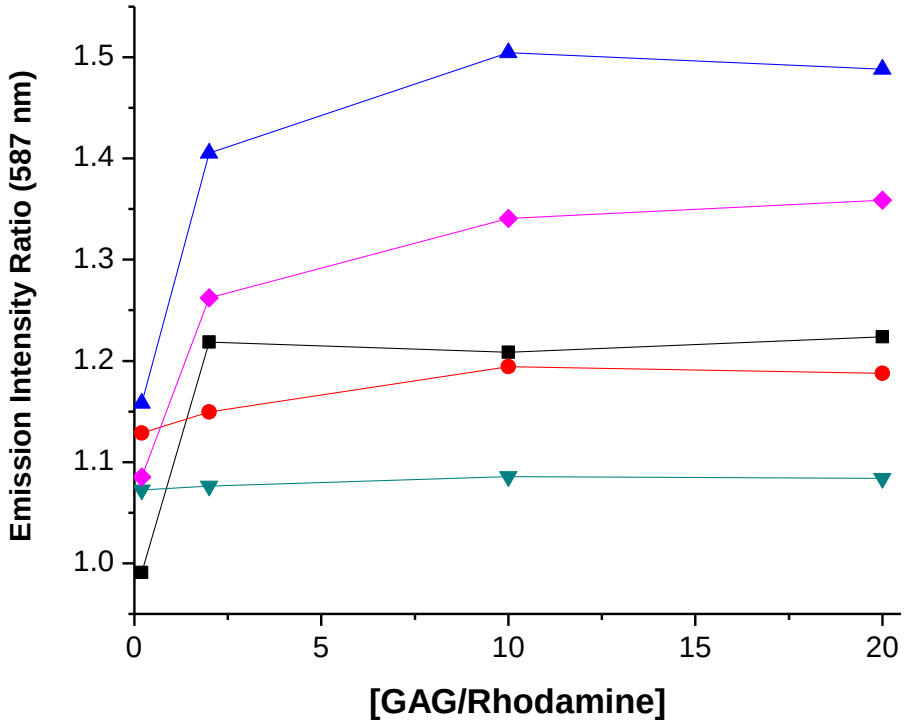


Figure S1. The emission intensity ratios for the rhodamine liposomes (λ_{ex} = 557 nm) in the absence and presence of added GAGs are shown. The GAGs include chondroitin sulfate (black squares), dextran sulfate (red circles), heparin sulfate (blue triangles), hyaluronic acid (dark cyan inverted triangles), and dermatan sulfate (magenta diamonds). The data points are connected by straight lines.

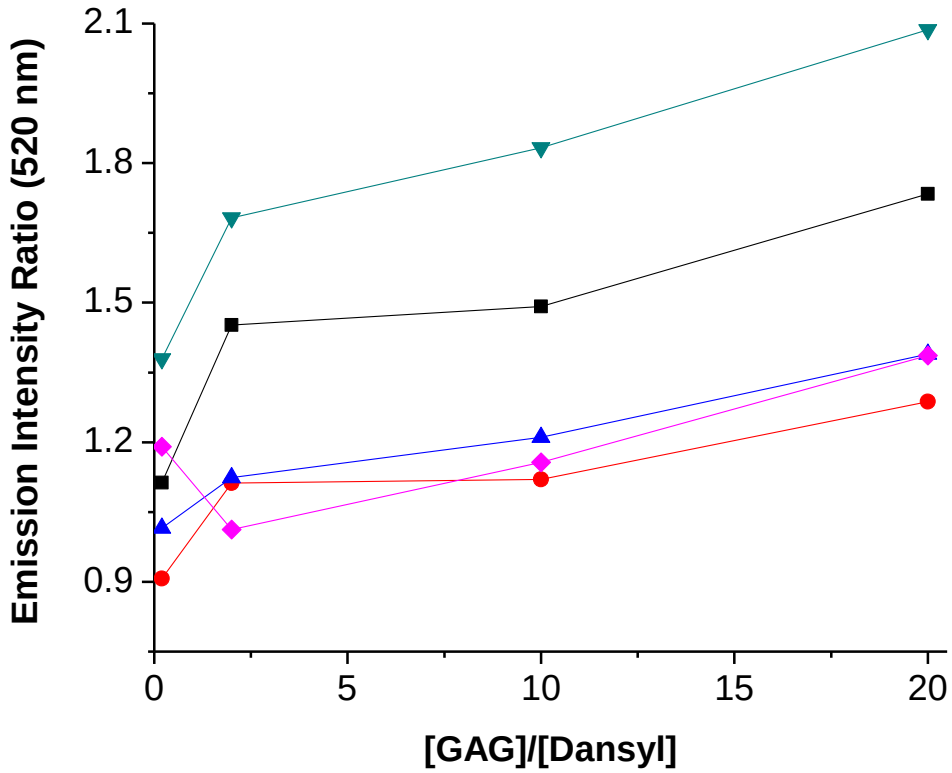


Figure S2. The emission intensity ratios for the dansyl liposomes (λ_{ex} = 336 nm) in the absence and presence of added GAGs are shown. The GAGs include chondroitin sulfate (black squares), dextran sulfate (red circles), heparin sulfate (blue triangles), hyaluronic acid (dark cyan inverted triangles), and dermatan sulfate (magenta diamonds). The data points are connected by straight lines.

3. Comparison of liposome fluorescence emission intensity changes in the presence of chondroitin sulfate of two molecular weights

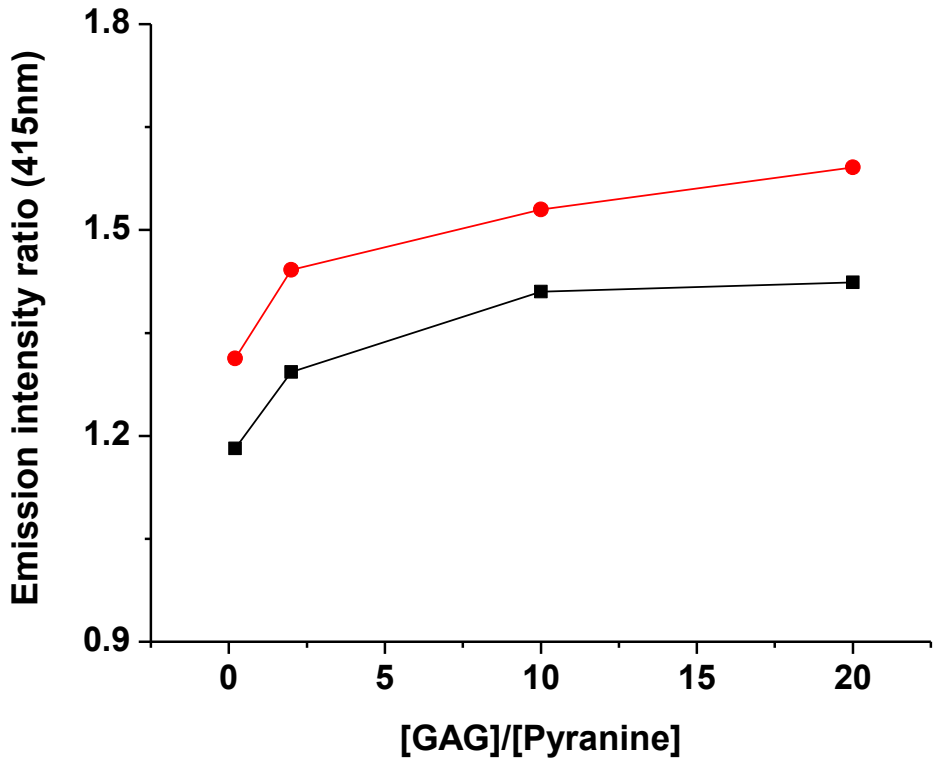


Figure S3. The emission intensity ratios for the pyranine liposomes (λ_{ex} = 415 nm) in the presence of 20 kDa MW chondroitin sulfate (black squares), and 35 kDa MW chondroitin sulfate (red circles). The data points are connected by straight lines.

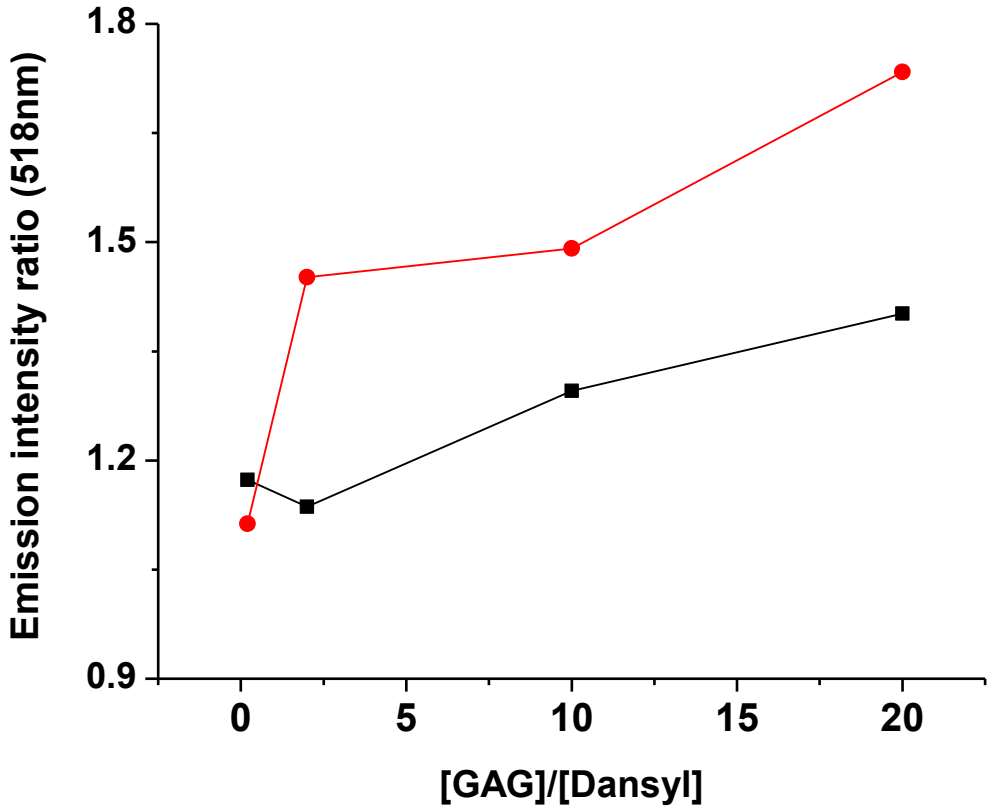


Figure S4. The emission intensity ratios for the dansyl liposomes (λ_{ex} = 587 nm) in the presence of 20 kDa MW chondroitin sulfate (black squares), and 35 kDa MW chondroitin sulfate (red circles). The data points are connected by straight lines.

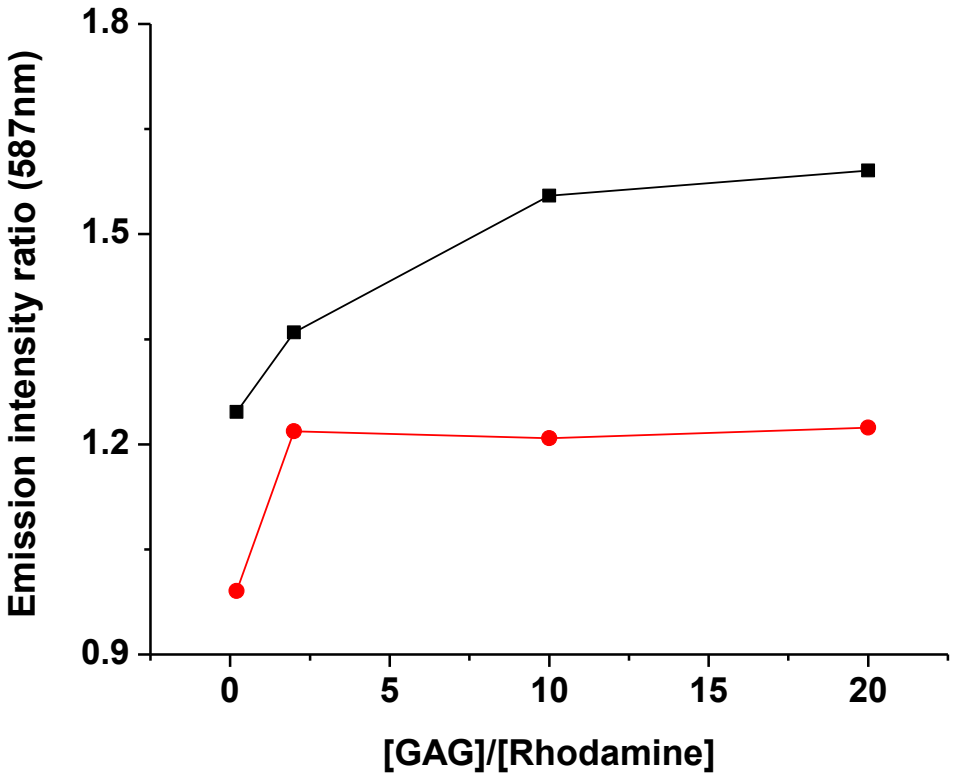


Figure S5. The emission intensity ratios for the rhodamine liposomes (λ_{ex} = 587 nm) in the presence of 20 kDa MW chondroitin sulfate (black squares), and 35 kDa MW chondroitin sulfate (red circles). The data points are connected by straight lines.

4. Statistical Data Analysis:

The following discussion presents the full set of LDA results for the analysis at 50 nM. For simplicity, these are labeled as Tables A1 – A5 and Figure A1. Note that several of these tables (A1 and A5), Figure A1 and some of the language are identical to those presented in the body of the text.

Linear discriminant analysis (LDA) is used to identify the predictive power of the liposomes. [12, 13] Emissions intensity data from the three liposomes (the predictor variables) and the five GAGs (the dependent variables) were replicated a total of six times, yielding a sample of 30 observations with four variables (one for each liposome and one identifying the GAG). Each liposome is included in the model using a stepwise procedure, where inclusion is based on minimizing the Mahalanobis D^2, or generalized squared inter-point distance, between each individual observation to the corresponding group centroid.

Once the appropriate number of liposomes is determined, LDA identifies canonical correlations (i.e., synthetic variables which are linear combinations of the predictor variables) which maximize the ratio of

the between-group variation to the within-group variation across the GAGs. That is, LDA identifies the set of predictive factors that leads to maximum discrimination between our GAGs. Note that, for K = 5 possible GAGs, it is possible to identify as many as K-1 = 4 possible canonical correlations, and by extension as many as K-1 = 4 discriminant functions, each of which relates a single canonical correlation to a linear combination of liposome fluorescence intensities. It is common practice to identify and interpret only those correlations that are both orthogonal and explain a significant portion of the variation in the GAGs. The coefficients characterizing the linear combination of the predictor variables can also be used (with a bit of algebraic manipulation) to identify the relative contribution of each liposome to a discriminant function. Liposomes with larger coefficient values (in absolute value) play a larger role in the formation of a given discriminant function, and by extension in predicting the GAGs. To characterize the contribution of each liposome to the model's overall ability to discriminate across GAGs (rather than the ability of a single canonical function), we utilize the correlations between each liposome and each of the discriminant functions to generate a structure matrix.[14] The elements of the structure matrix are subsequently combined with the eigenvalues of canonical functions to generate an overall "potency index" for each liposome. Higher values for each index signal the overall importance of each liposome to the model as a whole.

Overall model fit is assessed by examining canonical function plots to identify whether each of the group centroids (one for each of the five GAGs) is sufficiently distinct. Overlap between the data points of two or more groups indicates that the model does not adequately discriminate across these GAGs. Internal validity is assessed by comparing the percentage of GAG observations that are correctly predicted by the model. All model predictions are computed using both traditional and (leave one out) cross-validation techniques. Internally valid results should correctly predict a high percentage of observations, and display consistency in predicted values across both techniques. All statistical analyses were conducted using the PASW (formerly SPSS) Statistical Package, Version 18.

Table A1 contains means, F-statistics and Wilks' Lambda values for each liposome, disaggregated by GAGs. We note in passing that smaller values for the Wilks' Lambda indicate a greater potential for the liposome to discriminate across GAGs. All F-statistics have associated p-values less than 0.05, indicating significant differences exist across group means for each GAG. For the chondroitin sulfate, dextran sulfate, heparin sulfate and hyaluronic acid GAGs, the dansyl liposome has the highest mean fluorescence values. The pyranine liposomes have the second highest mean values, followed by rhodamine. The remaining GAG (dermatan sulfate) has the highest mean emission ratios when combined with pyranine, followed by dansyl and rhodamine. Wilks' Lambda values are lowest for pyranine, followed by rhodamine and dansyl.

Table A1. Tests of Equality of Group Means

GAG	Pyranine[a,b]	Rhodamine[a,b]	Dansyl[a,b]
Chondroitin Sulfate	1.562	1.297	1.563
Dermatan Sulfate	1.942	1.447	1.412
Dextran Sulfate	1.334	1.247	1.541
Heparin Sulfate	1.729	1.517	1.862
Hyaluronic Acid	1.487	1.124	2.243
Wilks' Lambda	0.068	0.214	0.560
F-Statistic [4,25]	85.829	22.898	4.904
P-Value	<0.001	<0.001	0.005

[a] first panel provides group-specific means [b] second panel provides statistics and p-values.

Table A2 contains a summary of the stepwise variable selection process. All three liposomes are found worthy of inclusion, and no variables are removed from the analysis. Table A3 identifies the number of significant canonical correlations, and by extension the number of significant canonical functions. Chi-square tests indicate that three canonical functions are sufficient to explain our 5 GAGs. Of these, the first canonical function is most important, as it explains 96.3% of the variation across GAGs. The remaining functions explain 3.0% and 0.7%, respectively. As such, we focus primarily on the first discriminant function.

Table A2. Variables in the Analysis[a]

Step Entered	Predictor	D^2	Between Groups[b]	F-Statistic	P-Value
1	Pyranine	1.475	1 and 5	4.426	0.046
2	Dansyl	6.150	3 and 5	8.857	0.001
3	Rhodamine	12.499	3 and 5	11.499	<0.001

[a] Variables are entered in a manner that maximizes the Mahalanobis D^2 between the two closest groups. [b] Each of five GAGs groups are: chondroitin sulfate (group 1), dermatan sulfate (group 2), dextran sulfate (group 3), heparin sulfate (group 4) and hyaluronic acid (group 5).

Table A3. Canonical Function Summary[a]

Fct.	Eigen-value	Pct. of Variance Explained	Canonical Correl.	Wilks' Lambda[a]	Chi-Square Statistic	P-Value
1	61.693	96.3	0.992	0.004[b]	139.166	<0.001
2	1.893	3.0	0.809	0.240[c]	35.710	<0.001
3	0.442	0.7	0.554	0.693[d]	9.151	0.010

[a] Lower values for Wilks' Lambda indicate greater discrimination. Wilks' Lambda and chi-square tests apply sequentially. [b] tests functions 1 – 3 cumulatively. [c] tests functions 2 – 3 cumulatively [d] tests function 3.

Figure A1 contains a canonical function plot of the first two canonical functions (explaining 99.3% of the variation in the GAGs). Note that each of the GAGs is clearly distinguished as a group in the plot. Moreover, traditional and cross-validated discriminant functions corrected predict 100% and 93.3% of the GAGs, respectively, indicating a high likelihood of interval validity.

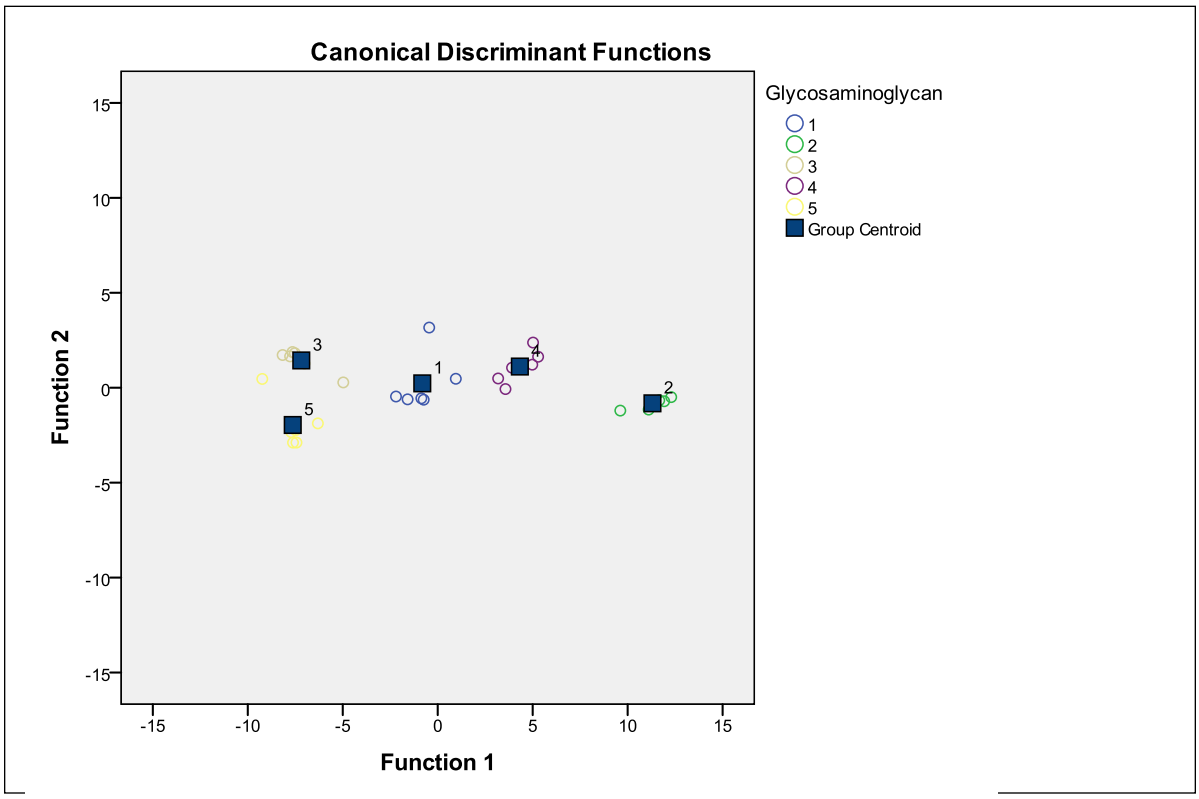


Figure A1. Canonical correlation plot between two largest canonical correlations and each of the five GAGs: chondroitin sulfate (group 1), dermatan sulfate (group 2), dextran sulphate (group 3), heparin sulphate (group 4) and hyaluronic sulphate (group 5).

Table A4 contains the standardized discriminant function coefficients, which measure the relative contributions of each liposome to a specific discriminant function. For function one, the dansyl liposome exhibits the largest coefficient in absolute value (signs merely denote the magnitude of the relationship) followed closely by the pyranine liposome. While still meaningful (coefficients with values above 0.3 are generally considered "significant" or meaningful), the rhodamine liposome is over twice as small as the other two coefficients.[14] Concomitantly, rhodamine carries the largest weight in the second canonical function, and is twice as large in absolute magnitude compared to the other coefficients. Lastly, the Dansyl liposome has the largest canonical weight in the third canonical function.

Table A4. Standardized Canonical Discriminant Function Coefficients

Predictor	Canonical Function 1	Canonical Function 2	Canonical Function 3
Pyranine	1.615	-0.478	-0.059
Rhodamine	0.795	0.924	0.352
Dansyl	-1.708	-0.426	0.811

To assess the overall contribution of each liposome to the discriminatory power of the LDA, we present Table A5, which contains the structure matrix and the cumulative potency indices. The potency indices suggest that pyranine provides the largest overall contribution to the model's ability to distinguish emission intensities across GAGs.

Table A5. Structure Matrix and Potency Index

Predictor	Canonical Function 1	Canonical Function 2	Canonical Function 3	Potency Index
Pyranine	0.456	-0.631	0.628	0.215
Rhodamine	0.209	0.613	0.762	0.057
Dansyl	-0.057	-0.312	0.948	0.012

On total, the LDA has a clear and intuitive interpretation. The results in Table 2 suggest that the first canonical function is, by far, the most important discriminant function. Tables 4 and 5 jointly suggest that pyranine is the largest contributor to this discriminant function, and to the model overall. This implies that the pyranine liposome is the "best" determinant of GAGs. Dansyl is identified as the least "potent" discriminator, even though its emission intensities are relatively high (Table 1). The Wilks' Lambda and structure matrix (Table 5) suggest (but do not prove) that this is at least partly attributable to excess variation in dansyl emission intensities, which offsets the high mean values.

The following tables present the full set of LDA results for the analysis at 100 nM. For simplicity, these are labeled as Tables B1 – A5 and Figure B1. Since the discussion of each of the following tables is analogous to what was described previously, we simply present the tables for the reader's consumption.

Table B1. Tests of Equality of Group Means

GAG	Pyranine[a,b]	Rhodamine[a,b]	Dansyl[a,b]
Chondroitin Sulfate	1.687	1.315	1.816
Dermatan Sulfate	2.035	1.495	1.712
Dextran Sulfate	1.388	1.253	1.728
Heparin Sulfate	1.766	1.530	2.093
Hyaluronic Acid	1.551	1.130	2.506
Wilks' Lambda	0.153	0.209	0.623
F-Statistic [4,25]	34.489	0.626	3.775
P-Value	<0.001	<0.001	0.016

[a] first panel provides group-specific means [b] second panel provides statistics and p-values.

Table B2. Variables in the Analysis[a]

Step Entered	Predictor	D^2	Between Groups[b]	F-Statistic	P-Value
1	Pyranine	0.615	1 and 4	1.845	0.187
2	Rhodamine	4.928	1 and 5	7.097	0.004
3	Dansyl	9.514	1 and 4	8.753	<0.001

[a] Variables are entered in a manner that maximizes the Mahalanobis D^2 between the two closest groups. [b] Each of five GAGs groups are: chondroitin sulfate (group 1), dermatan sulfate (group 2), dextran sulfate (group 3), heparin sulfate (group 4) and hyaluronic acid (group 5).

Table B3. Canonical Function Summary[a]

Fct.	Eigen-value	Pct. of Variance Explained	Canonical Correl.	Wilks' Lambda[a]	Chi-Square Statistic	P-Value
1	23.726	85.3	0.980	0.006[b]	126.396	<0.001
2	3.749	13.5	0.889	0.158[c]	46.199	<0.001
3	0.336	1.2	0.502	0.748[d]	7.250	0.027

[a] Lower values for Wilks' Lambda indicate greater discrimination. Wilks' Lambda and chi-square tests apply sequentially. [b] tests functions 1 – 3 cumulatively. [c] tests functions 2 – 3 cumulatively [d] tests function 3.

Table B4. Standardized Canonical Discriminant Function Coefficients

Predictor	Canonical Function 1	Canonical Function 2	Canonical Function 3
Pyranine	1.546	-1.117	-0.058
Rhodamine	0.438	1.412	0.281
Dansyl	-1.686	-0.245	0.830

Table B5. Structure Matrix and Potency Index

Predictor	Canonical Function 1	Canonical Function 2	Canonical Function 3	Potency Index
Pyranine	0.456	-0.308	0.835	0.199
Rhodamine	0.346	0.435	0.831	0.136
Dansyl	-0.085	-0.169	0.982	0.022

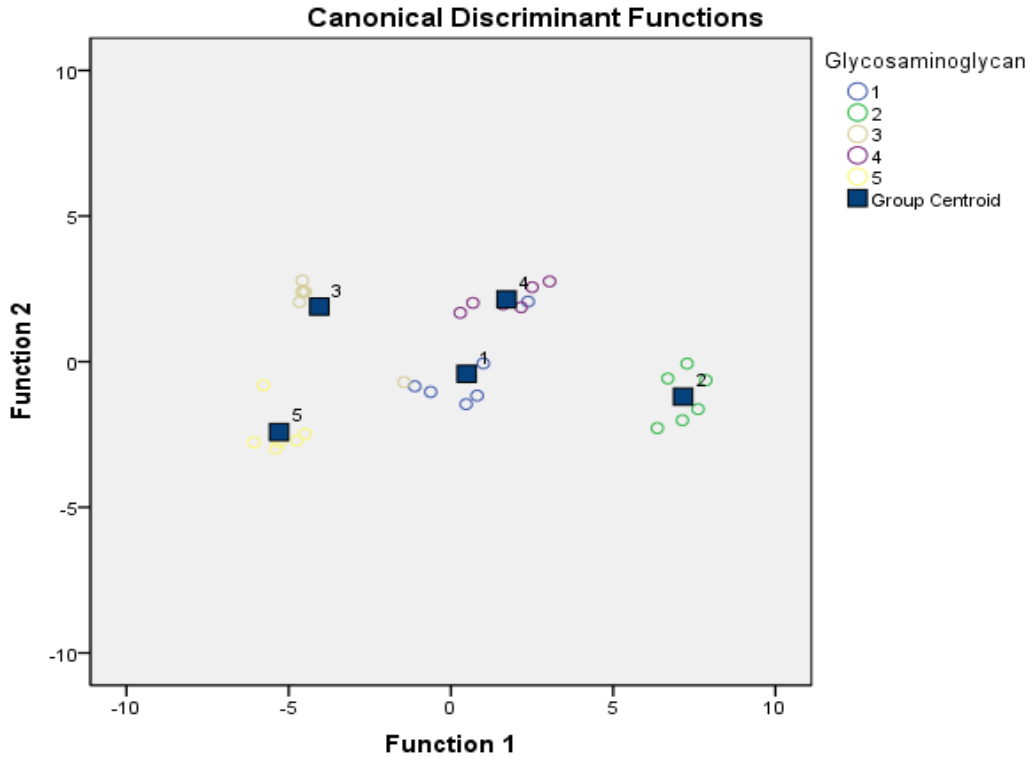


Figure B1. Canonical correlation plot between two largest canonical correlations and each of the five GAGs: chondroitin sulfate (group 1), dermatan sulfate (group 2), dextran sulphate (group 3), heparin sulphate (group 4) and hyaluronic sulfate (group 5).