Supporting information

Characterization of fluorescently labeled protein with ESI-MS and fluorescence spectroscopy: how random is random labeling?

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Abstract:

In this section, we included additional data of deconvoluted ESI-MS spectra of labeled NGAL; Detailed description of Incorporation ratio (I.R.) calculation by absorption and mass spectroscopy, and result comparison of the two methods; List of all the identified peptides from digested NGAL by ESI-MS.

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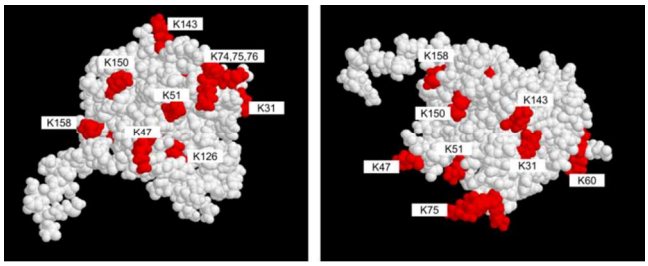
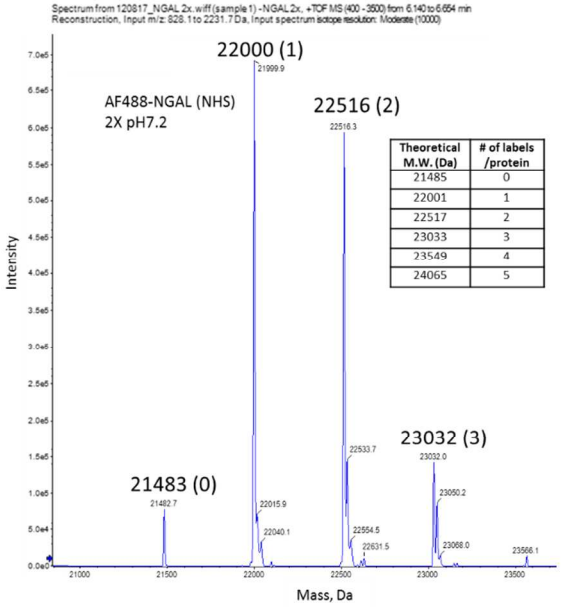
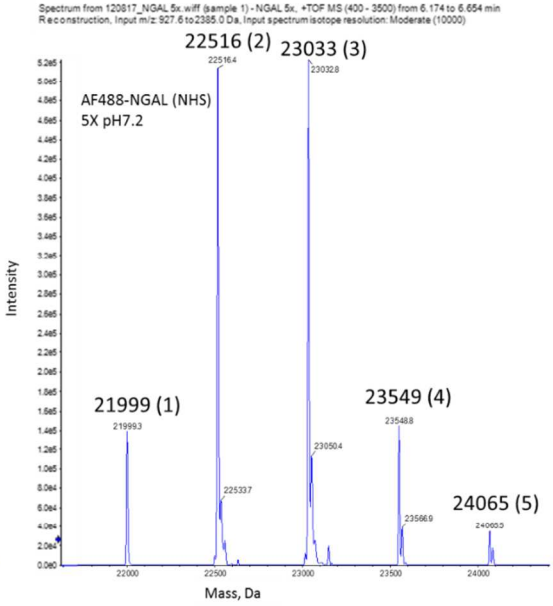
S1: 3D structure of NGAL determined by NMR spectroscopy, surface exposed lysines are shown in red.

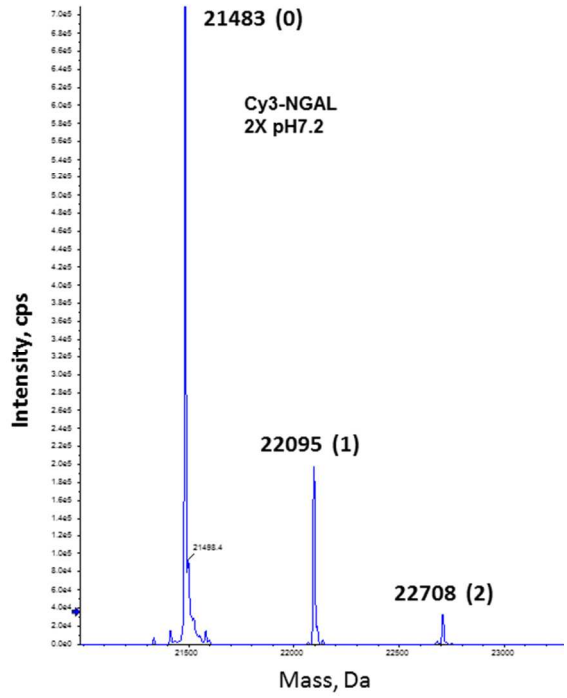


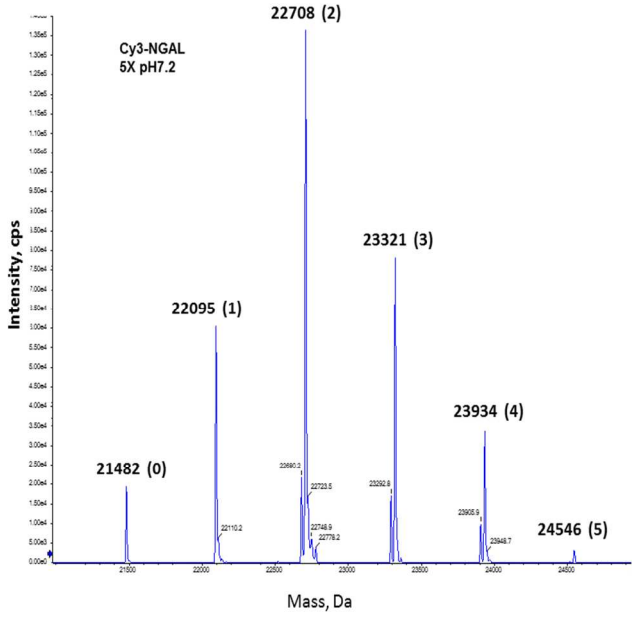
S2. Examples of deconvoluted ESI-MS spectra of AF488-NGAL (NHS) and Cy3-NGAL. The mass increment between each peak for AF488-NGAL (NHS) is 516 Da, which matches the molecular weight of AF488, confirming that the structure of AF488 was not modified during labeling. Similarly, the molecular weight of Cy3 is 613 Da, and the mass increments between each peak for Cy3-NGAL is also 613 Da, confirming structural integrity of Cy3 after its conjugation to NGAL.



Theoretical M.W. (Da)	# of labels /protein
21485	0
22001	1
22517	2
23033	3
23549	4
24065	5

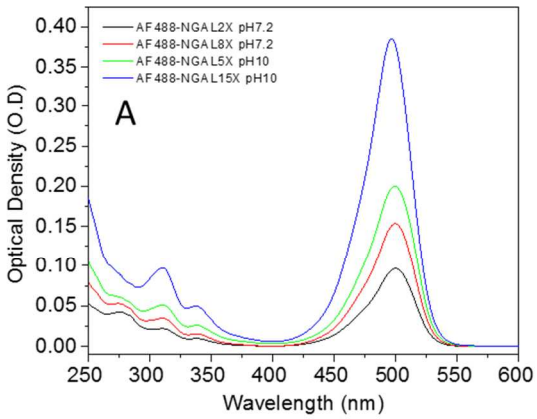


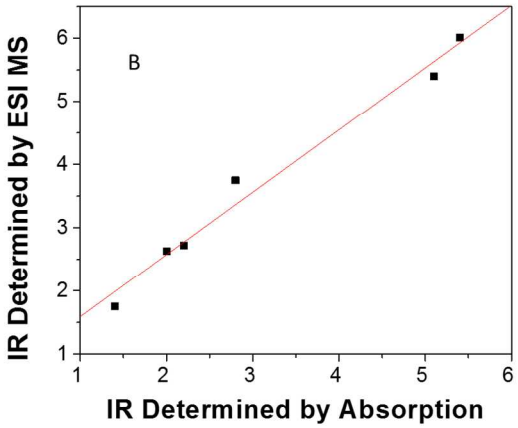




S3: Incorporation ratio (I.R.) determination: The average incorporation ratio (I.R.) in each labeled AF488-NGAL (SDP) sample can be calculated from deconvoluted ESI-MS spectra using the formula: ∑(number of fluorophores x peak intensity) / ∑(peak intensity). Alternatively, the I.R. of labeled NGAL can be determined by absorption spectrum. The absorption spectra of the labeled proteins were measured on a Cary UV-Vis spectrophotometer. The main absorption peak of AF488 at 496 nm (A_M = 71 000 M^{-1} cm^{-1}) was used to calculate fluorophore concentrations: $C_{AF488}=A_{496}/71000$; NGAL concentration in the complex was calculated using following equations: $C_{AF488\text{-}NGAL} = (A_{280}-A_{496}*0.16)/25300$, where 0.16 is correction factors reflecting spectral overlaps. The molar absorption coefficient of NGAL is A_M = 25 300 $M^{-1}cm^{-1}$. The average I.R. of the labeled protein is the ratio of the fluorophore and NGAL concentrations: $I.R._{AF488} = C_{AF488}/C_{NGAL}$

Figure, A): Absorption spectra of AF488-NGAL labeled at pH 7.2 and pH 10 varying the molar excess of AF488-SDP ester from 2X to 15X. The I.R. values 1, 2, 3 and 6 were calculated as described above. Figure B) Correlation of the AF488-NGAL I.R. value determined by absorption spectra and ESI-MS (r^2=0.976).





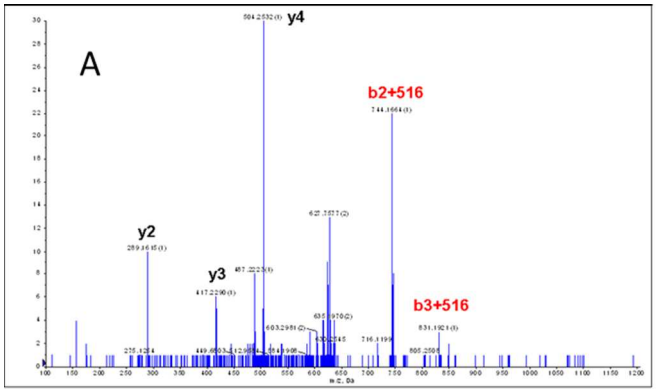
S4. The list of identified NGAL peptides without AF488 attachment from sample AF488-NGAL (SDP, 2X, pH7.2) and AF488-NGAL (NHS, 2X, pH7.2). The experimental M.W. matches with the theoretical M.W with most of the mass accuracy within 20 ppm. The ones with mass accuracy more than 20ppm have multiple peptides to confirm the sites. The overall peptide coverage is 98%.

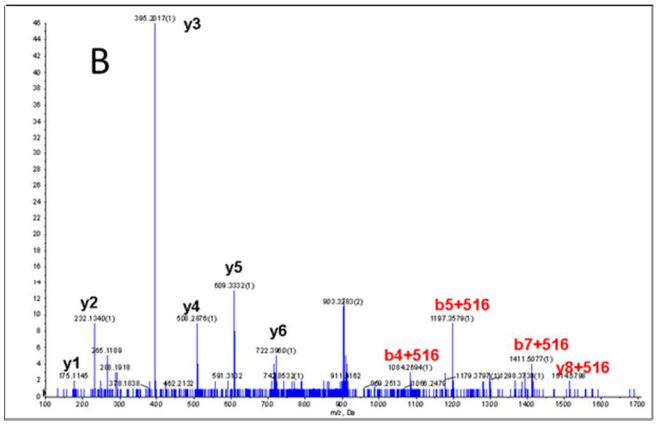
AF488-NGAL2X(SDP), pH7.2			
Peptide location	Experimental MW (Da)	Theoretical MW (Da)	Error (PPM)
132-135	585.3	585.3	3.9
151-155	677.4	677.3	2.8
136-141	721.4	721.4	-2.9
144-150	818.4	818.4	5.5
77-82	854.4	854.4	0.5
45-51	858.4	858.4	-27.3
76-82	982.5	982.5	6.9
142-150	1047.6	1047.6	8.1
75-82	1110.5	1110.6	-41.2
52-60	1130.6	1130.6	7.7
64-73	1184.6	1184.6	7.8
100-110	1254.7	1254.7	3.9
32-44	1430.8	1430.8	3.3
144-155	1477.8	1477.8	12.3
52-63	1502.8	1502.7	16.8
61-73	1556.8	1556.8	10.1
61-74	1684.9	1684.9	-16.8
1-16	1698.9	1698.8	6.9
142-155	1706.9	1706.9	13.1
111-125	1783.9	1783.9	10.7
17-31	1789.9	1789.9	4.4
83-99	1790.9	1790.9	-0.2
61-75	1812.9	1813.0	-32.4
127-141	1873.0	1873.0	20.6
100-125	3020.6	3020.5	19.6
159-185	3084.5	3084.5	16.2
100-126	3148.5	3148.6	-38.8
17-44	3202.7	3202.7	15.0
1-31	3470.8	3470.7	16.2
100-131	3733.0	3732.9	12.8
17-51	4043.1	4043.1	15.2
1-44	4883.6	4883.5	17.0

AF488-NGAL2X(NHS), pH7.2			
Peptide Location	Experimental MW (Da)	Theoretical MW (Da)	Error (PPM)
77-82	854.4	854.4	0.3
48-63	1971.0	1971.0	3
48-73	3137.6	3137.6	-4.7
48-74	3265.6	3265.7	-9.4
45-51	858.4	858.4	-2.5
45-60	1971.0	1971.0	3
45-73	3509.7	3509.7	-0.5
45-74	3637.8	3637.8	1
61-73	1556.8	1556.8	1.1
61-74	1684.9	1684.9	-8.4
61-75	1812.9	1813.0	-33.2
144-150	818.4	818.4	-0.1
144-155	1477.8	1477.8	1
151-155	677.4	677.3	3.4
132-135	585.3	585.3	3
132-141	1288.7	1288.7	0.5
132-143	1517.8	1517.8	-1.4
136-141	721.4	721.4	3.3
76-82	982.5	982.5	-0.1
75-82	1110.6	1110.6	2.1
74-76	402.3	402.3	6.2
74-82	1238.7	1238.7	4.8
126-131	730.4	730.4	2
1-16	1698.8	1698.8	0.7
1-45	4883.5	4883.5	6.7
1-47	5255.7	5255.7	0.1
1-51	5723.9	5723.9	5
1-73	8375.2	8375.2	-4.3
1-75	8631.5	8631.4	9.1
52-60	1130.6	1130.6	-0.6
52-63	1502.7	1502.7	0.9
52-73	2669.3	2669.3	-2.6
159-185	3084.5	3084.5	4.8
100-110	1254.7	1254.7	0.1
100-125	3020.5	3020.5	3.1
100-126	3148.6	3148.6	2.8
100-131	3732.9	3732.9	-3.5
100-141	5003.6	5003.6	2
83-99	1790.9	1790.9	-0.8

142-150	1047.6	1047.6	1.7
142-155	1706.9	1706.9	0
17-31	1789.9	1789.9	-0.5
17-44	3202.7	3202.7	2
17-51	4043.1	4043.1	-0.7
17-63	5527.8	5527.8	4.1
17-73	6694.4	6694.4	1.7
127-131	602.3	602.3	1.4
127-141	1873.0	1873.0	14.4
111-125	1783.9	1783.9	-0.5
111-126	1912.0	1912.0	0
32-44	1430.8	1430.8	0.3
32-73	4922.6	4922.5	9.8

S5: A) MS/MS of peptide 126-131 KVSQNR (624.2, 2+). The b ions are the fragment ions from N-terminal and y ions are the fragment ions from C-terminal. The b2 and b3 ions were observed to have one AF488 attachment and this confirmed that the AF488 was labeled on K126. B) MS/MS on peptide 132-141 EYFKITLYGR (903.4, 2+). The b4, b5, b7 and y8 were observed to have one AF488 attachment and this confirmed that the AF488 was labeled on K135.





S6: Structure of A: AlexaFluor488 and B: Cy3