Complement Activation and Cell Uptake Responses toward Polymer-Functionalized Protein Nanocapsules

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SUPPORTING INFORMATION

Materials and Methods

Construction of mutants. To perform site-directed mutagenesis, we cloned the E2-WT gene into pGEM-3Z (Promega) and followed a modified version of the Stratagene QuickChange protocol. The starting DNA template encodes the wild-type E2 scaffold (E2-WT) in a pGEM vector and includes amino acids 174-427, as described previously.[1] We used oligonucleotides with the sequence listed in Table S-1 as the primers for mutagenesis. Sequences listed in Table S-1 are forward oligonucleotides; the reverse sequences used are complementary to the forward primers listed here.

Table S-1. Sequences of forward oligonucleotides used for mutagenesis. Codons in bold denote mutated sites.

Mutant	Oligonucleotide sequences
T219C	5'– GCC ATG GTT CAC TCT AAA CAC **TGC** GCG CCA CAC GTT ACC CTG ATG – 3'
E279C	5'– CTG AAC ACC TCT ATT GAC GAC **TGC** ACC GAA GAA ATC ATC CAG – 3'
D278C	5'– GTT CTG AAC ACC TCT ATT GAC **TGC** GAG ACC GAA GAA ATC ATC CAG – 3'
T334C	5'– GCT CGT GAC GGT AAA CTG **TGC** CCT GGT GAA ATG AAA GGC – 3'
M338C	5'– C GGT AAA CTG ACT CCT GGT GAA **TGC** AAA GGC GCG TCT TGC – 3'

The resulting clones were screened and sequenced, and the mutated E2 genes were ligated into the expression vector pET-11a at the *Nde*I and *Bam*HI sites.

Functionalization with dye molecules. Reactivity of the engineered Cys thiols with small molecules was quantified by conjugating AlexaFluor 532 C5-maleimide (AF532M) to the surface-accessible side chains. Conjugation and analysis was performed according to protocol presented in Dalmau et al.[1]. Purified E279C (1.4 mg/ml) and E2-WT (1.3 mg/ml) were reduced with 8.5 molar excess of TCEP and reacted with AF532M at a 3:1 (dye:protein subunit) molar ratio. Unreacted dye was removed using drop dialysis ("V" series membrane, Millipore).

To label primary amines, proteins in phosphate buffer were reacted with AlexaFluor 488 succinimidyl ester (AF488) at a 3:1 and 4:1 (dye:protein subunit) for E2-WT and PEG-conjugated E279C, respectively. These ratios were chosen to yield an equimolar conjugation ratio of dye-to-subunit, enabling direct comparison in subsequent uptake assays. Samples were agitated in the dark at room temperature for 1.5 hours. Excess dye was removed using desalting columns (Zeba, 40 kDa MWCO), with phosphate buffer as the exchange solution. Dye conjugation was quantified by measuring absorbance at 495 nm and calibrated with a standard curve. Protein concentration was determined using micro-BCA, allowing for dye:protein subunit ratio quantification (average ± standard deviation for at least 3 batches).

Analysis of complement assay data. Analysis was performed as previously described.[2] Raw data from absorbance (Abs) measurements taken at 405 nm was converted to red blood cell % lysis using the equation

$$\%\ Lysis = \frac{Abs_{Sample} - Abs_{DVGB\ buffer\ control}}{Abs_{water\ control} - Abs_{DVGB\ buffer\ control}}$$

Next, the lysis data was normalized to a z-value using the equation

$$Z - value = -\ln(1 - \%\, lysis)$$

and z-values between 0.2 and 2 (the linear range on a graph of % lysis versus normal human serum dilution) were plotted again serum dilution on a log-log plot. The slope of the z-value data was used to determine the serum dilution at which z-value is equal to 1 (i.e. the point at which 50% cell lysis occurs), and from this dilution the percent C4 remaining protein consumption relative to the phosphate buffer was determined using the equation

$$\%\, C4\, remaining = 100 \times \frac{Dilution_{sample\ when\ z-value\ is\ 1}}{Dilution_{phosphate\ buffer\ when\ z-value\ is\ 1}}$$

Results & Discussion

Labeling PEGylated particles with AF 488. DLS analysis confirmed intact PEG1200-E279C protein particles conjugated with AF488, with an average particle size of 37.8 ± 2.2 nm. Using our labeling conditions, quantification of labeling revealed dye:protein subunit ratios of 1.0 ± 0.2, 1.1 ± 0.2, 1.4 ± 0.1, and 1.3 ± 0.5 for E2-WT, PEG1200-E279C, PEG2000-E279C, and PEG5000-E279C, respectively.

Summary of particle characterization assays. The E279C nanocapsule's chemical and physical properties were characterized before and after conjugation with PEG1200-maleimide, the results of which are summarized in Table S-2. The molecular weight of E279C and PEG1200-E279C was determined using electrospray ionization mass spectrometry (Figures S-1 and S-2), and comparison with the theoretical molecular

weights (28091 and 29330 Da theoretical for E279C and PEG1200-E279C, respectively) confirmed correct amino acid structure and successful PEGylation. Accessibility of the E279C's engineered cysteines was determined by conjugation with AF532M. Accessibility to the E279C's surface lysines was confirmed by reaction with AF488.

	E279C	**PEG1200-E279C**
Single Protein Subunit Mass (Da)	28091 ± 1	29330 ± 1
Average Particle Diameter (nm)	28.0 ± 0.8	37.3 ± 3.6
Particle Diameter at Histogram Peak Height (nm)	24-28	28-33
Molar Ellipticity Minima (nm)	208, 222	208, 222
Midpoint of Unfolding Temperature (°C)	89.5 ± 1.9	89.2 ± 1.3
Accessible Cysteines (AF532/E2)	58.3 ± 12.5	N/A
Endotoxin Level (ng/ml)	< 120	< 120

Table S-2. Summary of characterization of the PEGylated (PEG1200-E279C) and non-PEGylated (E279C) protein nanocapsules.

Particle size was determined using dynamic light scattering (DLS) and representative graphs are shown for E279C (Figure 3a) and PEG1200-E279C (Figure 4a), with additional confirmation of particle size through transmission electron microscopy (Figure 4c). Average particle diameter and diameter at peak height of the DLS histograms were recorded. The structural properties were analyzed with circular dichroism by performing an ellipticity wavelength scan (reported here are spectral minima for E279C and PEG1200-E279C) and a thermostability scan at the global minima (222 nm) to determine the melting temperatures. E279C and PEG1200-E279C exhibit the high thermostability profile that E2-WT displays (Figures 3c and 4b).

Endotoxin levels in our protein stocks used for *in vitro* studies were less than 120 ng/ml, as determined from the LAL gel clot assay. At concentrations used in the

complement assays, this is approximately 80 times lower than reported values of LPS that activate human complement *in vitro* (~5,000-10,000 ng/ml).[3]

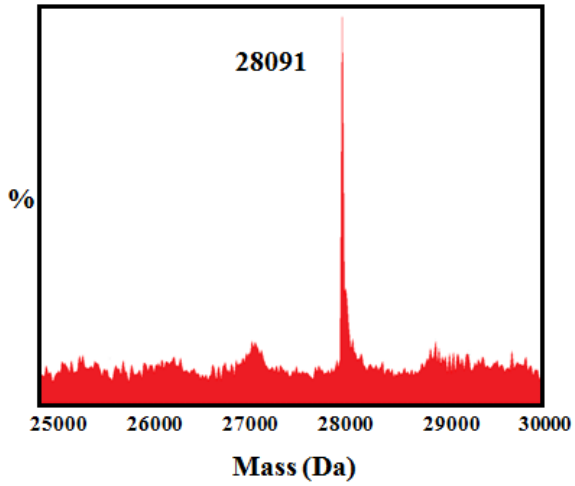

Figure S-1. Electrospray ionization mass spectrometry analysis of E279C mutantexhibits a single peak at 28091 Da (theoretical = 28091 Da).

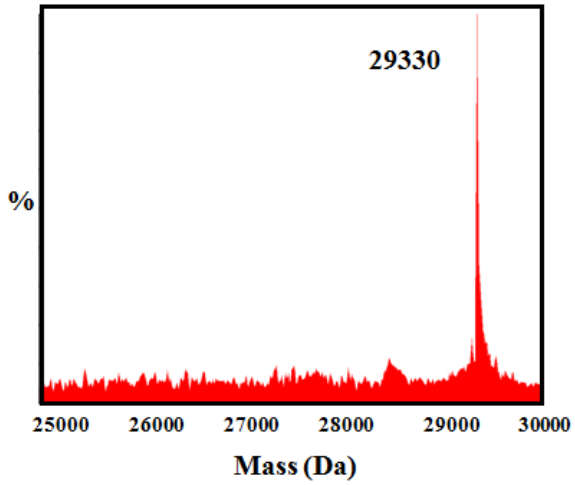

Figure S-2. Electrospray ionization mass spectrometry analysis of PEG1200-E279C exhibits a single peak at 29330 Da (theoretical = 29330 Da). The lack of a secondary peak at 28091 Da reveals no detectable unreacted E279C and indicates incorporation of a single PEG1200 chain on a single E279C subunit. ESI-MS analysis for PEG2000-E279C and PEG5000-E279C could not obtain a single observable peak; the commercial source reports a range of molecular weights within 10% of 2000 Da and 5000 Da for PEG2000-maleimide and PEG5000-maleimide, respectively.

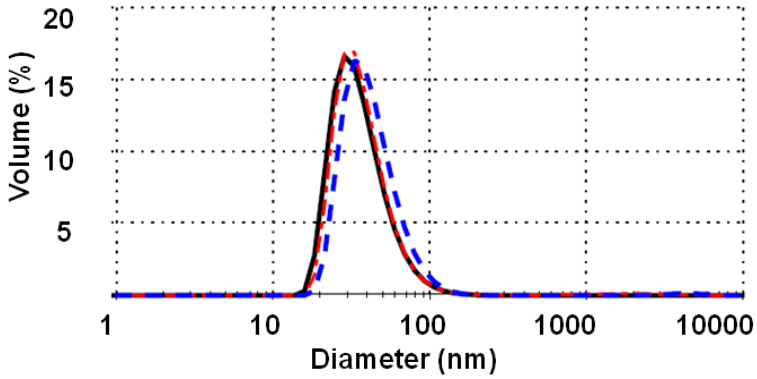

Figure S-3. DLS size measurement of E279C and PEGylated E279C mutants. DLS showing representative size measurements of PEG1200-E279C (37.3 ± 3.6 nm, black line), PEG2000-E279C (41.6 ± 6.4 nm, red dashes) and PEG5000-E279C (41.4 ± 4.6 nm, blue dashes).

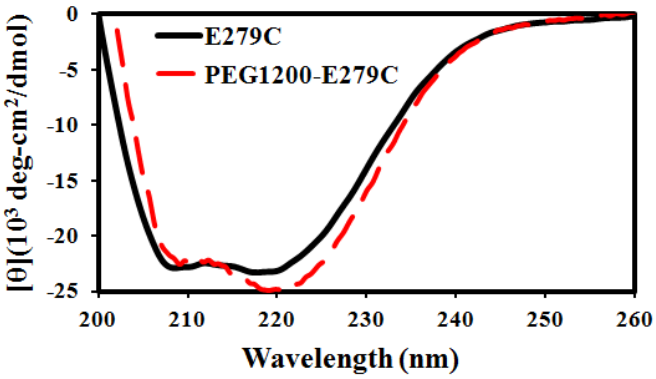

Figure S-4. Molar ellipticity versus wavelength shows characteristic minima at 208 and 222 nm for E279C and PEG1200-E279C. PEGylation of the E279C mutant does not disrupt the characteristic high alpha helical structure.

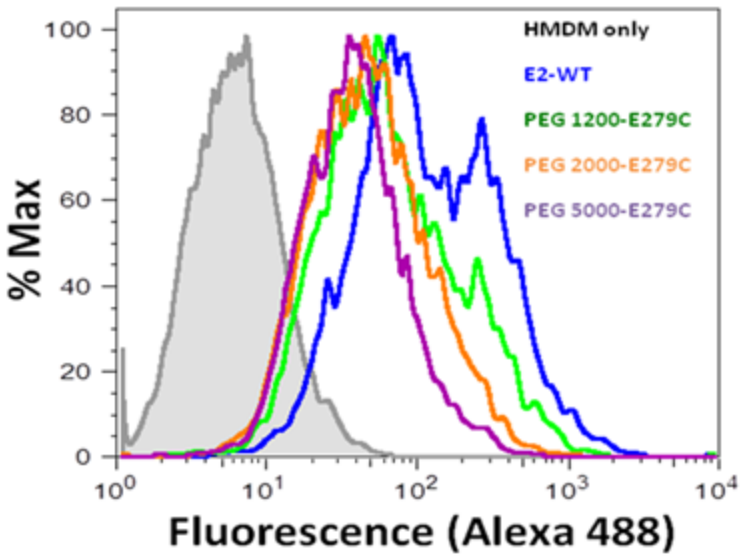

Figure S-5. Representative histogram of fluorescence intensity of human macrophages incubated with 10 mg/ml Alexa488-labeled PEGylated and non-functionalized E2 proteins. Measured by flow cytometry.

References

(1) Dalmau, M.; Lim, S.; Chen, H. C.; Ruiz, C.; Wang, S. W., *Biotechnol Bioeng* **2008,** 101, (4), 654-64.

(2) Webster, S. D.; Tenner, A. J.; Poulos, T. L.; Cribbs, D. H., *Neurobiol Aging* **1999,** 20, (3), 297-304.

(3) Kaca, W.; Roth, R., *Biochimica Et Biophysica Acta-General Subjects* **1995,** 1245, (1), 49-56.