Supporting Information:

Charge Group Surface Accessibility Determines Micelleplexes Formation, Stability and Cellular Interaction

*Yu Zhang[a], Yang Liu[b], Soumyo Sen[c], Petr Král[c,d], and Richard A. Gemeinhart[a,e,f]**

Departments of Biopharmaceutical Sciences[a], Medicinal Chemistry and Pharmacognosy[b], Chemistry[c], Physics[d], Bioengineering[e], and Ophthalmology and Visual Sciences[f], University of Illinois, Chicago, IL 60612, USA.

*Correspondence to: Richard A. Gemeinhart, 833 South Wood Street (MC865), Chicago, IL 60612-7231, USA. Email: rag@uic.edu

Materials

Carboxyl terminal-mPEG2000-PLA3000 was purchased from Advanced Polymer Materials (Montreal, Canada). Ac-CR_8-NH_2 and Ac-CR_{15}-NH_2 peptides were synthesized by VCPBIO (Shenzhen, China). Heparin sulfate, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Aldrich. 3-(2-pyridyldithio) proprionyl hydrazide (PDPH) crosslinker was obtained from Fisher. Single stranded anti-miRNA sequence 5'-AUCGAAUAGUCUGACUACAACU-3' was synthesized by Integrated DNA Technologies (IDT) with standard RNA chemistry. Cy-5 labeled single stranded anti-miRNA was from Invitrogen as previously reported.[1]

Polymer conjugate synthesis

Carboxyl terminal-mPEG2000-PLA3000, EDC and NHS (100, 40 and 24 mg, respectively) was dissolved in DMSO and stirred for 30 min, after which 14 mg PDPH crosslinker was added, thus modifying mPEG2000-PLA3000 with a pyridyldisulfide functional group at the previous carboxyl termini. Unreacted reagents were removed by dialysis against deionized water for 24 h with a molecular weight cut off of 1000 dialysis membrane. The sample was recovered from the dialysis tube and lyophilized to a white powder. Successful PDPH modification was confirmed with ^{1}H NMR in $CDCl_3$ using a 400 MHz Bruker DPX-400 spectrometer (Bruker BioSpin Corp., Billerica, MA). To couple mPEG-PLA-PDPH with peptide, 40 mg of mPEG-PLA-PDPH and 10 mg CR_{15} or 5.6 mg CR_8 (1:1 molar ratio) was dissolved in argon degased DMSO and reacted for 6 hours in argon environment before purification with a Sep-Pak C_{18} column. Successful conjugation was confirmed by ^{1}H NMR (**Figure S1**) in DMSO-d_6.[2] The R_x conjugation rate was calculated based on the intensity ratio between the proton peak of the guanidine group (δ 7.43, 4H) and that of the PLA segment (δ 5.19, 1H). The conjugation rate was controlled by varying functional group molar ratios. For example, a conjugation rate of approximately 28% (relative to total polymer) was achieved when the molar ratio of pyridyldisulfide group to sulfhydryl group in peptide was 1:1, and a 14% conjugation rate was achieved with a molar ratio of 2:1.

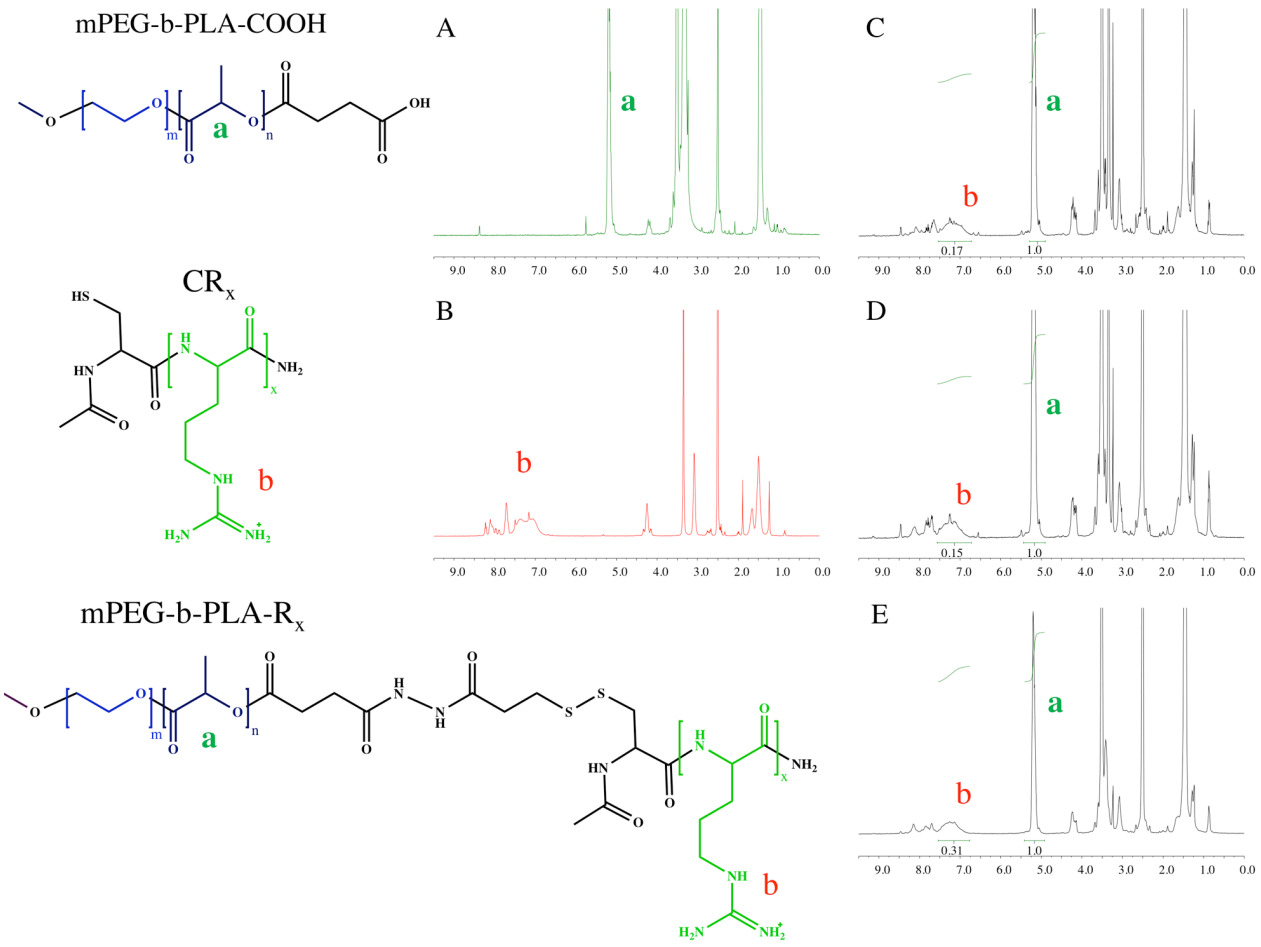

Figure S1. ^{1}H NMR spectra of (A) mPEG-PLA-COOH (B) CR_x (C) mPEG-PLA-R_8 (D) mPEG-PLA-R_{15}^{Low} (E) mPEG-PLA-R_{15}^{High}. The purified products have characteristic peaks from both PLA segment (a, δ 5.19, 1H) and guanidine group (b, δ 7.43, 4H) in R_x.

Micelleplex preparation

Micelles were prepared by the film hydration and sonication method as previously reported.[3] Briefly, 10 mg of copolymer was dissolved in 0.5 mL acetonitrile. Solvent was removed by rotovap to form a polymer matrix. RNAse free water (0.5 mL) was added to rehydrate the polymer film followed by sonication for 5 min to form empty micelles. To prepare micelleplexes, micelles were diluted with RNAse free water to the appropriate concentration and added dropwise to RNA solution (10 μM) and incubated for 15 min at room temperature.

Critical micelle concentration

Critical micelle concentration (CMC) was determined by using a pyrene fluorescence probe as previously reported.[4] Briefly, 100 μL of 1.2 $\times 10^{-5}$ M pyrene in acetone was added to a series of amber vials and evaporated in fume hood overnight to form a pyrene film. The final concentration of pyrene upon addition of 2 mL of polymer solution was 6×10^{-7} M. Different copolymer micelle solutions in water (0.1 – 500 mg/L) were added to vials containing pyrene film. The solutions were incubated at room temperature for 24 h on a shaker before fluorescence measurement. The emission wavelength was set at 390 nm and the excitation spectrum was scanned from 300 nm to 400 nm using a spectrofluorophotometer (RF 1501, Shimadzu, Japan) and the intensity ratio I335/I333 against log concentration was plotted. The CMC value was determined from the onset of the change in intensity ratio at high polymer concentrations as previously reported.[5]

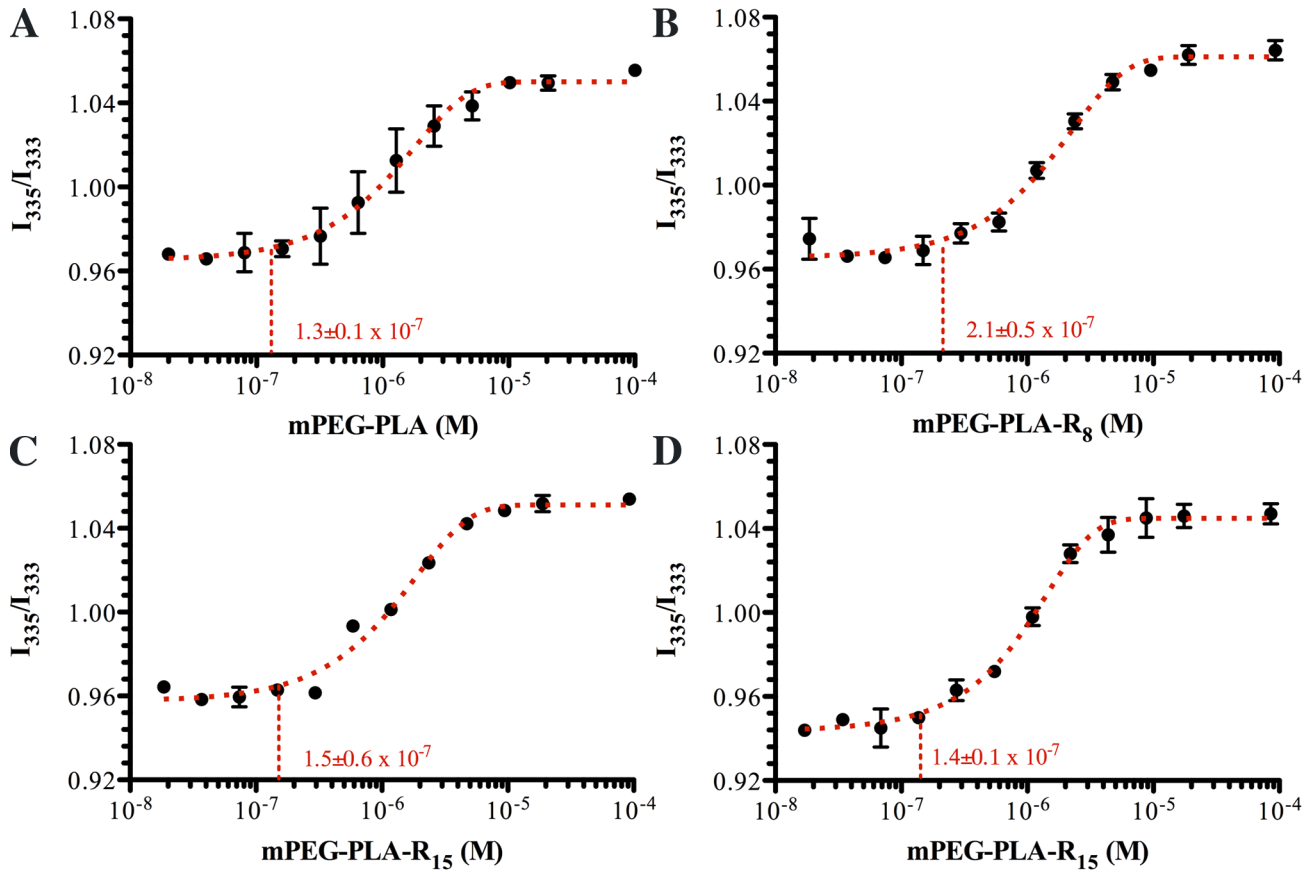

Figure S2. mPEG-PLA-R_x copolymers have CMC similar to mPEG-PLA. CMC plots of (A) mPEG-PLA (B) mPEG-PLA-R_8 (C) mPEG-PLA-R_{15}^{Low} and (D) mPEG-PLA-R_{15}^{High}. Data represent mean ± standard deviation, N=3.

Particle sizing and ζ-potential

Particle size and ζ-potential were measured in RNAse free water to obtain the parameters before RNA complexation (**Table S1**), using a Nicomp 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). The data represent the mean plus or minus (±) the standard deviation (SD) from three independent experiments.

Table S1. Micelle particle size and ζ-potential.

	Peptide conjugation (mol%)	Micelle size (nm)	Micelle ζ-potential (mV)
mPEG-PLA-R_8	27%	20.13±0.42	30.76±1.16
mPEG-PLA-R_{15}^{Low}	14%	25.00±1.51	19.65±4.7
mPEG-PLA-R_{15}^{High}	28%	20.43±1.50	30.46±1.88

To mimic a physiologically relevant conditions as previously reported, [6] the particle size and ζ-potential of micelleplexes were measured in 10 mM HEPES buffer (pH 7.4) following RNA complexation. (**Table S2**) The data represent mean plus or minus (±) the standard deviation (SD) from three independent experiments.

Table S2. Micelleplexes particle size and ζ-potential.

	Peptide conjugation (mol%)	Micelleplex size (nm)	Micelleplex ζ-potential (mV)
mPEG-PLA-R_8	27%	21.6±1.25	0.93±0.96
mPEG-PLA-R_{15}^{Low}	14%	22.37±4.50	6.01±3.57
mPEG-PLA-R_{15}^{High}	28%	19.9±2.98	4.95±0.43

Morphology characterization

The morphology of micelles and micelleplexes were analyzed by transmission electron microscopy (TEM, JEM-1220, JEOL Ltd., Japan). A drop of micelles (1 mg/mL) was placed on a carbon-coated 300 mesh copper grid. The sample was negatively stained with 2% (w/v) uranyl acetate solution, and then dried at room temperature.

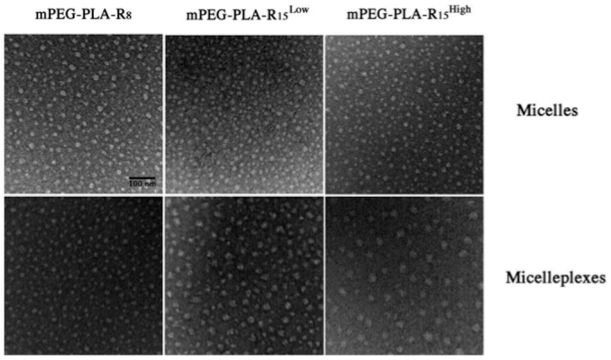

Figure S3. TEM images of mPEG-PLA-R_8, mPEG-PLA-R_{15}^{Low} and mPEG-PLA-R_{15}^{High} micelles and micelleplexes (+/- charge ratio 30).

Gel shift

The successful formation of micelleplexes was confirmed by a gel shift assay. Micelleplexes were prepared as described above at predetermined positive to negative (+/-) charge ratios. The resulting micelleplexes were analyzed by electrophoresis using a 20% non-denaturing polyacrylamide gel for 1 h at 80 V in TBE buffer (89 mM Tris-borate, 2 mM EDTA). Following SYBR gold staining, the gel was visualized using a gel documentation system (GelDoc 2000, Bio-Rad, Hercules, CA).

Heparin competition

With the RNA amount fixed, micelleplexes were prepared as described above at predetermined positive to negative (+/-) charge ratios. To compare the ability of different micelleplexes in resisting negatively charged macromolecule competition, appropriate amount of heparin solution (2 mg/mL) was added at 4:1 and 8:1 heparin to RNA weight ratios. The RNA was visualized by running the samples in 20% non-denaturing polyacrylamide gel as described in gel shift assay.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was carried out on a VP-ITC from Microcal (GE Healthcare Bio-Sciences, Milwaukee, WI, USA) with an active cell volume of 1.4 mL using a stirring rate of 307 rpm.[7] RNAse free water was degassed under vacuum for 10 min and equilibrated to room temperature before use. Measurements were performed at 25°C. The baseline was recorded by titrating an equivalent micelle concentration into water. All experiments were performed by titrating a 3 μL of micelles containing 4.0 mM arginines into the sample cell containing 5 μM anti-miRNA (0.11 mM phosphate). An initial data point from 2 μL first injection was always removed from data analysis to avoid systematic error from syringe filling. The following injections were maintained at 3 μL at intervals of 200 s until the anti-miRNA was saturated with micelles. Injection heat caused by anti-miRNA binding to micelles during each injection was obtained from the integral of the calorimetric signal after subtraction of the baseline. ITC data were analyzed with Microcal, LLC ITC package for Origin® version 7.0. Each experiment was repeated at least once to ensure reproducibility. The peaks were integrated and corrected with a single-site-binding model. The thermodynamic parameters binding sites, binding constant K, enthalpy (ΔH), entropy (ΔS) were reported (**Table S3**).

Table S3. Thermodynamic parameters for the binding between different micelles and anti-miRNA.

	N **(sites/arginine)**	***K*** **(x10^3)**	**Δ*H*** **(kcal/mol)**	**Δ*S*** **(cal/mol/deg)**	**Δ*G*** **(kcal/mol)**
mPEG-PLA-R$_8$	0.32±0.05	21.5±2.82	-7.74±1.48	-6.21	-5.88
mPEG-PLA-R$_{15}^{Low}$	0.41±0.22	18.8±9.53	-2.41±1.56	11.49	-5.83
mPEG-PLA-R$_{15}^{High}$	0.73±0.15	42.2±26.2	-0.40±0.11	19.83	-6.31

Cellular association

The cellular association was determined as we previously reported.[8] Briefly, 2×10^5 U251 cells were seeded in 12-well plates and incubated overnight. Micelleplexes were prepared at a charge ratio 30:1 with the final anti-miRNA concentration maintained constant at 100 nM. Micelleplexes were added to cells to make the total media volume 1 mL. Following addition of micelleplexes to cells, cells were incubated for an additional 4 h. Cells were then washed with cold PBS twice followed by trypsinization and centrifugation at 1500 rpm for 5 min. The pellets were then washed twice with cold PBS and centrifugation before resuspending in 200 μL of 1% formaldehyde and analyzing on Becton Dickinson Fortessa flow cytometer (San Jose, CA) at excitation 543 nm and emission 570 nm.

Micelle Modeling

The micelle structure was modeled by using an atomistic molecular dynamics (MD) simulation of mPEG-PLA-R$_8$ mixed with mPEG-PLA copolymers in water with different aggregation numbers, N_{agg}, as previously described.[4] Briefly, a molecular dynamics (MD) simulation of the micelle with NAMD[9] and CHARMM forcefield (CHARMM general forcefield [10, 11] and CHARMM protein forcefield[12]). Micelles were spherically distributed to form the basic micelle structure. Then, the micelle was compacted by a brief simulation in vacuum while applying force (25 pN) on the PLA of the micelle. Then, micelle structure was simulated in ionic solution (concentration 0.15 M) for 50 ns at a temperature of 300K and pressure of 1 atm. Nonbonding interactions were calculated using cut-off distance of 1 nm and long-range electrostatic interactions calculated by the PME[13] method in the presence of the periodic boundary condition. The systems were simulated with NPT ensemble, using the Langevin dynamics with a damping constant of 0.1 ps^{-1} and the timestep of 2 fs.

REFERENCES

1. Y. Zhang, M. Köllmer, J. S. Buhrman, M. Y. Tang and R. A. Gemeinhart, *Peptides*, 2014, **58**, 83-90.
2. Z. X. Zhao, S. Y. Gao, J. C. Wang, C. J. Chen, E. Y. Zhao, W. J. Hou, Q. Feng, L. Y. Gao, X. Y. Liu, L. R. Zhang and Q. Zhang, *Biomaterials*, 2012, **33**, 6793-6807.
3. E. Blanco, E. A. Bey, Y. Dong, B. D. Weinberg, D. M. Sutton, D. A. Boothman and J. Gao, *J. Control. Release*, 2007, **122**, 365-374.
4. J. W. Bae, R. M. Pearson, N. Patra, S. Sunoqrot, L. Vukovic, P. Kral and S. Hong, *Chem. Commun.*, 2011, **47**, 10302-10304.
5. J. Aguiar, P. Carpena, J. A. Molina-Bolivar and C. Carnero Ruiz, *J. Colloid Interface Sci.*, 2003, **258**, 116-122.
6. D. Gutsch, D. Appelhans, S. Hobel, B. Voit and A. Aigner, *Mol. Pharmaceutics*, 2013, **10**, 4666-4675.
7. M. Zheng, G. M. Pavan, M. Neeb, A. K. Schaper, A. Danani, G. Klebe, O. M. Merkel and T. Kissel, *ACS Nano*, 2012, **6**, 9447-9454.
8. Y. Zhang, M. Kollmer, J. S. Buhrman, M. Y. Tang and R. A. Gemeinhart, *Peptides*, 2014, **58**, 83-90.
9. J. C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kale and K. Schulten, *Journal of computational chemistry*, 2005, **26**, 1781-1802.
10. K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes, I. Vorobyov and A. D. Mackerell, Jr., *Journal of computational chemistry*, 2010, **31**, 671-690.
11. W. Yu, X. He, K. Vanommeslaeghe and A. D. MacKerell, Jr., *Journal of computational chemistry*, 2012, **33**, 2451-2468.
12. A. D. Mackerell, Jr., M. Feig and C. L. Brooks, 3rd, *Journal of computational chemistry*, 2004, **25**, 1400-1415.
13. T. A. Darden, D. M. York and L. G. Pedersen, 1993, **98**, 10089-10092.