Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Nanoparticle Cellular Uptake by Dendritic Wedge Peptides: Achieving Single Peptide Facilitated Delivery

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Peptide Dendrimer Synthesis.

Abbreviations.

AO	Amino-oxy
Boc	*t*-Butyloxycarbonyl
CHO	Formyl
DCM	Dichloromethane
DIEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylformamide
Fmoc	9-Fluorenylmethyloxycarbonyl
HCTU	O-(1H-6- chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
RP-HPLC	Reversed-phase high-performance liquid chromatography
MBHA	4-Methylbenzhydrylamine
MS	Mass spectrometry
MW	Molecular weight
r.t.	Room temperature
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid

Materials.

N,N-Dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (CH_3CN) and N,N-diisopropylethylamine (DIEA) were peptide synthesis grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). 4-Methylbenzhydrylamine (MBHA) resin and O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased from Peptides International (Louisville, K, USA) and trifluoroacetic acid (TFA) from NuGen Tec (Emerville, CA, USA). Boc-amino-hexanoic acid (Boc-6-Ahx-OH / Boc-hex) was obtained from Sigma Aldrich (St. Louis, MO, USA). Standard Boc-amino acids for SPPS were purchased from CS Bio (Menlo Park, CA, USA). Amicon® Ultra 4 mL Centrifugal Filter Unit with a 3 kDa cut-off membrane, Amicon® Ultra 15 mL Centrifugal Filter Unit with a 10 kDa cut-off membrane, Sartorius™ Vivaspin™ 6 Centrifugal Concentrators (6 mL) with a 3 kDa and a 5 kDa cut-off membrane and Sartorius™ Vivaspin™ 15R Centrifugal Concentrators (15 mL) with a 5 kDa and a 10 kDa cut-off membrane were purchased from Fisher Scientific and used for peptide dendrimer purification according to supplier's guidelines.

Analytical reversed-phase high performance liquid chromatography (RP-HPLC, preparative RP-HPLC, and mass spectrometry (MS) analysis.

Peptides were analyzed by analytical RP-HPLC (Phenomenex Jupiter C_{18} column, 90 Å, 4 μm, 150 mm x 4.6 mm) using a linear gradient of 0-60% B (solvent A, H_2O / 0.05% TFA; solvent B, 90% CH_3CN / 10% H_2O/0.043% TFA) in 60 min at 1 mL/min while monitoring UV absorbance at 214 nm. MS analysis was carried out on an API III and API 2000 from AB Sciex (Framingham, MA, USA) and high resolution mass of the final products were determined on a Sciex X500R QTOF system. Peptide purification was carried out by preparative RP-HPLC (Vydac C_{18} column, 300 Å, 10 μm, 250 mm x 21.2 mm) using a linear gradient of 0–60% B over 60 min at 8 mL/min while monitoring UV absorbance at 214 nm.

Chemical Synthesis and Peptide Dendrimer Assembly

Synthesis of poly-arginine binding motif by Boc-SPPS

Amino-oxy-GFR_9* (*...C-terminal amide) was assembled on a CS Bio CS336X peptide synthesizer using Boc chemistry and the in situ neutralization protocol.[1] Boc-SPPS was performed at 0.2 mmol scale on a MBHA resin with 1.0 mmol amino acid (5-fold excess) activated with 1 eq 0.4 M HCTU in DMF and 1.5 eq 1 M DIEA (*N*,*N*-diisopropylethylamine) in DMF. Removal of the N-terminal Boc group was carried out with neat TFA (2x 1 min) followed by a 1 min flow-wash with DMF and a 1 min flow-wash with DCM/MeOH before the resin was dried under N_2 atmosphere. Deprotection of the side-chain groups and release of peptide from the resin was carried out by standard HF cleavage and workup conditions.[2] The crude lyophilized peptide was purified by preparative RP-HPLC. [M+H] predicted: 1700.04 m/z; [M+H] observed: 1700.58 m/z.

Synthesis of the dendritic QD coordination scaffold by Boc-SPPS

The dendritic assembly scaffold 1x S-hex-hex-$WP_9G_2H_6$*, 2x (S-hex-$P_9G)_2$-K'WGH_6*, 4x $S_4K'_2K'WP_9G_2H_6$*, 8x $S_8K'_4K'_2K'WP_9G_2H_6$* and 16x $S_{16}K'_8K'_4K'_2K'WP_9G_2H_6$* (1-16x indicating multivalence, K' indicating branched lysine residues; excess of Boc-K'(Boc) and Boc-S(Bzl) amino acids were adjusted to the degree of branching) were assembled on a CS Bio CS336X peptide synthesizer using Boc-SPPS as described above. Trp, Pro and His residues were double coupled. Boc deprotection was carried out with neat TFA for 2x 5 min. After complete assembly, deprotection of the 2,4-dinitrophenyl (Dnp) group of histidine was carried out manually by treatment with 20% mercaptoethanol, 10% DIEA and DMF (2x 30 min) followed by a 1 min flow-wash with DMF. Removal of the N-terminal Boc group was carried out with neat TFA (2x 1 min) followed by a 1 min flow-wash with DMF. Removal of the formyl (CHO) group of Trp was carried out with a 5% (v/v) ethanolamine, 5% (v/v) water in DMF solution (2x 30 min) followed by a 1 min flow-wash with DMF and a 1 min flow-wash with DCM/MeOH before the resin was dried under N_2 atmosphere. Deprotection of the other side-chain groups and release of peptide from the

resin was carried out by standard HF cleavage and workup conditions.[2] The crude lyophilized peptides were purified by preparative RP-HPLC.

Valency	Sequence	$[M+H]_{predicted}$	$[M+H]_{observed}$	MW
1x	S-hex-hex-$WP_9G_2H_6$*	2328.18	2328.0	2327.65
2x	$(S\text{-hex-}P_9G)_2$-K'WGH_6*	3473.80	3473.5	3474.01
4x	$S_4K'_2K'WP_9G_2H_6$*	2747.39	2748.0	2747.09
8x	$S_8K'_4K'_2K'WP_9G_2H_6$*	3607.90	3608.0	3608.10
16x	$S_{16}K'_8K'_4K'_2K'WP_9G_2H_6$*	5327.91	5329.5	5328.11

**...C-terminal amide; K'...branched lysine; hex ... hexane spacer*

Conversion of N-terminal serine residues to aldehydes by $NaIO_4$ oxidation

The N-terminal serine residues of the dendritic assembly ligands were converted to N-terminal aldehydes with 1.2 eq of $NaIO_4$ per serine residue: 10 mL of an aqueous 5 mM dendritic assembly ligand solution was mixed with 1 mL for 1x, 2 mL for 2x, 4 mL for 4x, 8 mL for 8x and 16 mL for 16x serine multivalence of a 60 mM $NaIO_4$, 100 mM sodium acetate buffer solution at pH 4.5 that was degassed for 10 min with N_2 prior to addition. The reaction mixture was topped up with more N_2-degassed 100 mM sodium acetate solution at pH 4.5 to a total reaction volume of 30 mL. The reaction mixture was stirred at r.t. for 30 min and was then directly purified by preparative RP-HPLC (reaction can be quenched if needed with $Na_2S_2O_3$).

Valency	Sequence	$[M+H]_{predicted}$	$[M+H]_{observed}$	MW
1x	[CHO]-hex-hex-$WP_9G_2H_6$*	2297.14	2297.0	2296.59
2x	[CHO-hex-$P_9G]_2$-K'WGH_6*	3411.72	3412.0	3411.89
4x	$[CHO]_4K'_2K'WP_9G_2H_6$*	2623.22	2623.5	2622.86
8x	$[CHO]_8K'_4K'_2K'WP_9G_2H_6$*	3359.56	3359.5	3359.64
16x	$[CHO]_{16}K'_8K'_4K'_2K'WP_9G_2H_6$*	4833.25	4832.5	4833.20

**...C-terminal amide; K'...branched lysine*

Assembly of the multivalent poly$(Arg_9)_{1\text{-}16}$ ligand series by chemoselective oxime ligation

The amino-oxy-GFR_9* binding motif was ligated in 1.5 eq excess to the N-terminal aldehyde groups of the dendritic assembly ligands: 100 µL of the 5 mM aqueous dendritic assembly ligand solutions (1x, 2x, 4x, 8x, 16x) were mixed with the 40 mM aqueous amino-oxy-GFR_9* solution (19 µL for 1x, 38 µL for 2x, 75 µL for 4x, 150 µL for 8x and 300 µL for 16x aldehyde multivalence) and the reaction mixture volume was doubled by addition of 100 mM anilinium

acetate buffer with a pH of 4.5. The reaction was left for 12 h at r.t. and monitored by analytical RP-HPLC and MS. The multivalent peptide dendrimers were purified through centrifugal size-exclusion membrane filters using a 3 kDa cut-off for the 2x poly(Arg_9)$_2$ ligand, a 5 kDa cut-off for the 4x poly(Arg_9)$_4$ ligand and a 10 kDa cut-off for the 8x and 16x poly(Arg_9)$_{8/16}$ dendrimers with two solvent A washes. The 1x monomeric ligand was purified by RP-HPLC. All purified ligands were lyophilized and characterized by analytical RP-HPLC and MS.

Valency	Sequence	Cut-off	$[M+H]_{predicted}$	$[M+H]_{observed}$	MW
1x	[R_9FG-x]-hex-hex-W$P_9G_2H_6$*	HPLC	3978.17	3978.08	3978.59
2x	[R_9FG-x-hex-P_9G]$_2$-K'WGH_6*	3 kDa	6774.79	6775.60	6775.89
4x	[R_9FG-x]$_4$-K'$_2$K'W$P_9G_2H_6$*	5 kDa	9350.36	9350.60	9350.85
8x	[R_9FG-x]$_8$-K'$_4$K'$_2$K'W$P_9G_2H_6$*	10 kDa	16811.84	16812	16815.62
16x	[R_9FG-x]$_{16}$-K'$_8$K'$_4$K'$_2$K'W$P_9G_2H_6$*	10 kDa	31768.54	n.o.[&]	31767.53

**...C-terminal amide; K'...branched lysine; x ... oxime bond; N/A ... not applicable; & n.o. ... not observed: with increasing size, branching generation and increasing numbers of positive charged arginine units (144 arginine residues for the 16x dendrimer) it becomes difficult to identify the correct detection parameters (i.e. ionization energy and declustering potential) to clearly detect the predicted masses as the mass detection is suppressed by poly(Arg)$_9$ fragments (i.e. 842 Da) during the ionization process. This is not the case with 16x non-poly(Arg)$_9$ dendrimers that have been synthesized as a control to verify quantitative ligation and the purification method suggesting it is a MS specific problem.*

Figure S1. Analytical RP-HPLC and MS data of purified 1x poly(Arg) monomeric ligand [R_9FG]-hex-hex-W$P_9G_2H_6$.

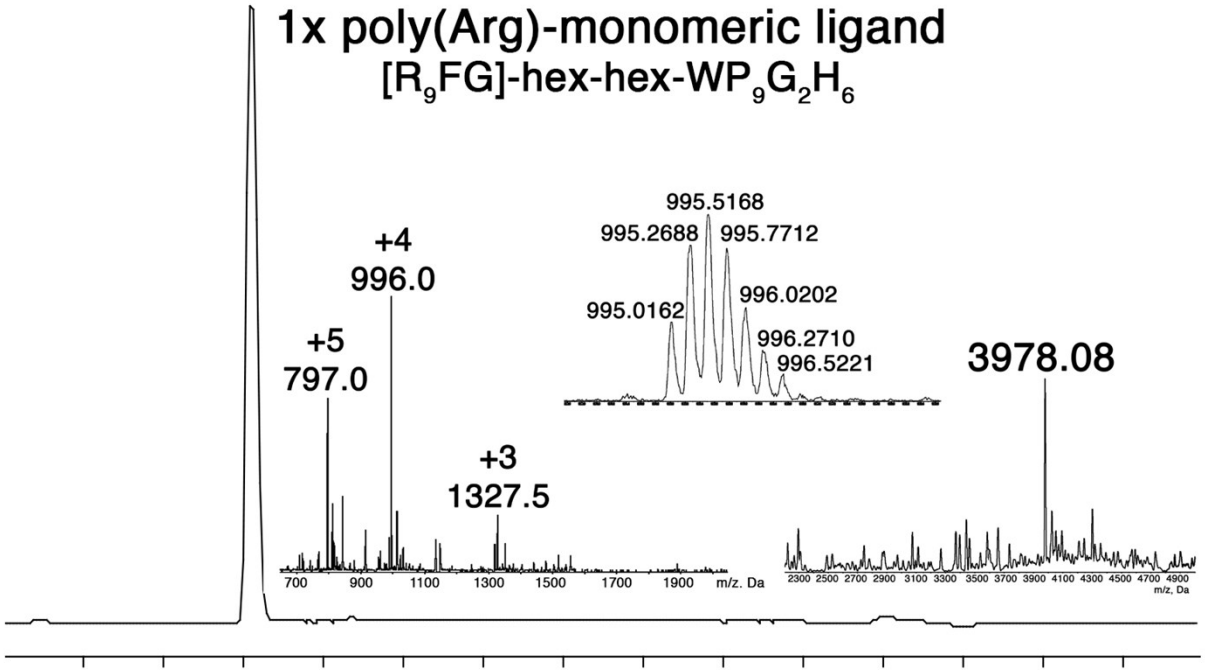


Figure S2. Analytical RP-HPLC and MS data of purified 2x poly(Arg) peptide dendrimer [R_9FG-hex-P_9G]$_2$-K'WGH_6.

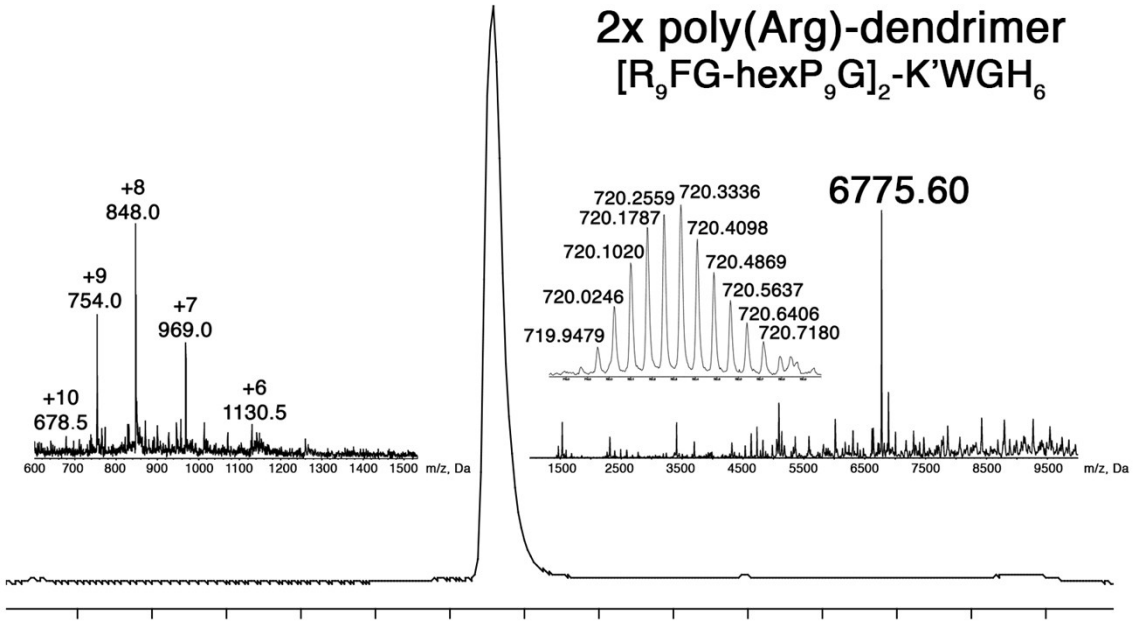


Figure S3. Analytical RP-HPLC traces and mass spectra for the assembly of the 4x poly(Arg) peptide dendrimer $[R_9FG]_4$-K'$_2$K'WP$_9$G$_2$H$_6$. A. Oxime ligation of the poly(Arg) binding motif equipped with an N-terminal amino-oxy (AO) group to react with the N-terminal aldehyde (CHO) residues of the dendritic QD coordination scaffold. Aniline catalyzed oxime ligation was complete after 3 h. The assembled peptide dendrimer was purified by centrifugal size exclusion with a 5 kDa membrane filter. **B.** The first three washes of the centrifugal size exclusion purification were monitored by analytical RP-HPLC showing that two washes were sufficient to remove excess binding motif and other impurities. **C** Mass spectrometry data showing the positive electrospray ionization series and the reconstructed monoisotopic mass observed. High resolution mass (zoom inserts) was run separately on a Sciex X500R QTOF to validate the predicted mass.

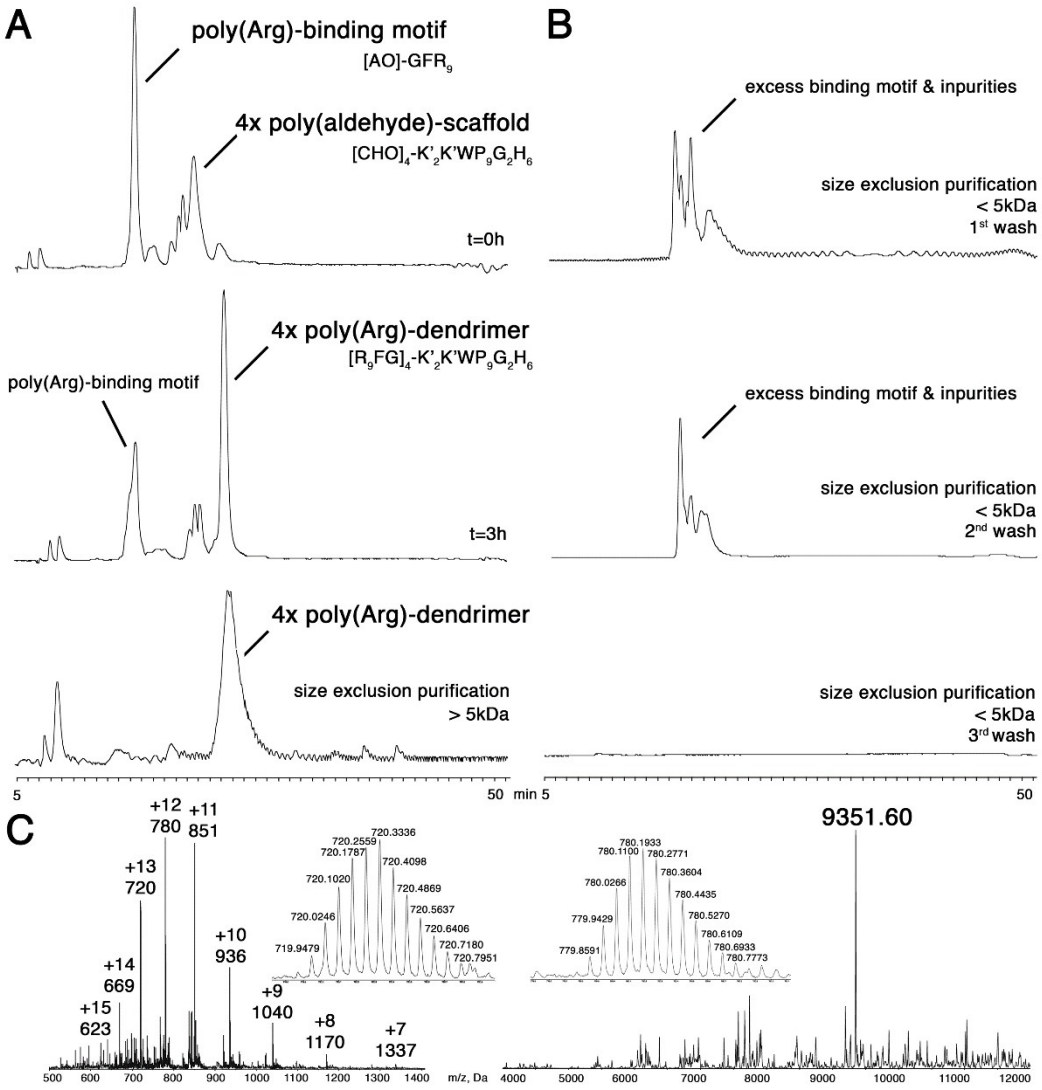


Figure S4. Analytical RP-HPLC traces and mass spectra for the assembly of the 8x poly(Arg) peptide dendrimer $[R_9FG]_8$-K'$_4$K'$_2$K'WP$_9$G$_2$H$_6$. A. Oxime ligation of the poly(Arg) binding motif equipped with an N-terminal amino-oxy (AO) group to react with the N-terminal aldehyde (CHO) residues of the dendritic QD coordination scaffold. The assembled peptide dendrimer was purified by centrifugal size exclusion with a membrane filter of 10 kDa. **B.** Analytical RP-HPLC of the purified peptide dendrimer. **C.** Mass spectrometry data showing the positive electrospray ionization series and the reconstructed monoisotopic mass observed.

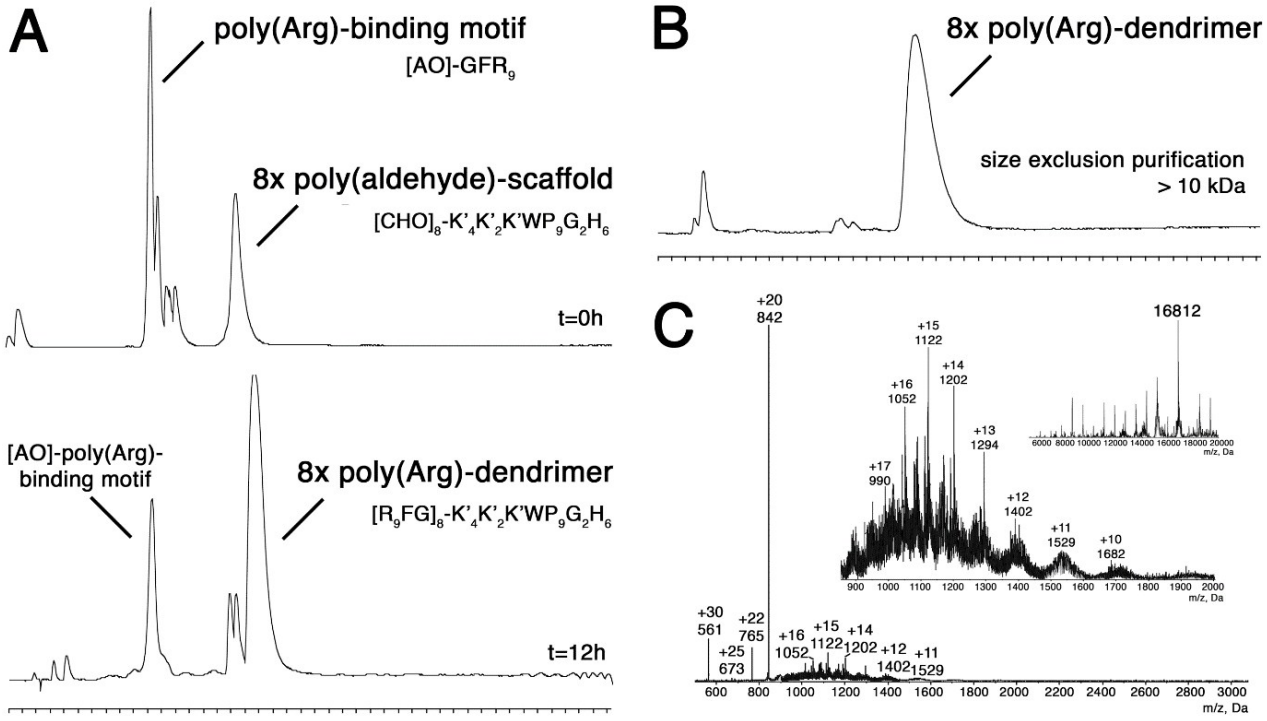


Figure S5. Analytical RP-HPLC trace of purified 16x poly(Arg) peptide dendrimer $[R_9FG\text{-}x]_{16}\text{-}K'_8K'_4K'_2K'WP_9G_2H_6$.

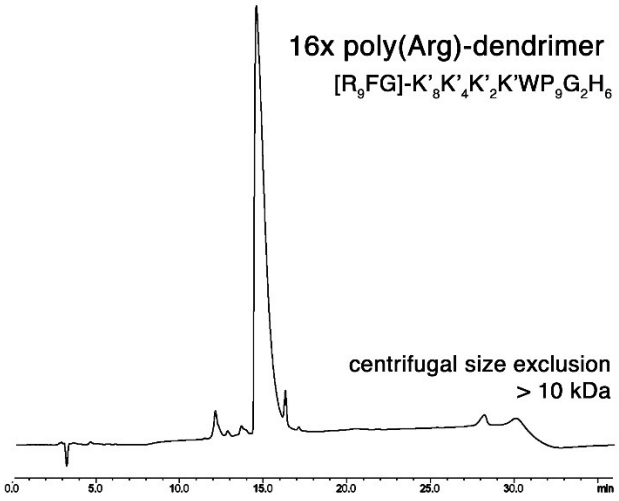


Figure S6. **Cellular Toxicity of High Ratio Dendritic Peptides to QDs or Free In Solution.** The ratio of dendritic peptide to QD was maintained at 25 or the equivalent free in solution for QD concentrations 3 to 200 nM and COS-1 cells exposed for 1 h or 24 hrs. Proliferation of COS-1 cells was measured 72 h after exposure. After 1 h exposure, dendritic peptides attached to QDs cause no noticeable decreases in cell viability while dendritic peptides with branch numbers above 4 to exhibit some cytotoxic affect at the highest concentration tested. After 24 h of exposure for both dendritic peptides attached to QD or free in solution there is a correlation between increasing branch number and cytotoxic effect at the highest concentration of QD or equivalent QD tested. Typical QD delivery concentration is 100 nM, where, at all the time points and ratios, no discernable cytotoxic effect was observed for any of the dendritic peptides attached to QD. Slight toxicity is seen with the highest dosages at the longest exposure times and this is not unexpected even for the bare QD only controls.[3]

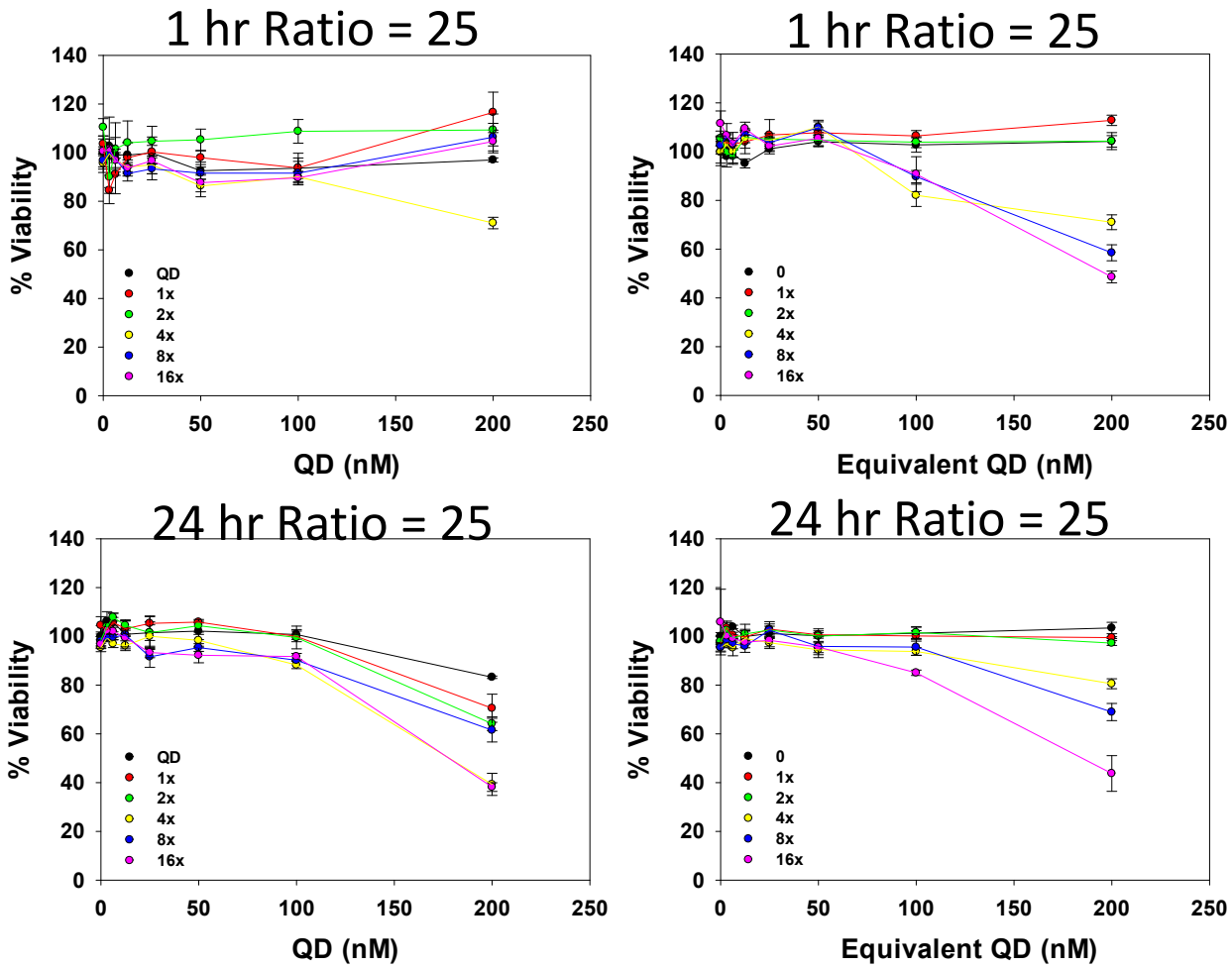
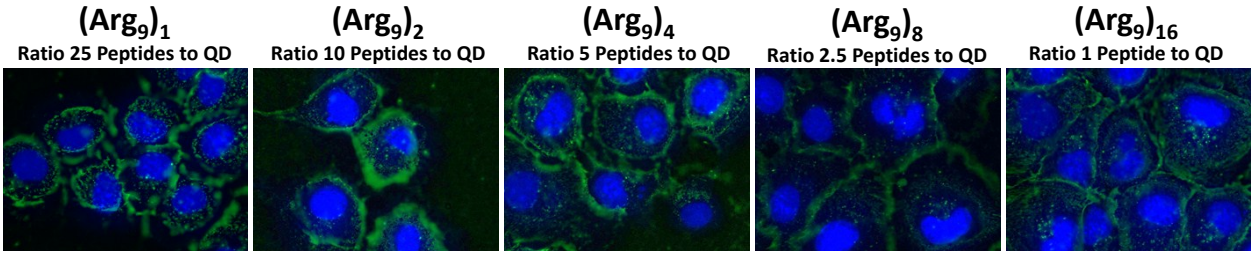


Figure S7. **Comparison of Cellular Uptake Efficiency with Equivalent Number of $(Arg_9)_n$ Moieties per QD.** QDs were assembled with ratios of 25 $(Arg_9)_1$, 10 $(Arg_9)_2$, 5 $(Arg_9)_4$, 2.5 $(Arg_9)_8$, and 1 $(Arg_9)_{16}$ per QD. These were then delivered to cells using the standard protocol described in the Materials and Methods for 1 h. Following fixing and DAPI staining, the average number of endosomes per cell was quantified and tabulated. The results indicate a generalized equivalency in QD uptake efficiency within error using at least these ratios.



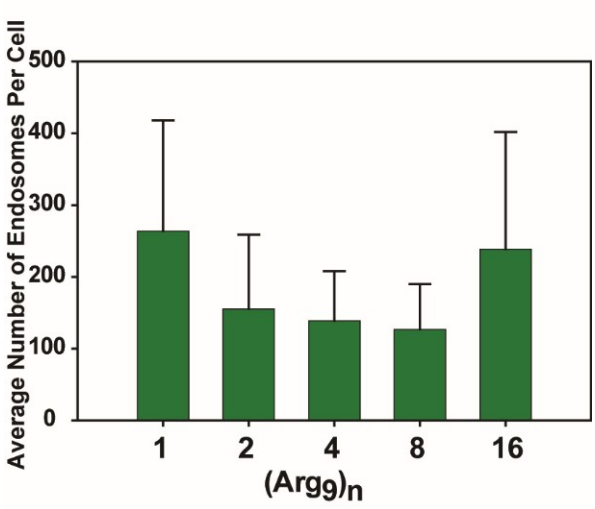


Figure S8. Selected individual confocal image slices through the cells of data presented in manuscript Figure 3.

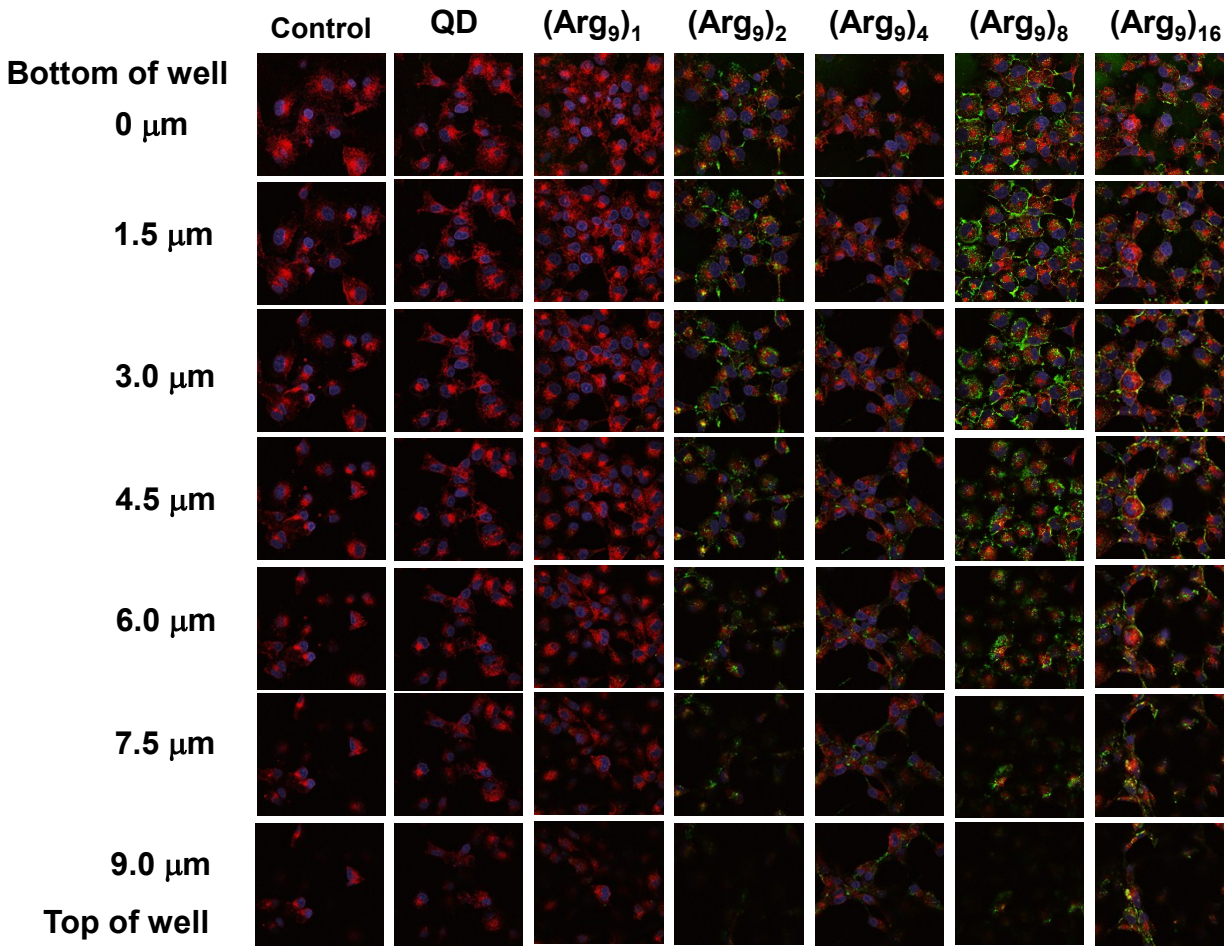


Figure S9. Supplemental to Manuscript Figure 4 showing individual channels along with combined image for each time point. A. Ratio of 5 Dendritic Peptides per QD: 30 min.

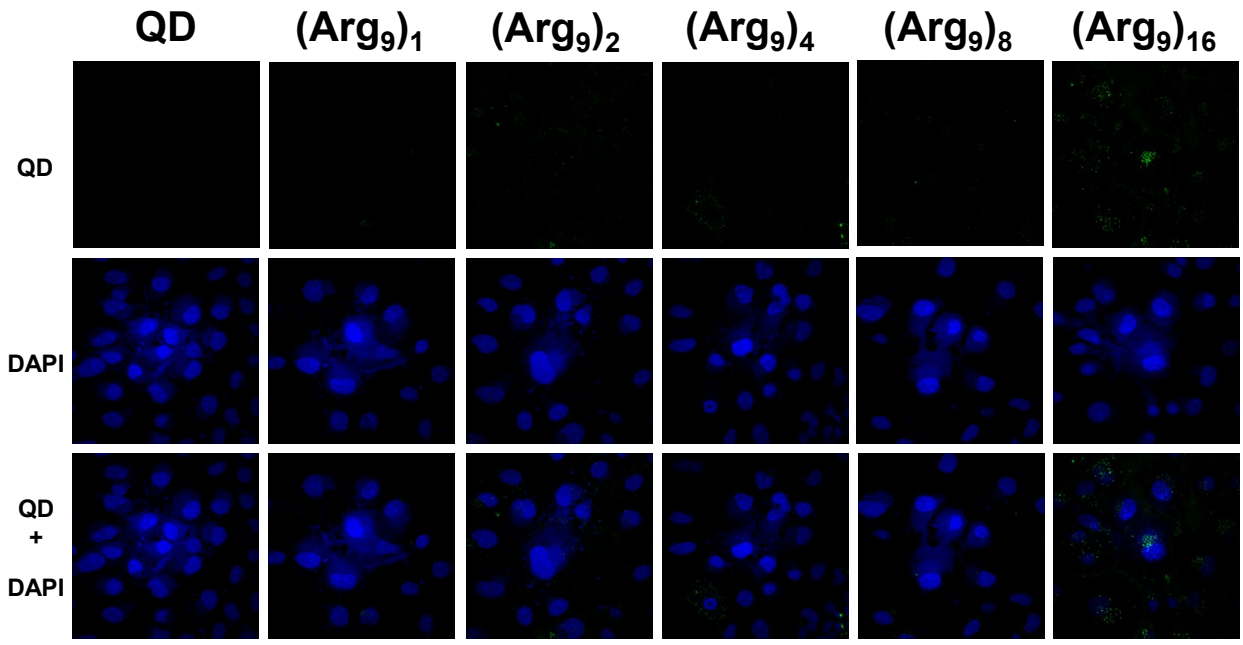


Figure S9. B. Ratio of 5 Dendritic Peptides per QD: 1 hr.

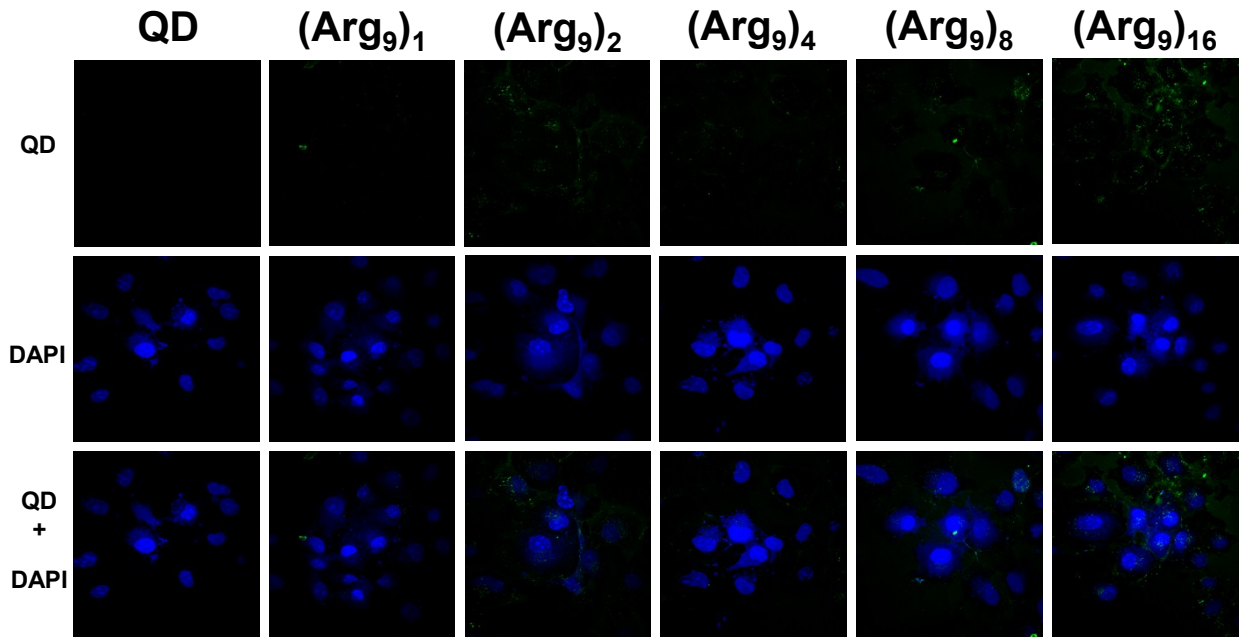


Figure S9. C. Ratio of 5 Dendritic Peptides per QD: 4 hr.

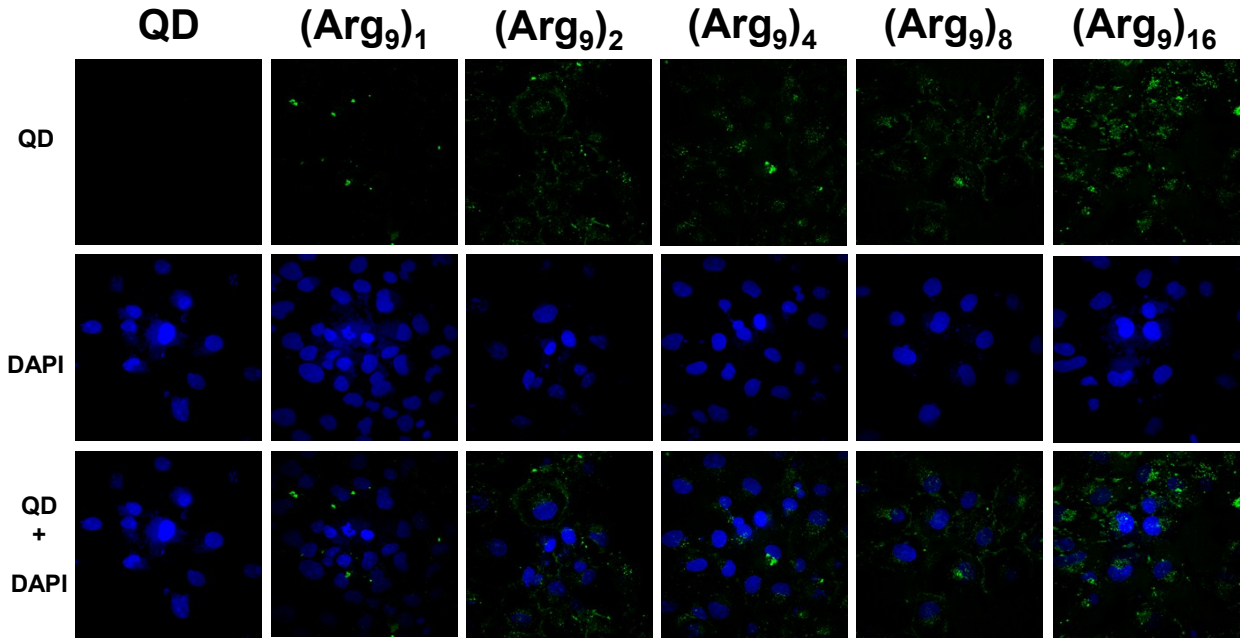


Figure S9. D. Ratio of 5 Dendritic Peptides per QD: 8 hr.

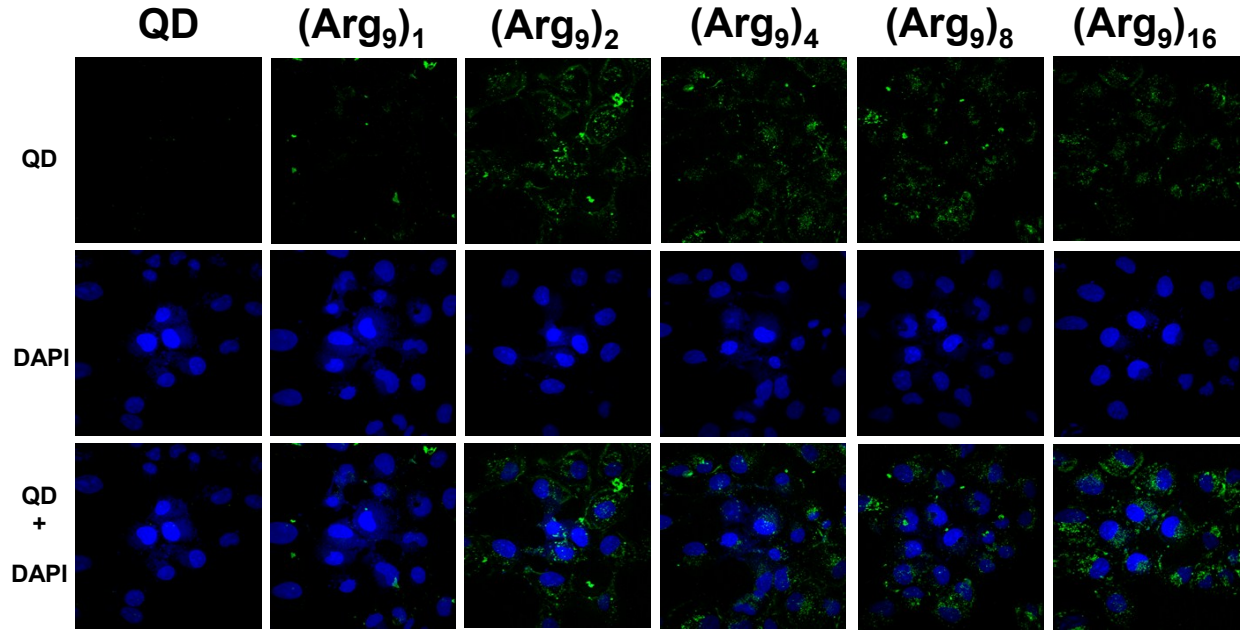


Figure S9. E. Ratio of 5 Dendritic Peptides per QD: 24 hr.

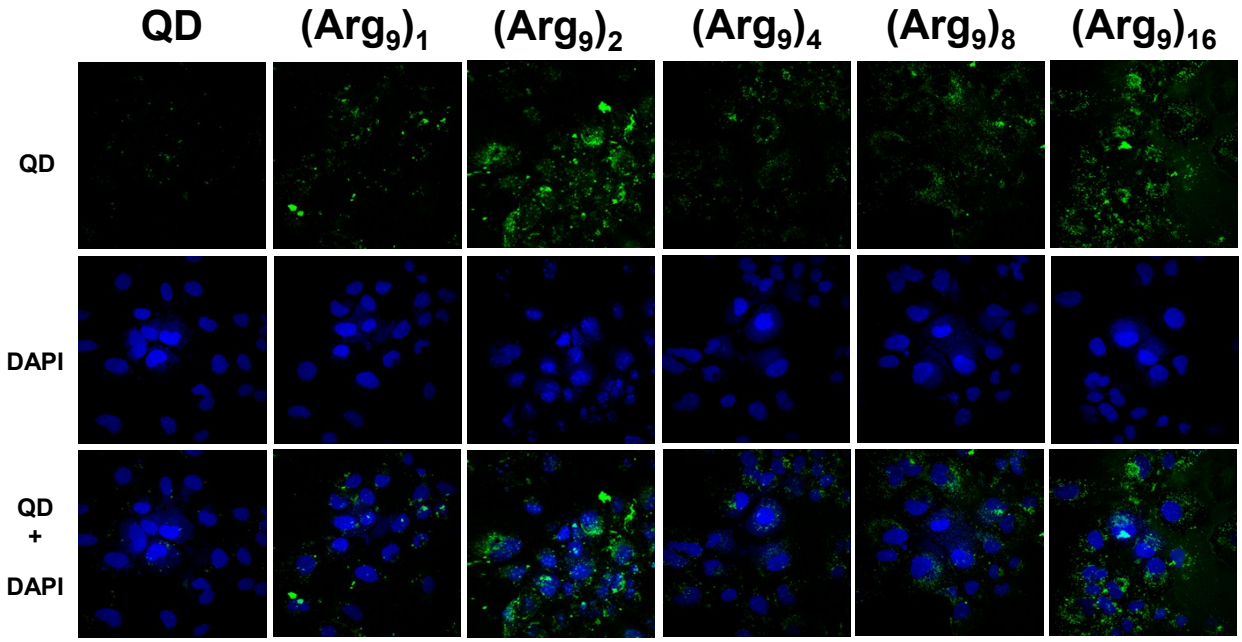


Figure S10. Maximum projection summary for manuscript Figure 4.

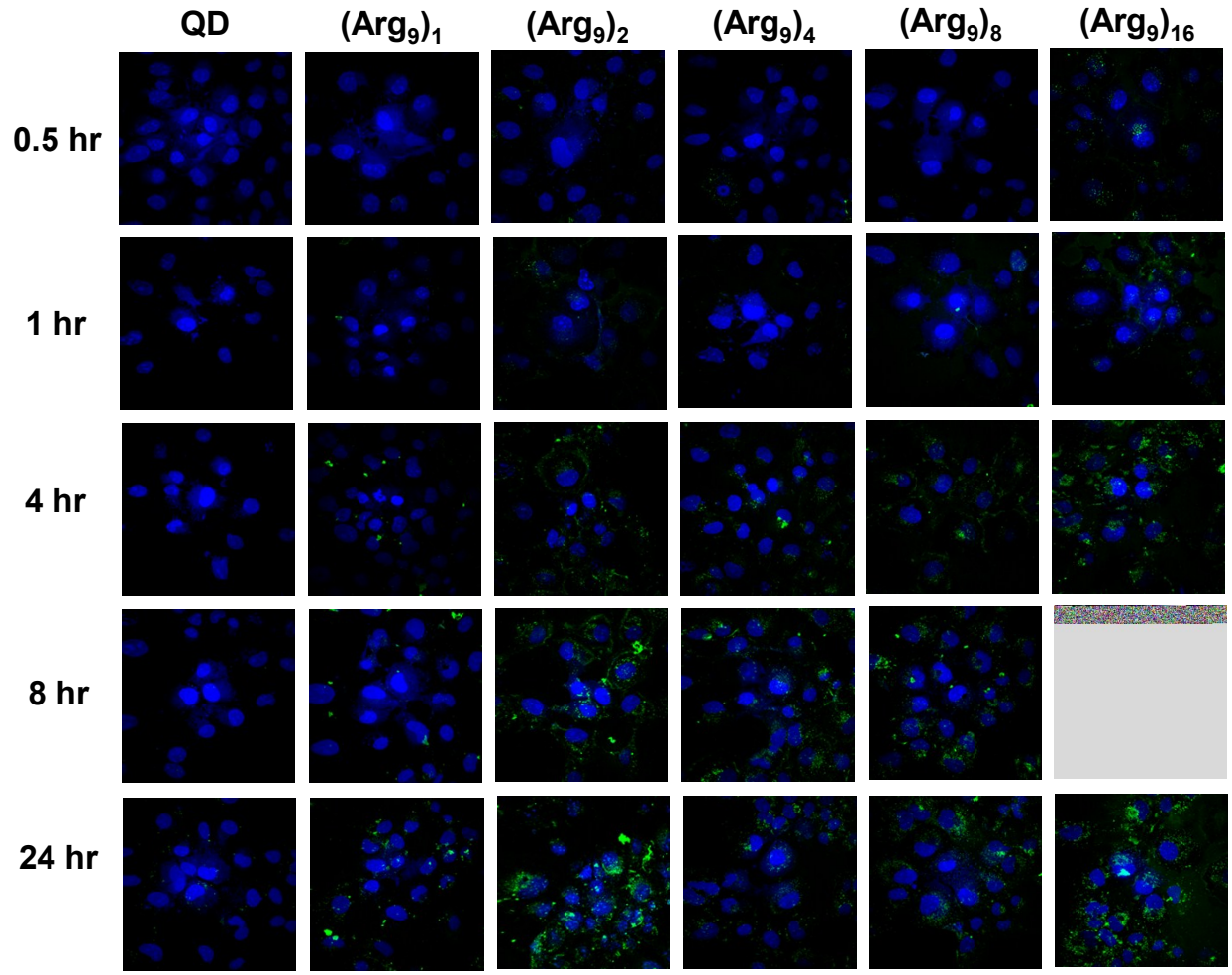


Figure S11. Selected individual confocal image slices through the cells of data presented in manuscript Figure 7B.

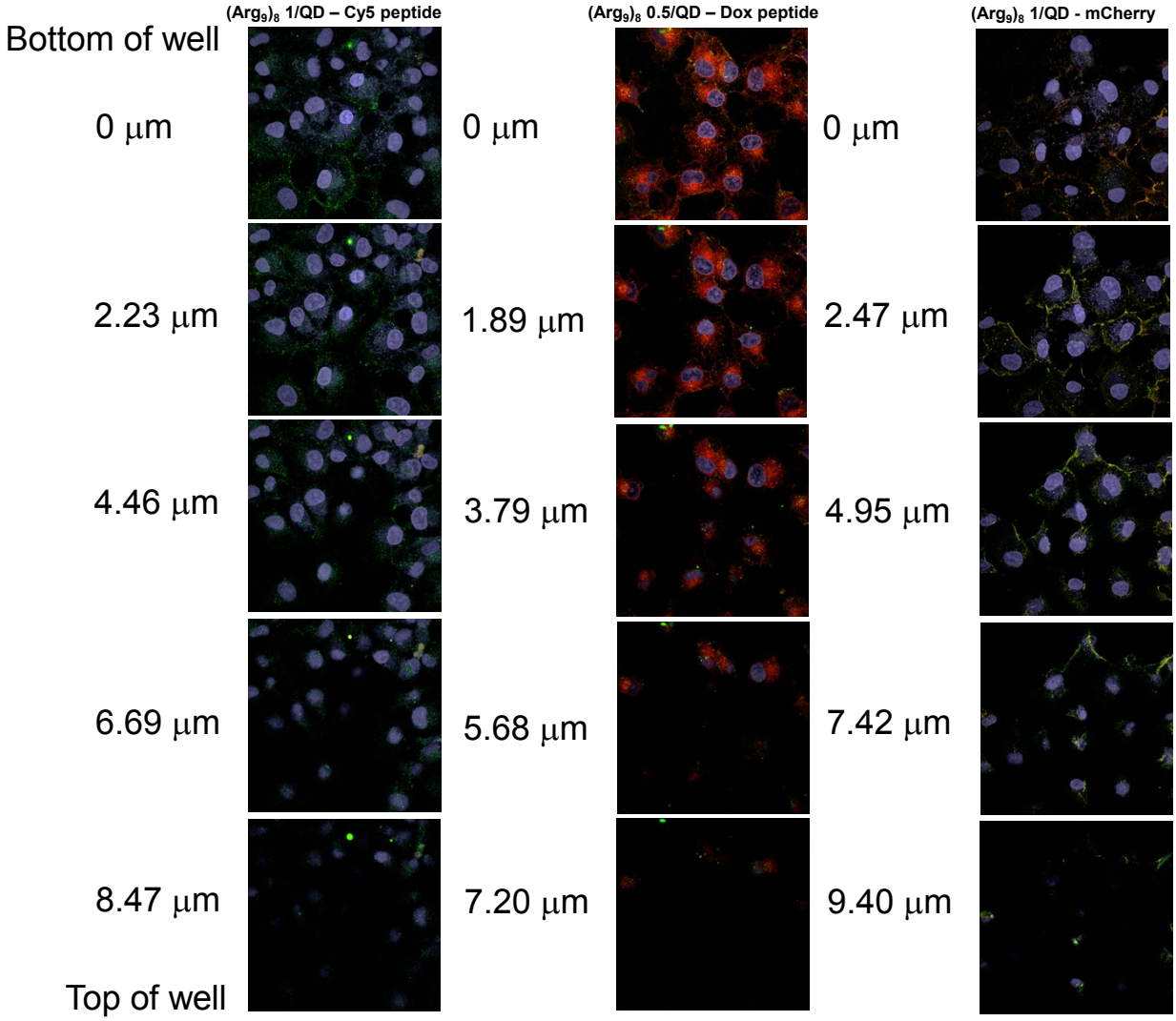
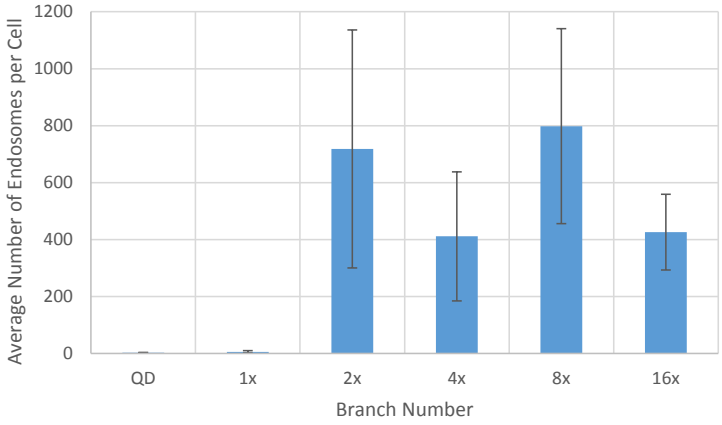
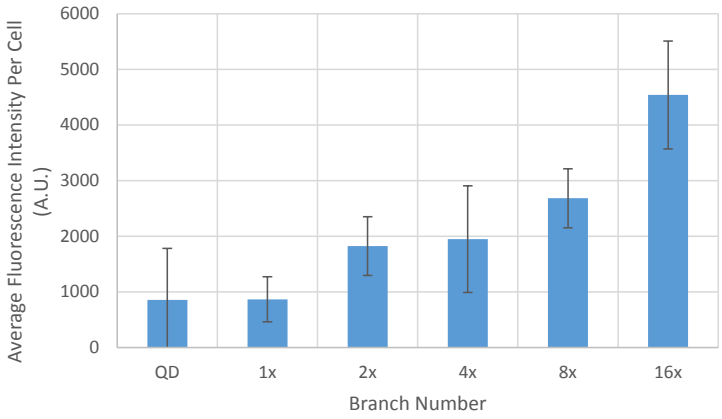


Figure S12. Selected analytical metrics from the experiments shown in manuscript Figure 7A. Data were collected as in manuscript Figure 5.





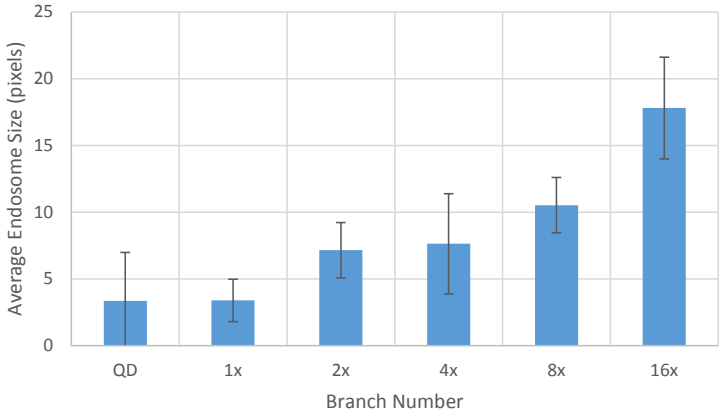


Table S1. Selected analytical metrics from the experiments shown in manuscript Figure 7B. Data were collected as in manuscript Figure 5.

Sample	Average Endosome Size (Pixels)	Average QD Intensity per Cell	Average # of QD Endosomes per Cell	Average Cargo Intensity per Cell	Average Number of Cargo Endosomes per Cell
$(Arg_9)_8$ 1/QD-Cy5 Peptide	3 ± 2	821 ± 413	354 ± 137	1139 ± 774	108 ± 35
$(Arg_9)_8$ 0.5/QD-Dox Peptide	5 ± 3	1179 ± 670	236 ± 108	5247 ± 2707	292 ± 122
$(Arg_9)_8$ 1/QD-mCherry Peptide	6 ± 2	1602 ± 469	280 ± 93	2540 ± 660	251 ± 93

References

1. Schnölzer, M., Alewood, P., Jones, A., Alewood, D. & Kent, S.B.H. In situ neutralization in boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res.* 40, 180-193 (1992).

2. Muttenthaler, M., Albericio, F. & Dawson, P.E. Methods, setup and safe handling for anhydrous hydrogen fluoride cleavage in boc solid-phase peptide synthesis. *Nat. Protoc.* 10, 1067-1083 (2015).

3. (a) Delehanty, J.B., Bradburne, C.E., Boeneman, K., Susumu, K., Farrell, D. Mei, B.C. Blanco-Canosa, J.B., Dawson, P.E., Mattoussi, H. and Medintz, I.L. Delivering quantum dot-peptide bioconjugates to the cellular cytosol: Escaping from the endolysosomal system. *Integrative Biology* 2, 265-277 (2010). (b) Delehanty, J.B., Medintz, I.L., Pons, T., Brunel, F.M., Dawson, P.E. and H. Mattoussi. Self-assembled quantum dot-peptide bioconjugates for selective intracellular delivery. *Bioconjugate Chemistry* 17, 920-927 (2006). (c) Oh, E., Liu, R., Nel, A., Boeneman Gemmill, K., Bilal, M., Cohen, Y., Medintz, I.L. Meta-Analysis of Cellular Toxicity for Cadmium Containing Quantum Dots. *Nature Nanotechnology* 11,479-486 (2016).