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Authors

Howitz, William
Guaglianone, Gretchen
McKnelly, Kate
et al.

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Macrocyclic Peptides Derived from Familial Alzheimer's Disease Mutants Show Charge-Dependent Oligomeric Assembly and Toxicity

William J. Howitz[a], **Gretchen Guaglianone**[a], **Kate J. McKnelly**[a], **Katelyn Haduong**[a], **Shareen N. Ashby**[a], **Mohamed Laayouni**[a], **James S. Nowick**[a,b]

[a]Department of Chemistry, University of California, Irvine, Irvine, California 92697, United States

[b]Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697, United States

Abstract

This work probes the role of charge in the oligomeric assembly, toxicity, and membrane destabilization of a series of peptides derived from Aβ and the E22Q and E22K familial mutants. In the mutant Aβ peptides, an acidic residue (E) is replaced with either a neutral or basic residue (Q or K), thus altering the net charge of the peptide. Acetylation at peripheral positions permits modulation of charge of the peptides and allows investigation of the role of charge in their oligomeric assembly, cytotoxicity, and membrane disruption. Peptides with the same net charge generally behave similarly even if the amino acid residue at position 22 differs. As the net charge of the peptide decreases, so does the extent of assembly, cytotoxicity, and membrane destabilization, which were determined using SDS-PAGE, LDH-release assays with SH-SY5Y cells, and dye leakage assays using liposomes. These findings suggest that the charge of the amino acid side chain, rather than its size or hydrophobicity, accounts for the differences in the oligomeric assembly and toxicity of the E22 familial mutants of Aβ.

Graphical Abstract

Corresponding Author: James S. Nowick – Department of Chemistry and Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697-2025, United States; jsnowick@uci.edu.

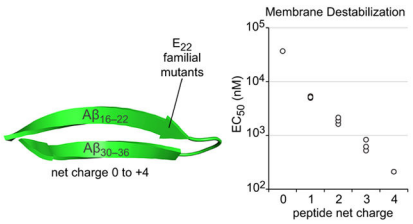
Author Contributions

W. J. H. led the synthesis and characterization of peptides **1–3**, performed SDS-PAGE and cytotoxicity experiments. G. E. G. and W. J. H. collaborated on the dye leakage assays. K. J. M. conceived of use of acetylation to prove the role of charge in the familial mutants of Aβ in consultation with J. S. N. K. H., S. A., and M. L. helped synthesize and purify peptide **1–3.** W. J. H. and J. S. N. wrote and edited the manuscript.

The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org/.

Procedures for peptide synthesis, SDS-PAGE, cytotoxicity, and dye leakage assays. (PDF)

The authors declare no competing financial interests.



INTRODUCTION

Charge plays an essential role in molecular recognition. Phosphorylation of receptor-tyrosine kinases triggers the transduction of signals that allow the release of growth factors and hormones like insulin by converting neutral hydroxy groups to anionic phosphate groups.[1] Removal of these phosphate groups by phosphatases halts downstream signaling.[2] C-terminal amidation increases the binding affinity of corticotropin-releasing hormone, thyrotropin-releasing hormone, and other peptide hormones to their G-protein coupled receptors and thus enhances cell signaling by eliminating the negative charge of the C-terminal carboxylate group.[3] Acetylation of the positively charged ammonium groups of the lysine residues of histone proteins by acetyltransferases increases gene expression by reducing the electrostatic interactions between those proteins and DNA.[4] Removal of the acetyl groups by histone deacetylases has the opposite effect.[4]

In the current study, we set out to use acetylation to study the impact of charge on the oligomerization and toxicity of peptides derived from familial Alzheimer's disease (FAD) mutants of Aβ. Amyloid oligomers are the result of protein misfolding and aggregation, and these oligomers are critical to study because they have been implicated in the pathogenesis of amyloid diseases such as Alzheimer's disease.[5,6,7] Due to the transient and heterogeneous nature of these oligomers, evaluating how they assemble and exert their toxic effects has been challenging.[8]

We had previously found that a β-hairpin peptide derived from $A\beta_{16–36}$, peptide **1a,** assembles in both the crystal state and in SDS-PAGE to form a hexamer (Figure 1).[9] The formation of a hexamer is significant, as hexamer formation has been shown to be an early step in the self-assembly of Aβ.[10–16] We recently found that introduction of familial Alzheimer's disease mutations at position 22 of peptide **1a** alters its oligomeric assembly and cytotoxicity.[17] In this study, we compared a peptide derived from wild-type Aβ (peptide **1a**) to peptides containing the E22Q and E22K mutations (peptides **2a** and **3a**) and found increasing toxicities across this series of peptides, all of which form hexamers. Each of these macrocyclic peptides is composed of two heptapeptide strands ($A\beta_{16–22}$ and $A\beta_{30–36}$) joined by δ-linked ornithine turn units ($^{\delta}$Orn).[18,19] *N*-Methylation of the peptide backbone attenuates uncontrolled aggregation.[20] The peptides differ only in the residue at position 22. The resulting increasing charge of the peptides at physiological pH (+2, peptide **1a;** +3, peptide **2a;** +4 peptide **3a**) correlates with increasing toxicity toward SH-SY5Y cells in an LDH-release assay and increasing membrane disruption of negatively charged LUVs in a dye leakage assay.

Familial mutations at position 22 have also been studied extensively by other researchers. The E22G, E22Q, and E22K mutants of $A\beta_{1-40}$ and $A\beta_{1-42}$ aggregate into oligomers at faster rates than wild-type Aβ and have enhanced cellular toxicity.[21–36] Some studies have attributed the enhanced aggregation rates and toxicity of the E22G and E22Q mutants in part to the greater hydrophobicity of the glycine and glutamine residues relative to the charged glutamic acid residue.[22,32,34,36] A separate study has attributed the increased aggregation rates of the E22G, E22Q, and E22K mutants primarily to differences in the size of the amino acid residues.[28] The authors of that study suggest that as the steric bulk of the side chain decreases, the rate of aggregation increases. Still other studies suggest that the charges of the amino acid residues are responsible for the differences in the aggregation rates and toxicities of the peptides.[21,33] Replacement of the charged glutamic acid residue with a neutral glycine or glutamine may reduce the electrostatic repulsion between adjacent peptides in the oligomeric state, and thus stabilize the oligomer.[21] Although replacement of glutamic acid with lysine might not eliminate electrostatic repulsion among adjacent peptides, the researchers suggest that the greater conformational freedom of the lysine side chain lessens this repulsion.

Mutations in the E22 residue concurrently alter the hydrophobicity, size, and charge of the side chain, making it challenging to identify the principal factor responsible for the observed differences in the aggregation rates and toxicities of the E22 mutants. Methods other than amino acid mutation are needed to understand the contribution of the individual factors. Herein, we probe the role of charge in the oligomeric assembly and toxicity of the E22 mutants of Aβ by varying the charges of the peptides **1a, 2a,** and **3a** without varying the residue at position 22. To alter the net charge of the peptides, we acetylated the α-amino group of either or both of the $^{\delta}$Orn residues in peptides **1a, 2a,** and **3a** (Figure 2).[37] Peptides **1b, 2b,** and **3b** are acetylated on the top strand, peptides **1c, 2c,** and **3c** are acetylated on the bottom strand, and peptides **1d, 2d,** and **3d** are acetylated on both strands. This approach allows for a controlled decrease of net charge without altering amino acid sequence.

RESULTS AND DISCUSSION

We evaluated the effects of charge on the assembly, toxicity, and membrane destabilization of peptides **1–3** by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), LDH-release assays with SH-SY5Y cells, and dye leakage assays using liposomes.

SDS-PAGE.

We assessed how the net charges of the twelve peptides affect the propensity of these peptides to assemble into oligomers using SDS-PAGE. In SDS-PAGE, non-acetylated peptides **1a, 2a,** and **3a** all migrate near the 10 kDa marker band at molecular weights consistent with hexamers (~10.6 kDa) although the shapes of the bands differ (Figure 3). Peptide la forms a band in which the higher molecular weight region is rounded and intense and the lower molecular weight region is more diffuse, suggesting a hexamer in equilibrium with lower-order oligomers. The comet-like appearance of this band suggests that lower-order oligomers are in rapid equilibrium with the hexamer and migrate more quickly as the band migrates down the gel. As the lower-order oligomers migrate more

quickly, they become more dilute, peeling away from the more concentrated region of the band. The greater dilution shifts the equilibrium further toward lower order oligomers, leading to yet more rapid migration and the formation of an elongated diffuse region. In contrast to peptide **1a,** peptides **2a** and **3a** form tighter bands, suggesting the formation of more stable hexamers. The more compact shape of these bands suggests that the equilibrium more strongly favors the hexamer over lower-order oligomers.

The diacetylated peptides **1d, 2d,** and **3d** migrate lower on the gels than their non-acetylated counterparts. Peptide **1d** forms a tight band that migrates near the 4.6 kDa marker band at a molecular weight consistent with a trimer (~5.6 kDa). Peptides **2d** and **3d** form elongated bands that migrate slightly below the 10 kDa marker band. The elongated bands formed by peptides **2d** and **3d** have a more intense higher molecular weight region and a more diffuse lower molecular weight region, similar to the band formed by peptide **1a.** The different net charges of peptide **1a** (+2) and peptide **1d** (0) appear to be responsible for the differences in the oligomeric assemblies of the two peptides on the gel. Differences in net charge also appear to be responsible for the different shapes of the bands formed by peptides **2a** and **2d,** and by peptides **3a** and **3d.** The change in the shape of the bands from tighter to more elongated also correlates with a reduction in the net charge of the peptides (+4, peptide **3a** vs. +2 peptide **3d;** +3, peptide **2a** vs. +1, peptide **2d**), suggesting that greater net charge imparts greater stability to the hexamers in SDS.

The monoacetylated peptides **1b** and **1c** migrate differently in SDS-PAGE, even though both peptides have the same net charge (+1). Peptide **1b** migrates as an elongated band above the 10 kDa marker band and slightly above peptide **la,** while peptide **1c** migrates as an elongated band at the 4.6 kDa marker band. Peptide **2b** also migrates as an elongated band above the 10 kDa marker band and slightly above peptide **2a,** while peptide **2c** migrates as a comet-shaped band below the 10 kDa marker band. Peptides **3b** and **3c** show similar, but less pronounced differences in migration. The differences in migration of each of these pairs of peptides is surprising, because both members of each pair have comparable net charge. The differences in their behavior suggest that the position of acetylation may also have an effect upon the oligomeric assembly of the peptides.

Cytotoxicity.

We treated SH-SY5Y cells with peptides **1–3** to assess how the cytotoxicity of the peptides correlates with their net charges. Of the non-acetylated peptides, peptide **1a** proved the least cytotoxic, exhibiting toxicity at 50 μM (Figure 4). Peptide **2a** is more toxic, exhibiting toxicity at 25 μM. Peptide **3a** is the most toxic of the non-acetylated peptides, exhibiting toxicity at 12.5 μM. The increasing cytotoxicity correlates with the increasing net charge of the peptides: +2, peptide **1a;** +3, peptide **2a;** +4 peptide **3a.**

The acetylated variants of peptides **1a, 2a,** and **3a** are less toxic than their non-acetylated counterparts. Peptides **1b, 1c,** and **1d** do not exhibit toxicity at any of the concentrations tested. Peptide **2b** exhibits toxicity at 25 μM, and peptides **2c** and **2d** exhibit toxicity at 50 μM. Peptides **3b** and **3c** exhibit toxicity at 25 μM, and peptide **3d** exhibits toxicity at 50 μM.[38] The cytotoxicities thus increase with the increasing net charges of the peptides. The

peptides with net charges of +1 or 0 do not exhibit toxicity at any of the concentrations tested (peptides **1b, 1c,** and **1d**), except for peptide **2d** which exhibits toxicity at 50 μM. The peptides with a net charge of +2 (peptides **la, 2c,** and **3d**) exhibit toxicity at 50 μM, except peptide **2b** which also exhibits toxicity at 25 μM. The peptides with a net charge of +3 (peptides **2a, 3b,** and **3c**) exhibit toxicity at 25 μM.

Membrane Destabilization.

The cytotoxicity of Aβ is associated with the binding to and disruption of cell membranes, and previous studies have demonstrated that Aβ binds and disrupts negatively charged lipid bilayers to a greater extent than neutrally charged ones.[39–49] To investigate whether the cytotoxicity of peptides **1–3** in our study also correlates with membrane disruption, we assessed the extent to which the peptides destabilize the membranes of negatively charged large unilamellar vesicles (LUVs) composed of 1:1 phosphatidylcholine :phosphatidylserine (PC:PS) in a dye leakage assay. In a dye leakage assay, LUVs encapsulating a fluorescent dye are exposed to increasing concentrations of peptide. Destabilization of the membranes of the LUVs by the peptides releases the encapsulated dye, and the reduced self-quenching and increased fluorescence is detected spectrophotometrically.[50] Lysis buffer is used as a positive control and is normalized to 100% dye leakage; water is used as a negative control and is normalized to 0% dye leakage. A nonlinear regression curve is fit to the normalized data points for each peptide. The effective concentrations at which the non-acetylated peptides cause 50% dye leakage (EC_{50}) are 1.63 μM for peptide **1a,** 0.52 μM for peptide **2a,** and 0.21 μM for peptide **3a** (Figure 5). The membrane destabilization by these peptides follows the same trend as their cytotoxicities and correlates with their increasing net charge (+2, peptide **1a;** +3, peptide **2a;** +4 peptide **3a**).

The acetylated variants of peptides **1a, 2a,** and **3a** cause membrane destabilization at higher concentrations than their non-acetylated counterparts. The EC_{50} values for peptides **1b, 1c,** and **1d** are 5.35 μM, 4.95 μM, and 36.87 μM, respectively. The EC_{50} values for peptides for peptides **2b, 2c,** and **2d** are 2.14 μM, 1.87 μM, and 5.12 μM, respectively. The EC_{50} values for peptides **3b, 3c,** and **3d** are 0.83 μM, 0.62 μM, and 2.15 μM, respectively.

The concentrations at which peptides **1–3** destabilize negatively charged membranes correlate strongly with their net positive charges, suggesting a direct relationship with the degree of electrostatic interaction between the peptide and the membranes. The peptides with a net charge of +3 (peptides **2a, 3b,** and **3c**) have EC_{50} values below that of the peptide with a net charge of +4 (peptide **3a**) and above those of the peptides with a net charge of +2 (peptides **1a, 2b, 2c,** and **3d**). The peptides with a net charge of +2 have EC_{50} values below those of the peptides with a net charge of +3 and above those of the peptides with a net charge of +1 (peptides **1b, 1c,** and **2d**). The peptides with a net charge of +1 have EC_{50} values below those of the peptides with a net charge of +2 and above that of the peptide with a net charge of 0 (peptide **1d**).

SUMMARY AND CONCLUSION

Acetylation permits the modulation of charge within a series of peptides derived from Aβ familial mutants and allows the investigation of the role of charge in their oligomeric

assembly, cytotoxicity, and membrane disruption. By SDS-PAGE all of the peptides ran on the gel with molecular weights consistent with a hexamer except peptides **1c** and **1d** which were consistent with a trimer. Differences in the oligomeric assemblies between the non-acetylated and diacetylated peptides correlate with the differences in the net charges of the peptides. However, the position of acetylation also appears to affect the oligomeric assemblies, because each pair of monoacetylated peptides (**1b** and **1c, 2b** and **2c, 3b** and **3c**), despite having the same net charge, migrated differently. In cytotoxicity studies, the peptides exhibited increasing cytotoxicity with increasing net charge. Dye leakage experiments revealed that a greater propensity to cause membrane destabilization correlates strongly with net charge.

From these studies, a model emerges in which the net charge of peptides **1–3,** rather than the residue at position 22, is the primary determinant of oligomer stability in SDS-PAGE, membrane interaction, and cytotoxicity. The greater interaction of the more positively charged peptides with the membranes of liposomes may explain the greater cytotoxicity of the peptides through greater interaction with cell membranes. The similar behavior of the peptides with the same net charge, indicates that the differences in size and hydrophobicity of the different amino acids at position 22 are relatively unimportant in peptides **1–3.** These results further suggest that differences in charge imparted by E22 mutation are the primary driving force in differences in the oligomeric assembly and toxicity of the E22 familial mutants of Aβ.

METHODS

Procedures for the synthesis of peptides **1–3,** SDS-PAGE, cytotoxicity, and dye leakage are included in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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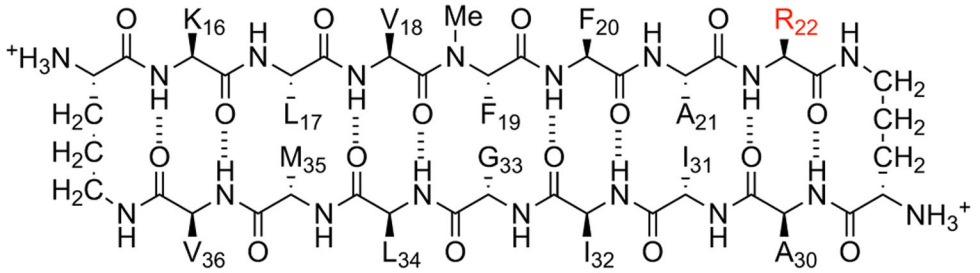


peptide **1a** (R_{22} = E); **2a** (R_{22} = Q); **3a** (R_{22} = K)

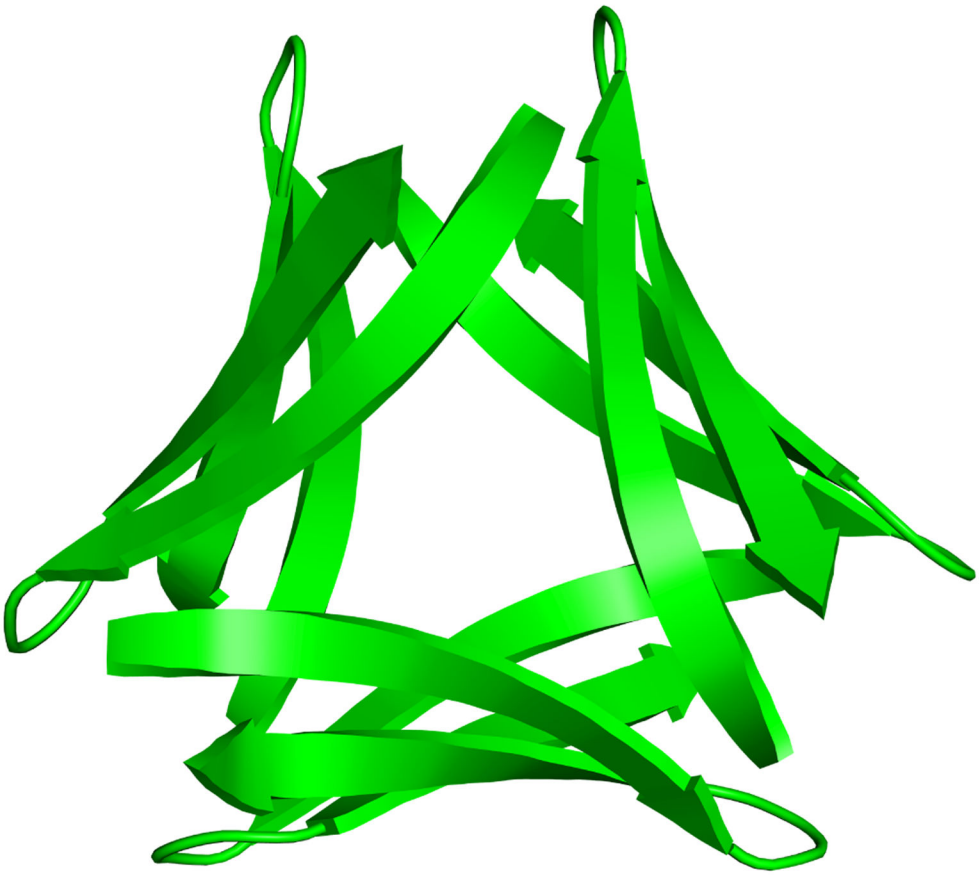
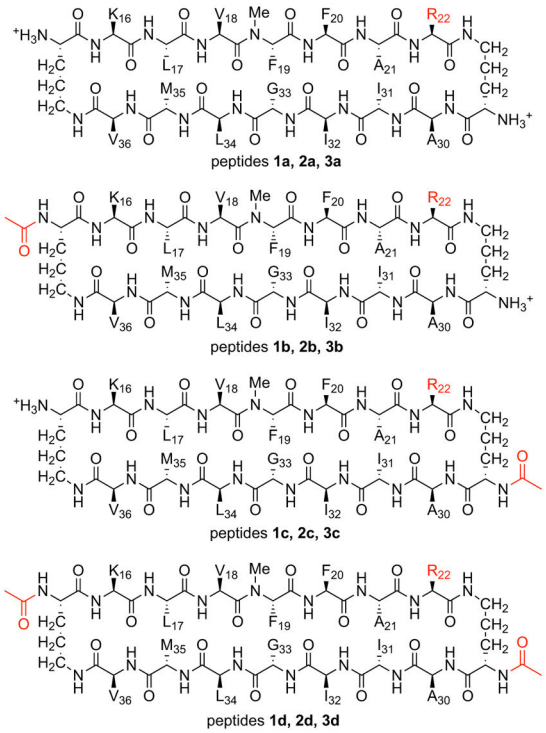


Figure 1.

Top: Chemical structure of peptides **1a, 2a,** and **3a.** Bottom: X-ray crystallographic structure of peptide **1a** reveals a hexameric assembly (PDB 5W4H).



peptide	R_{22}	acetylated postion	net charge
1a	E	none	+2
1b	E	top strand	+1
1c	E	bottom strand	+1
1d	E	top and bottom strands	0
2a	Q	none	+3
2b	Q	top strand	+2
2c	Q	bottom strand	+2
2d	Q	top and bottom strands	+1
3a	K	none	+4
3b	K	top strand	+3
3c	K	bottom strand	+3
3d	K	top and bottom strands	+2

Figure 2.
Structures, location of acetylation, and net charges of peptides **1–3.**

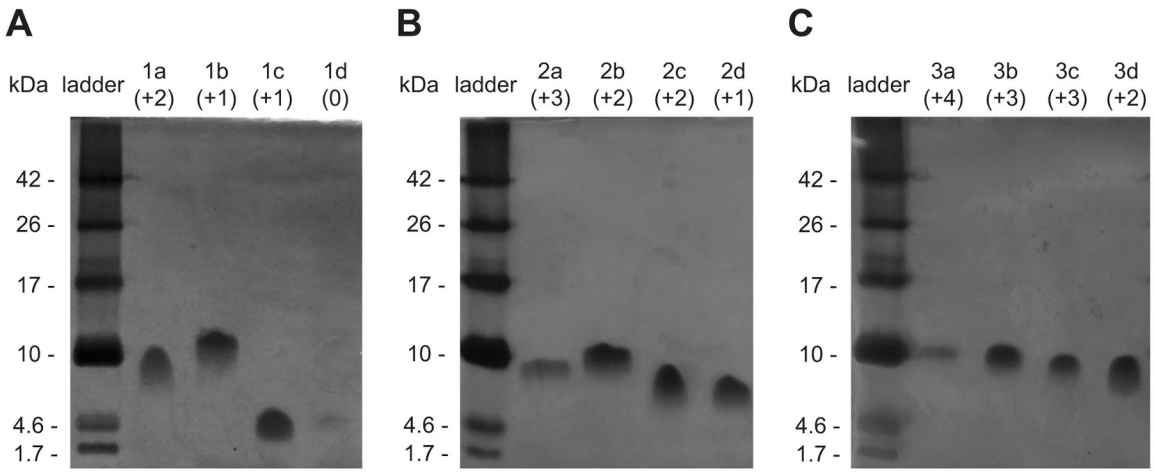


Figure 3.

Oligomeric assembly of peptides **1–3** observed in SDS-PAGE. All peptides were run at a concentration of 0.2 mg/mL and visualized by silver staining. (A) Peptides **1a** (+2), **1b** (+1), **1c** (+1), and **1d** (0). (B) Peptides **2a** (+3), **2b** (+2), **2c** (+2), and **2d** (+1). (C) Peptides **3a** (+4), **3b** (+3), **3c** (+3), and **3d** (+2).

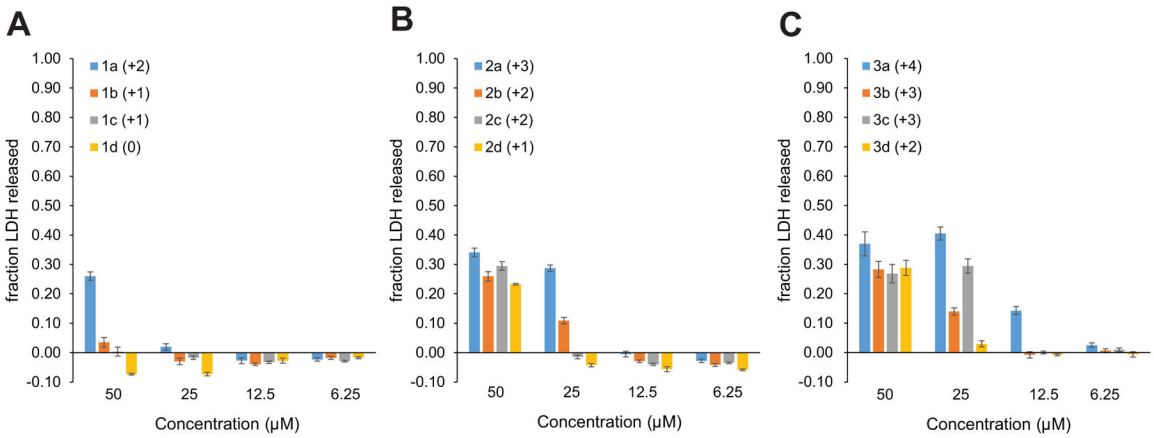


Figure 4.

Cytotoxicity of peptides **1–3** determined by an LDH-release assay. Concentrations of peptides ranging from 6.25 to 50 μM were tested with water (vehicle) as the negative control and lysis buffer as the positive control. (A) Peptides **1a** (+2), **1b** (+1), **1c** (+1), and **1d** (0). (B) Peptides **2a** (+3), **2b** (+2), **2c** (+2), and **2d** (+1). (C) Peptides **3a** (+4), **3b** (+3), **3c** (+3), and **3d** (+2).

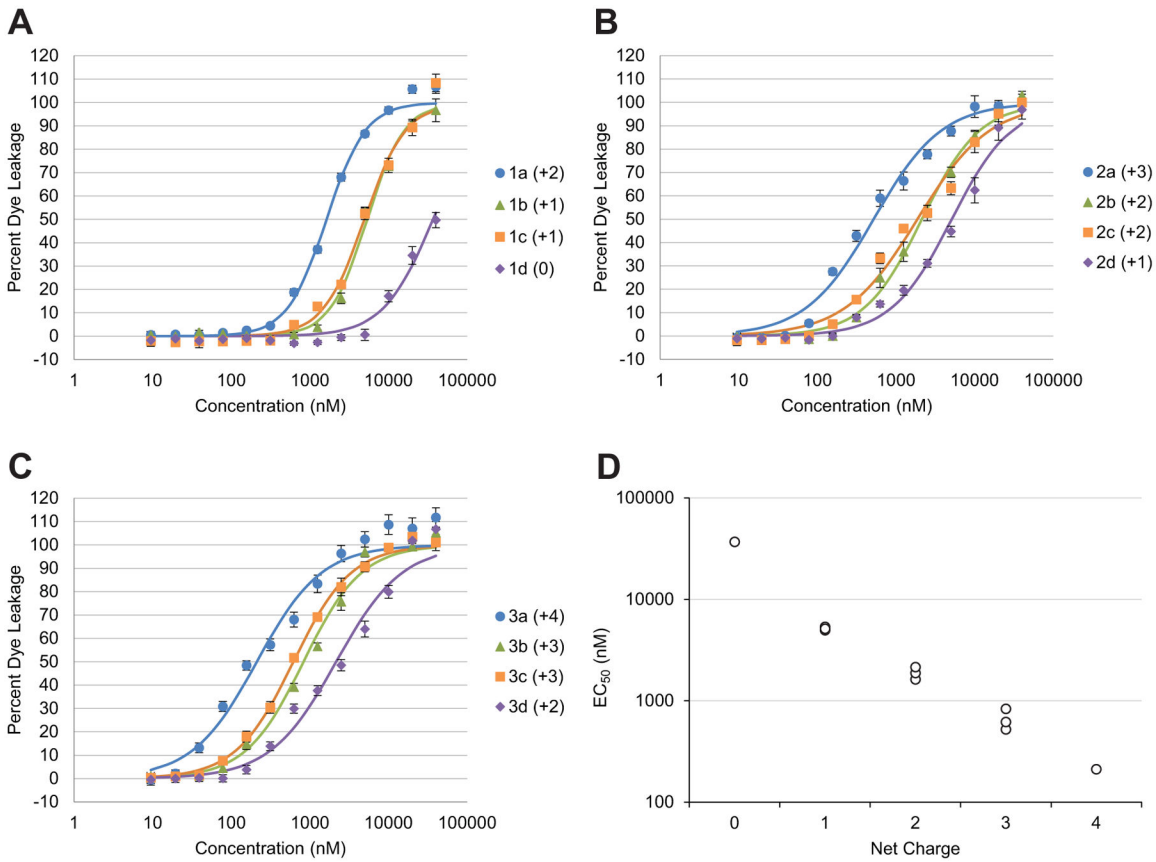


Figure 5.

Propensity of peptides **1–3** to cause membrane destabilization of negatively charged LUVs determined using a dye leakage assay. (A) Peptides **1a** (+2), **1b** (+1), **1c** (+1), and **1d** (0). (B) Peptides **2a** (+3), **2b** (+2), **2c** (+2), and **2d** (+1). (C) Peptides **3a** (+4), **3b** (+3), **3c** (+3), and **3d** (+2). (D) EC_{50} values of peptides **1–3** grouped by net charge.