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## NMEGylation: A novel modification to enhance the bioavailability of therapeutic peptides

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### Abstract

We have evaluated “NMEGylation”—the covalent attachment of a oligo-*N*-methoxyethylglycine (NMEG) chain—as a new form of peptide/protein modification to enhance the bioavailability of short peptides. OligoNMEGs are hydrophilic PEG-like molecules made by solid-phase synthesis, typically up to 40 monomers in length. They have been studied as non-fouling surface coatings, and as monodisperse mobility modifiers for free-solution conjugate capillary electrophoresis. However, polyNMEGs have not been demonstrated prior to this work as modifiers of therapeutic proteins. In prior published work, we identified a short peptide, “C<sub>20</sub>”, as a potential extracellular inhibitor of the fusion of human respiratory syncytial virus (hRSV) with mammalian cells. The present study was aimed at improving the C<sub>20</sub> peptide's stability and solubility. To this end we synthesized and studied a series of NMEGylated C<sub>20</sub> peptide-peptoid bioconjugates comprising different numbers of NMEGs at either the *N*- or *C*- terminus of C<sub>20</sub>. NMEGylation was found to greatly improve this peptide's solubility and serum stability; however, longer polyNMEGs ( $n > 3$ ) deleteriously affected peptide binding to the target protein. By incorporating just one NMEG monomer, along with a glycine monomer as a flexible spacer, at C<sub>20</sub>'s *N*-terminus (NMEG-Gly-C<sub>20</sub>), we increased both solubility and serum stability greatly, while recovering a binding affinity comparable to that of unmodified C<sub>20</sub> peptide. Our results suggest that NMEGylation with an optimized number of NMEG monomers and a proper linker could be useful, more broadly, as a novel modification to enhance bioavailability and efficacy of therapeutic peptides.

### Keywords

NMEGylation; bioavailability; peptide-based therapeutics

### Introduction

With the monumental advances in biotechnology over the past few decades, peptides and proteins have become key players in the drug market, as therapeutic candidates with undeniably high specificity and low toxicity compared to conventional synthetic small-molecule drugs, resulting in more than 60 biotherapeutics, which have sales reaching US

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\$40 billion.<sup>1,2</sup> However, clinical uses, manufacturing, and storage of peptide-based medicines are challenged by several unfavorable biophysical properties. One major obstacle to peptide-based therapeutics is that they are susceptible to proteolytic degradation, which can result in rapid renal clearance and short *in vivo* circulation half-lives (~ 2-5 min). Efforts have been made to increase the half-lives of biotherapeutics without sacrificing efficacy, but these efforts have yielded only a few notable successes.<sup>3-8</sup>

PEGylation, the conjugation of PEG (polyethylene glycol) chains to druggable materials, has been studied intensively and clinically proven and is an acceptable method of modifying peptide/protein-based medicines. The consequent increase in proteolytic stability reduces the rate of renal clearance and enhances therapeutic efficacy. PEGylation is known to improve the physical and thermal stability and the solubility of biopharmaceuticals, which makes this modification suitable for applications in drug delivery and formulation.<sup>9-12</sup> Since Adagen® (PEGylated adenosine deaminase) was introduced as the first FDA-approved PEGylated therapeutic agent in 1990, several PEGylated protein-based drugs have appeared, including PEGasys® (PEGylated  $\alpha$ -interferons, for Hepatitis C treatment) and Cimzia® (PEGylated Anti-TNF Fab, for rheumatoid arthritis and Crohn's disease).<sup>13</sup> However, PEGylation has limitations, such as the heterogeneity of PEG (lack of complete monodispersity). As a result, detailed bioanalytical aspects of the resultant “mixtures” of PEGylated products are an issue in the FDA approval of and clinical uses of PEGylated drugs.<sup>14</sup> The avoidance of variable extents of PEGylation requires a complicated, multi-step manufacturing process, which increases costs.<sup>15</sup> In addition to other approaches to improve specificity and homogeneity in PEGylation,<sup>16,17</sup> efforts to develop alternatives to PEG are also being pursued, so far resulting in limited but promising outcomes.<sup>18-20</sup>

*N*-methoxyethylglycine (NMEG) is a hydrophilic peptoid monomer that bears a similar chemical side-chain moiety (an ethylene oxide unit) to the PEG monomer (Figure 1). Peptoids are peptidomimetics based on a peptide backbone, and resist proteolysis because their side chains are attached to backbone nitrogens rather than to the  $\alpha$ -carbons.<sup>21</sup> Since 1998, peptoids have been demonstrated for uses in gene delivery,<sup>22</sup> drug delivery,<sup>23</sup> and as a scaffold for the design of novel protein ligands with improved binding affinities.<sup>24-26</sup> Virtually any primary amine can be incorporated into oligopeptoids as a side chain using the “sub-monomer” approach, enabling the use and exploration of a diverse set of biomimetic monomers in peptoid research.<sup>27,28</sup>

Previously, the Barron group has reported that oligoNMEGs (with  $n = 10, 20$  and  $30$ ) are promising as (1) antifouling agents, which prevent non-specific protein and cell binding to surfaces<sup>29</sup>, and (2) mobility modifiers enabling free-solution DNA separations by bioconjugate capillary electrophoresis.<sup>30</sup> Although NMEGylation, *i.e.*, covalent attachment of an oligoNMEG, should be anticipated to enhance protease resistance,<sup>31,32</sup> this has not yet been investigated. Thus, we have explored oligoNMEG as a PEG-like but more homogeneous modification.

We have previously identified a particular 20mer polypeptide (“C<sub>20</sub>”) as a potential new antiviral agent using a protein-based (five-helix bundle, 5HB) fluorescence polarization (FP) screening assay, as part of an effort to identify potential viral entry inhibitors that could prevent human respiratory syncytial virus (hRSV) infections.<sup>33</sup> As a lead compound, the C<sub>20</sub> peptide shows low micromolar binding affinity to the 5HB; however, neither the solubility nor the stability of this peptide is ideal. Therefore, using C<sub>20</sub> as the parent peptide, we prepared a series of NMEGylated C<sub>20</sub> analogues, all completely monodisperse and comprising different numbers of NMEG monomers, and evaluated the biophysical properties and biological activity of each bioconjugate. We were able to identify interesting new compounds, and thus believe that oligopeptoids, oligoNMEGs in particular, are

promising as novel modifiers of biotherapeutics, and an approach that could have a broad usefulness in biological and medical research.

## Methods and Materials

### General materials

Reagents for peptide and peptoid synthesis were purchased from Applied Biosystems (Foster city, CA, USA) or Sigma-Aldrich (Milwaukee, WI, USA). Resins and Fmoc-protected amino acids were purchased from NovaBioChem (San Diego, CA, USA) or Anaspec (San Jose, CA, USA). Solvents for analytical and preparation RP-HPLC were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and were used without additional purification.

### NMEGylated therapeutics and C<sub>20</sub> peptide synthesis

The C<sub>20</sub> peptide and its NMEGylated analogues were synthesized using standard Fmoc chemistry and submonomer peptoid synthesis<sup>28</sup>, each in a single synthesis, using an automated ABI 433A peptide synthesizer (Life Technologies, Foster city, CA USA). Final purities of synthetic peptides and therapeutics were confirmed by analytical RP-HPLC, and the molar masses of the purified products were confirmed by electrospray mass spectrometry (ESI) at the Stanford University Mass Spectrometry (SUMS) facility.

### Percent acetonitrile at RP-HPLC elution as a relative measure of bioconjugate hydrophilicity

The relative hydrophilicities (the percent acetonitrile at elution in RP-HPLC) of NMEGylated peptides were compared. Previously this has been reported as a reliable method for determining the contribution of the hydrophilicity of constituent amino acids of a polypeptide, based on the retention time of peptides.<sup>34,35</sup> The purified C<sub>20</sub> peptide and its NMEGylated analogues were analyzed using RP-HPLC on a Phenomenex (Torrance, CA, USA) C18 column (250 mm × 2.00 mm) with 5 μm resin size using a linear gradient of 5-95% solvent B over 30 or 60 min (solvent A is water with 0.1% (v/v) TFA and solvent B is acetonitrile with 0.1% (v/v) TFA).

### Serum stability assay<sup>36</sup>

Peptides (~ 0.4 mg) were dissolved in PBS pH 7.6 (400 μL, containing 0.1% v/v benzyl alcohol as internal standard). Equal volumes of 50% human serum plasma (Sigma-Aldrich, Milwaukee, WI, USA) and peptide/bioconjugate solutions were combined, and incubated at 37°C. Samples were taken in triplicate (3 × 50 μL) at 0, 30, and 240 min and placed on ice. Each sample (including the t = 0 time point) was prepared for HPLC analysis in the following way. First, 20 μL of 0.5M lysine monohydrochloride was added, followed by 65 μL of acetonitrile. The samples were cooled on ice for 10 min and subsequently centrifuged for 10 min at 3000 rpm. A 50 μL aliquot of resulting samples was taken from the supernatant, diluted with 100 μL of water, and analyzed by analytical RP-HPLC. The ratio of the disappearing peptide or bioconjugate peak area to the benzyl alcohol peak area was calculated for each time point, and normalized against the ratio obtained at the t = 0 time point (representing 100% peptide remaining) as a percentage peptide remaining.

### Competitive fluorescence polarization (FP) binding assay

Competitive FP binding assays of NMEGylated C<sub>20</sub> therapeutics were carried out as previously reported. Briefly, using a 5-helix bundle (5HB) protein construct and a fluorescently labeled 35-amino acid-long polypeptide (FI-C<sub>35</sub>):

YDPLVFPSEDFDASISQVNEKINQSLAFIRKSDEL) as a target and tracer, respectively, the binding affinities of NMEGylated therapeutics were each evaluated individually. Both the C<sub>20</sub> and C<sub>35</sub> peptide sequences are derived from HRB domain of hRSV fusion (F) protein, are meant to mimic the 6-helix bundle formation of hRSV F protein by occupying the one empty binding site on the 5HB protein. The binding of the Fl-C<sub>35</sub> peptide to the 5HB in the absence of test ligands provided us with our relative “100% 5HB·Fl-C<sub>35</sub> bound” for use as a positive control. Each well in a black 96-well plate (Corning Inc. Lowell, MA, USA) contained an aliquot of 20 nM 5HB solution, and increasing concentrations of each NMEGylated bioconjugate in FP buffer (20 mM PBS at pH 7.4, 500 mM NaCl, 0.01% (v/v) Tween-20, and 0.05 mg/ml bovine gamma globulin) in a final volume of 185  $\mu$ L with 1 hr incubation at room temperature. Fl-C<sub>35</sub> was then added to a final concentration of 5 nM followed by 30 min incubation at room temperature. FP response was monitored in a Synergy4 (Biotek, Winooski, VT, USA) plate reader with an excitation wavelength of 485 nm and emission wavelength of 530 nm. All experimental data were obtained in duplicate and the percentage of 5HB·Fl-C<sub>35</sub> bound was calculated using the following equation: % of 5HB·Fl-C<sub>35</sub> bound =  $100 \times [1 - (mP - mPf) / (mPb - mPf)]$ , where unbound Fl-C<sub>35</sub> (mPf), bound Fl-C<sub>35</sub> (mPb) and the bound inhibitor to the 5HB (mP) are used accordingly.

## Results and discussion

### Synthesis, purification and characterization of NMEGylated C<sub>20</sub> therapeutics

The C<sub>20</sub> peptide was previously identified from peptide truncation studies as having low-micromolar binding affinity to a 5-helix bundle (5HB) protein, a genetically engineered protein construct derived from the hRSV fusion protein (F); however, because of its short length and unstructured conformation,<sup>37</sup> the C<sub>20</sub> peptide ligand has high susceptibility to proteases and moreover, because of its amino acid sequence, poor solubility in aqueous solution. Although PEGylation might also improve the desirable biophysical features of the C<sub>20</sub> peptide, any attempt to PEGylate the C<sub>20</sub> peptide was discouraged by the following concerns: the large increase in molecular weight of PEGylated C<sub>20</sub> products and potential steric shielding would likely impede the binding of C<sub>20</sub> to the 5HB. (Monodisperse oligoethylene glycols available from Quanta BioSciences would likely also “work”, but are expensive and thus were not tested here.) Instead we decided to explore NMEG as a potential alternative to PEG, because NMEG is hydrophilic, like conventional PEG, but after HPLC purification is completely monodisperse, as compared with other commercially available PEG derivatives (even low-polydispersity PEGs, activated for reaction with amines, are expensive). Importantly, because *N*-substituted glycines such as the, oligoNMEGs cannot be recognized or cleaved by proteases<sup>38</sup>, we anticipated that the peptoid-peptide hybrids (therapeutics) formed by NMEGylation would be less susceptible to proteolytic degradation. As shown in Table I, a series of NMEGylated C<sub>20</sub> therapeutics were prepared by attaching NMEG oligomers (n = 1 – 10) to either the *N*- or *C*- terminus of the C<sub>20</sub>. The resulting NMEGylated therapeutics were found to be extremely soluble in aqueous buffer (up to > 10 mg/mL) as compared to C<sub>20</sub> (< 2 mg/mL). The relative hydrophilicity of NMEGylated C<sub>20</sub> analogues, based on percent acetonitrile at elution, was evaluated by RP-HPLC (see Table I).<sup>34,35</sup> A decrease in the percent acetonitrile at which modified peptides eluted demonstrates that NMEGylation, even with just a few monomers, dramatically enhances the hydrophilicity of the C<sub>20</sub> peptide.

### Biological activities of NMEGylated C<sub>20</sub> peptides

While greatly increasing peptide hydrophilicity and solubility, NMEGylation of C<sub>20</sub> with greater numbers of NMEG monomers (n = 3, 5, and 10) led to a significant loss of binding affinity to the 5HB target protein, as determined with the use of our previously reported fluorescence polarization (FP)-based assay.<sup>39</sup> Since the binding of the C<sub>20</sub> peptide to the

5HB protein involves a large interface, steric hindrance of binding could be caused by the NMEG oligomers, suggesting a need to optimize the precise number of NMEG oligomers incorporated. NMEGylated C<sub>20</sub> peptides comprising just one NMEG at either, or both, termini were prepared, and their biophysical properties and binding affinities toward the 5HB construct were evaluated. The addition of even a single NMEG to the C<sub>20</sub> peptide greatly improved its solubility and hydrophilicity. However, the binding affinity was significantly diminished, even by just one or two terminal NMEG monomers (Figure 2 and Table I).

To recover the biological activity of NMEGylated C<sub>20</sub> peptides, we decided to use an NMEG modifier attached through a flexible linker, to allow C<sub>20</sub> enough “space” to properly form its required  $\alpha$ -helical conformation upon binding to the 5HB. On the other hand, a linker region that is too flexible might lose too much entropy upon binding and consequently might also deleteriously affect the binding affinity. Glycine—one or a few—was chosen, because we anticipated that one glycine would provide the needed flexibility while maintaining a minimally sized peptide structure. NMEG-glycine-C<sub>20</sub> (NMEG-Gly-C<sub>20</sub>) showed significantly tighter binding affinity to the 5HB than the rest of NMEGylated C<sub>20</sub> analogues, with greatly improved solubility and hydrophilicity (Table I, Figure 2, and Supporting Information). Because glycine is known to be a helix breaker,<sup>40,41</sup> it is possible that the longer glycine linker might impede the binding affinities of therapeutics to the 5HB by increasing the flexibility of the peptide backbone structure (Figure S1 and S2, Supporting Information). The benefits of NMEGylating potential biotherapeutics should increase when the NMEG attachment is “pointed” directly away from the likely binding interface. Since the C<sub>20</sub> peptide likely forms a  $\alpha$ -helix upon binding to the helical bundle (5HB),<sup>42</sup> it makes sense that the direct attachment of *N*-substituted glycine (peptoid) monomers could disrupt the  $\alpha$ -helix due to the presence of the *N*-methoxyethyl side chain.<sup>43</sup>

### Serum stability of NMEGylated C<sub>20</sub> peptides, and unexpected results

As discussed earlier, PEGylation is known to prolong the half-life of biotherapeutics, thereby improving *in vivo* efficacy. We therefore examined whether NMEGylation can provide a similar beneficial effect, focusing on singly-NMEGylated therapeutics with the added glycine spacer; this small modification proved to be sufficient to greatly improve the C<sub>20</sub> peptide's aqueous solubility. NMEGylated C<sub>20</sub> peptides were incubated with human serum, which contains many proteases such as trypsin and elastase, at room temperature, and then analyzed by RP-HPLC to determine the amount of remaining intact C<sub>20</sub> peptide. Our resulting data (Figure 3), indicate that NMEGylation indeed enhances the serum stability of NMEGylated peptides as compared to the unmodified peptide. Interestingly, the protease stability of NMEG-Gly-C<sub>20</sub> was most greatly improved, suggesting that NMEG-Gly-C<sub>20</sub> could present comparable therapeutic efficacy to the C<sub>20</sub> peptide, but with a prolonged half-life.

### Conclusions

In this study, we demonstrate that NMEGylation potentially enhances multiple therapeutically desirable properties including the water solubility, hydrophilicity and serum stability of a polypeptide therapeutic. Our data also suggest that if this NMEGylation approach is taken, a minor modification of the target peptide, C<sub>20</sub>, changing its molar mass by less than < 5%, can be sufficient to achieve quite favorable biophysical properties. Furthermore, optimizing the length of the mono- or oligoNMEG modifier and the inclusion of glycine linker enabled the NMEGylated bioconjugate to substantially recover the binding affinity of the unmodified peptide to its biological target. While PEGylation is still a useful technique for large therapeutic proteins, it requires post-synthetic conjugation and multiple, subsequent purification steps to remove the undesired polydisperse PEGylated products.<sup>13</sup>

By contrast, NMEGylation can be used to modify short therapeutic peptides as well as proteins, quickly yielding highly monodisperse products at lower costs. Since NMEGylation is intrinsically and quite simply compatible with solid-phase peptide/peptoid synthesis, this approach has the advantage of low cost, negligible extension of the synthesis and purification time for the peptide, and of course, site-specific incorporation as part of the synthesis protocols. NMEGylated peptide/peptoid libraries could be designed at an early stage of molecular optimization by varying both the number and position of NMEG monomers and glycine or other flexible linker units. Based on these findings, we propose that NMEGylation may prove to be a broadly useful modification of peptide and protein therapeutics.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

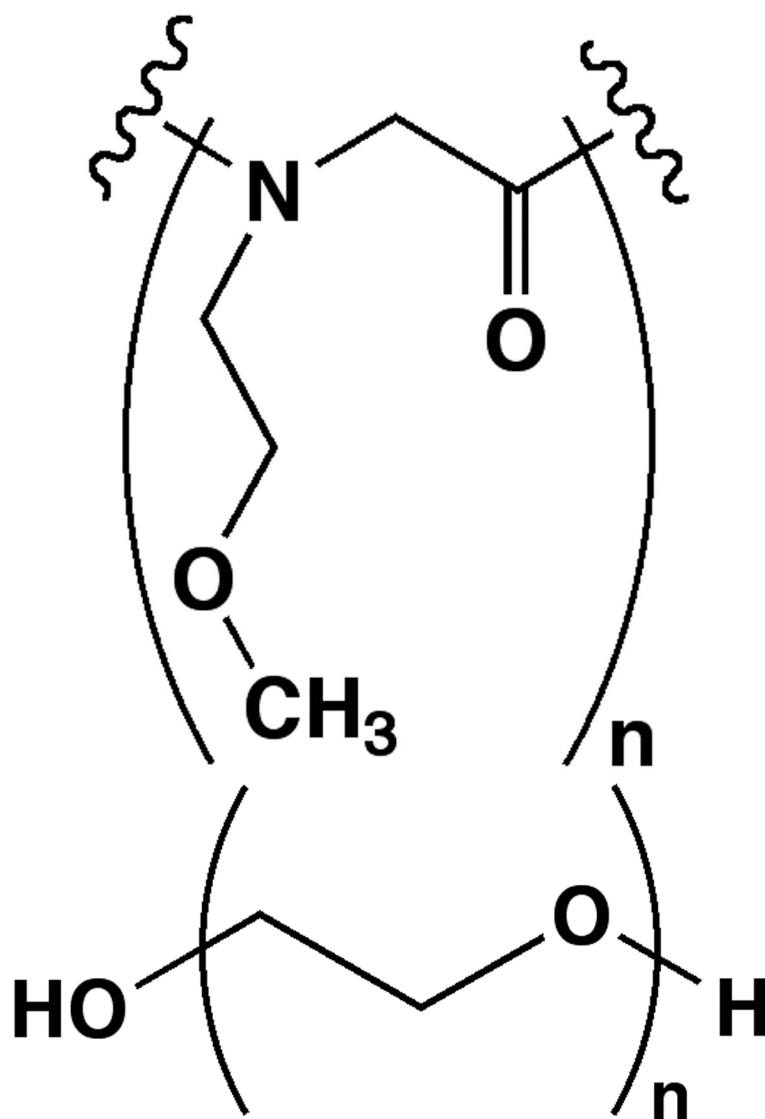
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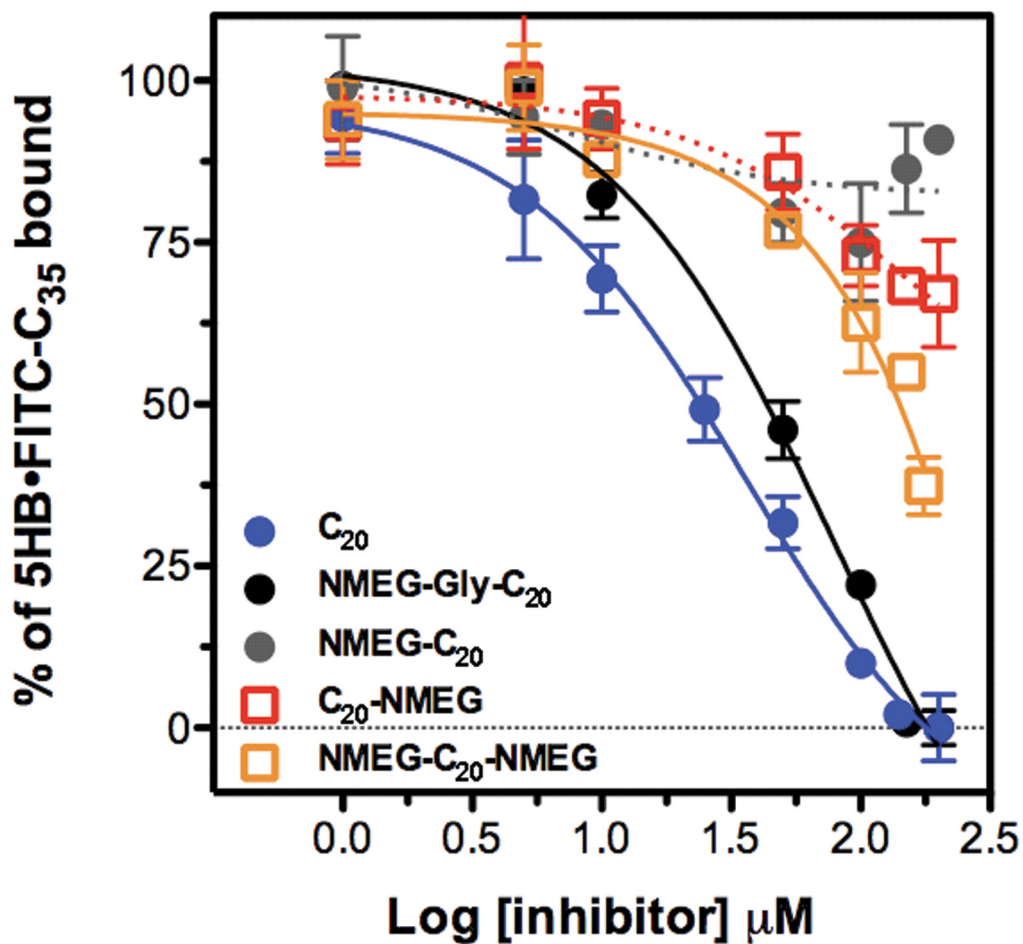
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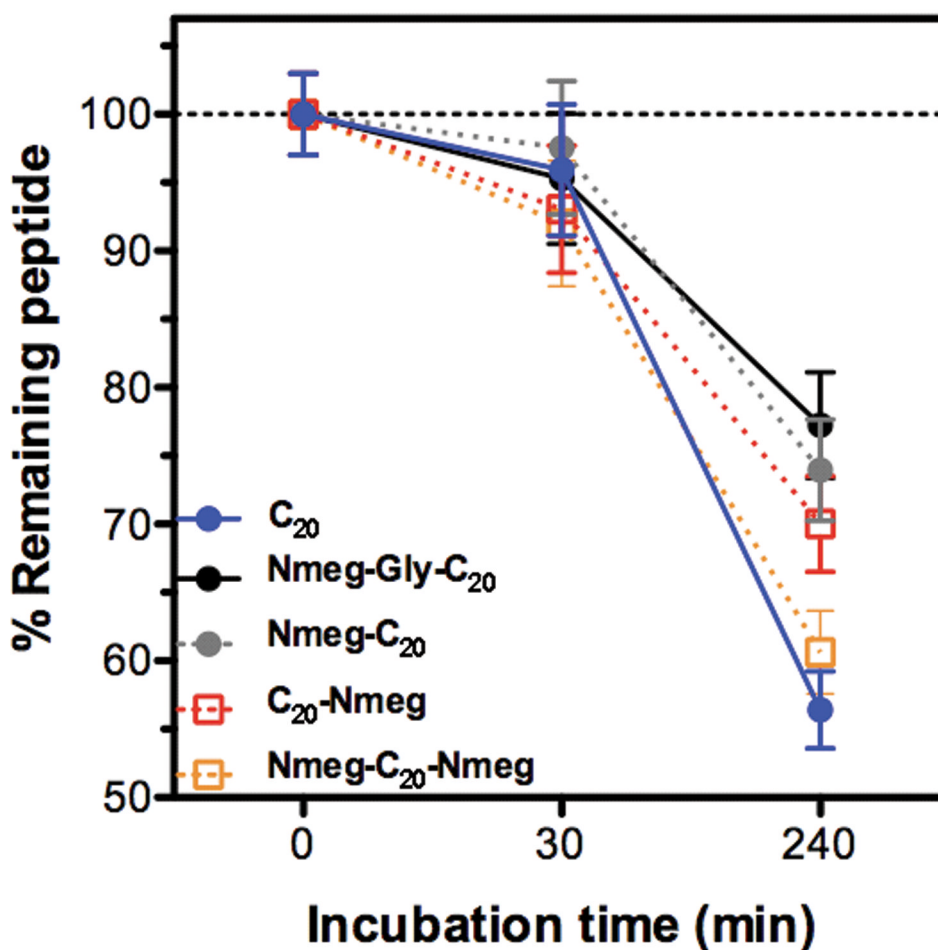
**FIGURE 1.** Structural comparison of (A) *N*-methoxyethylglycine (NMEG) and (B) polyethylene glycol (PEG).





**FIGURE 2. Data resulting from competitive FP assays**

The binding affinities of NMEGylated  $\text{C}_{20}$  peptide to the 5HB were determined by the use of a competitive FP assay. The “% 5HB-FI- $\text{C}_{35}$  bound” represents the capability of NMEGylated bioconjugates to displace a tracer (FI- $\text{C}_{35}$ ) peptide that binds to the target protein (5HB), and is presented as a function of the concentration of NMEGylated  $\text{C}_{20}$  peptides.



**FIGURE 3. Bioconjugate stability in the presence of serum**  
NMEGylated therapeutics were incubated in human plasma at 37 °C and sampled at certain time points, followed by RP-HPLC analysis. The amount of remaining peptide or peptide-peptoid conjugate at each time point was quantified against an enzyme-stable internal organic standard (benzyl alcohol).

**TABLE I**  
**NMEGylated C<sub>20</sub> bioconjugate sequences tested in this study with molar mass, purity, solubility, hydrophilicity and their IC<sub>50</sub> values**

Compounds	Sequences (amino to carboxy)	Molar mass (Da)	Calc./Observ.	Purity (%)	% AcN at elution <sup>a</sup>	IC <sub>50</sub> <sup>b</sup>
C <sub>20</sub>	ISQVNEKINWSLAFIRKSDE	2319.6/2319.0		> 95	78	36 ± 1
NMEG <sub>3</sub> -C <sub>20</sub>	NMEG <sub>3</sub> -ISQVNEKINWSLAFIRKSDE	2664.9/2664.4		> 95	60	> 200
NMEG <sub>10</sub> -C <sub>20</sub>	NMEG <sub>5</sub> -ISQVNEKINWSLAFIRKSDE	3470.3/3471.8		> 95	57	> 200
C <sub>20</sub> <sup>2</sup> -NMEG <sub>10</sub>	ISQVNEKINWSLAFIRKSDE-NMEG <sub>10</sub>	3468.9/3469.8		> 95	57	> 200
C <sub>20</sub> <sup>1</sup> -NMEG <sub>5</sub>	ISQVNEKINWSLAFIRKSDE-NMEG <sub>5</sub>	2894.2/2894.1		> 95	54	> 200
C <sub>20</sub> <sup>2</sup> -NMEG <sub>3</sub>	ISQVNEKINWSLAFIRKSDE-NMEG <sub>3</sub>	2663.9/2663.7		> 95	52	> 200
NMEG <sub>1</sub> -C <sub>20</sub>	NMEG <sub>1</sub> -ISQVNEKINWSLAFIRKSDE	2434.7/2334.0		> 95	52	> 200
C <sub>20</sub> <sup>2</sup> -NMEG <sub>1</sub>	ISQVNEKINWSLAFIRKSDE-NMEG <sub>1</sub>	2433.7/2432.9		> 95	52	> 200
NMEG <sub>1</sub> -C <sub>20</sub> -NMEG <sub>1</sub>	NMEG <sub>1</sub> -ISQVNEKINWSLAFIRKSDE-NMEG <sub>1</sub>	2548.8/2547.9		> 95	55	> 150
NMEG <sub>1</sub> -Gly-C <sub>20</sub>	NMEG <sub>1</sub> -G-ISQVNEKINWSLAFIRKSDE	2491.7/2491.1		> 90	55	81 ± 2

<sup>a</sup>% AcN = percent acetonitrile at RP-HPLC elution of each compound.

<sup>b</sup>The mean of IC<sub>50</sub> with standard error in μM.