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## Multivalent Fertilin $\beta$ Oligopeptides: the Dependence of Fertilization Inhibition on Length and Density

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### Summary

The sperm protein fertilin $\beta$ , a member of the ADAM family of proteins is implicated in sperm-egg binding in all mammals studied to date. Multivalent inhibitors containing the three-amino acid binding sequence of fertilin $\beta$ , ECD, have been shown previously to be more effective inhibitors of fertilization than their monovalent counterparts. In this work, we probed sperm-egg interactions by examining the potency of fertilization inhibition by polymers that contained from 3 to 70 ECD pharmacophores in densities ranging from 10-100%. These polymers were synthesized by ruthenium-catalyzed ring opening metathesis polymerization (ROMP). Evaluation of the polymer potencies, and synthesis of a triblock copolymer from 2 building blocks, revealed that two multivalent contacts are sufficient for maximal inhibition, and that the distance between ECD pharmacophores required is 7-9 monomers spanning 4-5 nm. We conclude that inhibition requires recruitment of two receptors on the egg surface into an inhibitory complex.

### Introduction

Fertilization of a mammalian oocyte by a single sperm is an extremely complex event. Many of the protein to protein and protein to carbohydrate interactions in the pathway are still unknown. The organization, localization, and binding properties of receptor-ligand complexes between sperm and egg still need to be characterized. Elucidation of the pathway and the mechanisms involved is critical for the design and synthesis of pharmaceuticals, which target population control and treat infertility. Sperm-egg recognition, binding and fusion events are dictated by a variety of multivalent receptor-ligand contacts. Using ring-opening metathesis polymerization (ROMP), we have developed synthetic ligands which mimic the multivalent display of sperm protein fertilin $\beta$  [1]. Here, we demonstrate the value of ROMP chemistry for discerning the optimal fertilin $\beta$  ligand presentation.

Fertilin $\beta$  is located in the equatorial region of the sperm head and is involved in sperm binding to the egg plasma membrane during fertilization [2-5]. Fertilin $\beta$  is a member of the ADAM (A Disintegrin and A Metalloprotease domain) family of proteins [6]. A tripeptide, glu-cys-asp (ECD) is the minimal recognition element necessary for the binding of fertilin $\beta$  to its egg receptor [7-10]. This recognition motif is taken from the sperm protein's disintegrin loop. Peptides containing the ECD motif inhibit sperm-egg adhesion *in vitro* [1,7,8,11-13]. For example, the IC<sub>50</sub> for inhibition of fertilization by the ECD monomer **1** is approximately 500  $\mu$ M. Peptides containing this sequence have been assayed in a diverse range of species, and the peptides inhibit fertilization in all of them [7,8,12,14-16]. Thus, ECD is a small pharmacophore around which inhibitors of fertilization may be designed.

Supplementary data including NMR spectra are available on line.

Regardless of their length or flanking sequence, all monomeric ECD peptides are poor inhibitors. Knowing that multivalent display of ligands often improves their affinity [17-20], we developed multivalent presentations of the ECD ligand [1,13,21]. Liposomes presenting 8 copies of the ECD ligand are 100-fold better inhibitors than the corresponding monomer [13, 21]. Polymers containing 10 copies of the ECD motif, e.g., **2**<sub>10</sub>, show 50 to 70-fold increased inhibition over the corresponding monomer [1]. These are the highest potencies reported to date for an ECD inhibitor. Incorporation of a fluorophore into the probes established that the target of the polyvalent ECD inhibitors is on the surface of the egg, and not on the sperm surface [21,22]. Previously, we synthesized an <sup>125</sup>I-labeled DECD peptide which both inhibited fertilization, and photoaffinity labeled integrin  $\alpha_6\beta_1$  [23]. Several other laboratories have also reported integrin  $\alpha_6\beta_1$  as being the receptor [12,24,25]. However, Myles and coworkers have established that mice with a conditional  $\beta_1$  integrin knockout in the egg are fertile [26] and that  $\alpha_6$  null eggs are still fertilized by wild-type sperm [27]. Little information has been obtained about the differences of the egg surface proteome between the integrin knockout eggs and wild-type eggs because of the extraordinarily small amounts of protein material that may be obtained. Clearly chemical methods that can be used to investigate the proteome are required. In this work, we advance ring-opening metathesis polymerization (ROMP) of peptide bearing monomers to define the optimal presentation of ECD ligands for future development of proteome probes that are of high affinity, and that have useful handles for identification of cell surface receptors, and co-receptors.

We employed ruthenium-catalyzed ROMP [28] for several reasons. Using ROMP, it is possible to prepare polymers of well-defined length. Ruthenium catalysts are very stable, readily available, and functional group tolerant. In addition, ROMP allows for the multivalent presentation of one or more functional groups along a polymer backbone. Monomer building blocks are constructed from strained cyclic alkenes, in our case 5-norbornene-*exo*-carboxylic acid. The utility of ROMP is that one can easily manipulate the length of a polymer by varying the monomer to catalyst ratio. Moreover, the pharmacophore density of a polymer is readily adjusted by feeding 2 simple monomer building blocks, one a pharmacophore and one a spacer, to the catalyst in varying ratios. The pharmacophore and spacer may be mixed in a random fashion [29-31]. In this work, we take advantage of the living nature of the polymerization catalyst to prepare block copolymer. Thus, using 1 or 2 simple monomer building blocks and ROMP chemistry, a family of polymers may be generated to determine the length and spacing requirements for maximal polymer affinity or avidity.

We synthesized a family of structurally diverse fertilin $\beta$  peptide polymers derived from **2**<sub>10</sub> using a combination of solution phase peptide synthesis and ROMP to define the best inhibitor structure (Figure 1). Polymers **3**<sub>10</sub>, **3**<sub>70</sub>, **4**<sub>10</sub>, **4**<sub>70</sub>, **3**<sub>8/472</sub>, **3**<sub>3</sub>, **3**<sub>6</sub>, and **3**<sub>2-47-3</sub><sub>2</sub> were tested as inhibitors in a mouse *in vitro* fertilization assay. These polymers were chosen to present a diversity of size and densities to the egg. With their assay, we explored the role of inhibitor length, and pharmacophore spacing. The chemical approach developed here is general and will be of use for exploring receptor-ligand interactions in many different biological systems.

## Results

*Synthesis of Polymers.* The peptide monomers **3** and **4** were synthesized using standard solution phase procedures with t-butyl/trityl based side-chain protection and Fmoc/Cbz  $\alpha$ -amino protection. Solution phase methods were used in order to obtain large quantities of pure peptide for ROMP. Polymers were prepared by ROMP using fully protected peptides and (H<sub>2</sub>IMes)(PCy<sub>3</sub>)Cl<sub>2</sub>Ru=CHPh or (H<sub>2</sub>IMes)(3-BrPyr)<sub>2</sub>Cl<sub>2</sub>Ru=CHPh as the catalyst in dichloromethane/methanol (3/1) with 3M LiCl to solubilize the polymer as previously described (Figure 1) [32]. Polymers were deprotected with a trifluoroacetic acid/water/triisopropylsilane cocktail and precipitated with diethyl ether. They were reduced with tris(2-carboxyethyl)phosphine,

precipitated with dilute acid, and resuspended in aqueous ammonium hydroxide at pH 7 before use in assays. Isolated yields were 63-95%. Integration of NMR spectra confirmed that the number of monomers incorporated into each polymer was the same as the initial monomer:catalyst ratio. Gel permeation chromatography (GPC) was used to analyze and monitor the molecular weight distribution of the polymer block intermediates in the synthesis of **3<sub>2</sub>-4<sub>7</sub>-3<sub>2</sub>**. The synthetic precursors **3<sub>2</sub>'** and **3<sub>2</sub>'-4<sub>7</sub>'**, and product **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** were analyzed with side-chain protection intact (Figure 2). For each reaction, the disappearance of monomer was monitored by TLC before addition of the subsequent monomer. Then, a portion of the polymerization reaction was quenched with ethylvinyl ether after each monomer addition for analytical purposes. The three protected polymers were analyzed by <sup>1</sup>H NMR spectroscopy and their monomer compositions corresponded to the monomers fed to the polymerization reaction. GPC of **3<sub>2</sub>'**, **3<sub>2</sub>'-4<sub>7</sub>'**, and **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** confirmed that none of the precursor block remained after addition of the subsequent monomer and that the number-average molecular weights shifted as expected (Figure 2). The final triblock copolymer **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** had a narrow polydispersity index of 1.21 consistent with a controlled, living polymerization.

*Assay of Polymers.* Polymers were assayed as inhibitors in a mouse in vitro fertilization assay. Sperm fusion was used as an endpoint to measure inhibition of sperm binding. Two measures of fertilization were calculated: fertilization rate (FR) and fertilization index (FI) [7]. The FR is the total number of eggs fertilized, divided by the total number of eggs. The FI is the total number of sperm fused divided by the total number of eggs. Inhibitor concentrations were varied over at least a 400-fold range to determine their IC<sub>50</sub>'s by both FR and FI. Concentrations were considered both in peptide ligand and in polymer (Table 1). For example, a 10 μM (in polymer) solution of **3<sub>10</sub>** contains 100 μM ECD ligand. Peptide ligand concentrations are useful for comparing the inhibition efficiencies of polymers containing different numbers of ligands.

*Sperm Susceptibility to Polymers.* Sperm were allowed to capacitate and acrosome react then treated with polymer and observed under a light microscope. The percentage of live, motile sperm remaining after 45 min was the same in control incubations, as in 500 μM polymer incubations (70 % ± 7 for **3<sub>10</sub>**, 76 % ± 11 for **3<sub>70</sub>**, 74 % ± 11 for **3<sub>2</sub>-4<sub>7</sub>-3<sub>2</sub>**, and 74 % ± 8 for the control). Sperm were also incubated with 125 μM **3<sub>2</sub>-4<sub>7</sub>-3<sub>2</sub>** for 45 min prior to insemination of eggs. In these experiments the final concentration of **3<sub>2</sub>-4<sub>7</sub>-3<sub>2</sub>** in the sperm-egg incubation buffer was below the IC<sub>50</sub>. There was no difference in FR or FI with pre-treated sperm compared to fertilization by control sperm that were incubated in buffer alone (Figure 3). Lastly, no inhibition of inhibition was observed in the presence of 500 μM negative control polymers **4<sub>10</sub>** or **4<sub>70</sub>** (Table 1).

## Discussion

Understanding protein-protein interactions in fertilization is of fundamental importance for elucidating the molecular display required for biological function. However, this biological system is not amenable to many methods typically used to probe protein interactions, for example phage display or mutagenesis of intact egg proteins, because of the limited quantities of material available to study gametes in mammalian systems and the lack of cell culture models. We have developed ROMP chemistry in order to advance our understanding of fertilization using polymer probes.

Previously in our laboratory, we demonstrated that a norbornyl polymer displaying the peptide sequence ECDVT, **2<sub>10</sub>**, is a significantly more potent inhibitor of fertilization than a simple monomeric peptide [1]. The first step to optimize polymer structure was to simplify the ligand appended to the polymer backbone. We prepared and tested **3<sub>10</sub>** and compared its potency to **2<sub>10</sub>**. Inhibition by **3<sub>10</sub>** was equipotent to that of **2<sub>10</sub>** (Table 1). These results demonstrate that, as expected, removal of valine and threonine to shorten the ligand sequence to ECD did not

impair inhibition. The polymer behaved similarly to **2<sub>10</sub>**. The shorter ligand improved solubility of the ROMP monomer precursors, as well as the polymer, and reduced the number of synthetic steps required.

In order to be certain that the inhibition observed was caused by the ECD binding motif, we prepared control polymers **4<sub>10</sub>** and **4<sub>60</sub>** with the mutant sequence ESA. We chose to mutate ECD rather than scramble it because two of the amino acids contain carboxylates. We were concerned that scrambling the peptide might not completely eliminate binding, and multivalent presentation would restore significant inhibition. We chose to mutate cysteine and aspartate because mutagenesis work by White and coworkers [33] and Evans and coworkers [9] showed that these residues were critical for binding. We retained the glutamate residue for solubility reasons. No inhibition of fertilization by polymers **4<sub>10</sub>** and **4<sub>70</sub>** was observed at polymer concentrations of 50  $\mu$ M and 8  $\mu$ M, respectively. Thus, the inhibition by **3<sub>10</sub>** observed is due to a specific interaction of the ECD peptide with a cell surface molecule.

Previous work had demonstrated that fluorescently labeled polymer **2<sub>10</sub>** binds to the surface of the egg, and not the sperm surface [22]. We tested whether the inhibition observed could be due to deleterious effects of polymer upon sperm. Polymers that only contained ESA peptide substituents: e.g. **4<sub>10</sub>** and **4<sub>70</sub>** showed no inhibition of fertilization. Incubation of sperm with ECD containing polymers did not affect their motility or viability. Sperm pre-treated with polymer **3<sub>2</sub>-4<sub>7</sub>-3<sub>2</sub>** before insemination in M-16 buffer were able to fertilize eggs normally (Figure 3). Therefore, treatment of sperm with our polymers does not affect sperm viability, motility, or penetrability. Targeting of the ECD polymers for the egg cell surface is responsible for inhibition.

A longer polymer should cluster many more receptors on the oocyte and have a statistical advantage that favors rebinding of ligand over dissociation, and therefore be a more potent sperm inhibitor. For example in studying glycopolymer inhibition of hemagglutination, Kanai and coworkers have shown that inhibition potency (as measured on a per residue basis) saturates at a length of about 50 residues [18]. Inhibition potency does not decrease as the polymer length increases beyond the length required to span binding sites as would be expected if only chelation were important [18]. Hence, we synthesized polymer **3<sub>70</sub>** which contained approximately 70 ECD ligands, and polymer **3<sub>8</sub>/4<sub>72</sub>**. Polymer **3<sub>8</sub>/4<sub>72</sub>** was approximately the same length polymer as **3<sub>70</sub>**, but only 10% of the peptides presented (randomly) were ECD ligand, the remainder were the ESA mutant sequence. To our surprise, we observed that the longer polymers showed decreased inhibition potency compared to **3<sub>10</sub>** when compared on the basis of peptide concentration (Table 1). When compared on the basis of polymer concentration **3<sub>10</sub>**, and **3<sub>70</sub>**, are essentially equipotent and **3<sub>8</sub>/4<sub>72</sub>** is less effective.

Previous investigations had demonstrated that if the cysteine residue of the ECD sequence is protected as a disulfide, no inhibition is observed [8,10]. We investigated the rate of thiol oxidation in the polymers under the conditions of the *in vitro* fertilization assay. If significant oxidation of the polymer thiols were to occur faster in the longer polymers, their potency would be reduced compared to the shorter polymers. We measured reduced thiol concentration as a function of time using 5,5'-dithiobis(2-nitrobenzoic acid) [34] in M16 buffer at 37 °C. For all of the polymers, less than 20% of the thiols were oxidized over the 45 minute incubation period of the assay, and the oxidation rate of **3<sub>10</sub>** did not differ significantly from the oxidation rates of **3<sub>70</sub>** or **3<sub>8</sub>/4<sub>72</sub>**.

We concluded that increasing the number of ligands presented and/or increasing the distance between ligands did not improve inhibitor potency. Our data imply that chelation of the receptor is more important than statistical factors favoring ligand rebinding. A polymer length of 10 represented the maximum length required for efficient inhibition. When modeled in the most

extended backbone configuration (*trans*-syndiotactic), the distance spanned by **3<sub>10</sub>** is at most 5.3 nm. We next sought to determine the minimum length of polymer required for inhibition.

We synthesized fully substituted ECD ligand 3-mers and 6-mers. Polymers **3<sub>3</sub>** and **3<sub>6</sub>** were both poor inhibitors, and showed no more potency than the ECD monomer **1**. These results indicated that multivalency was required for effective binding and inhibition, and that the 3-mer and 6-mer did not have sufficient length to span the distance between binding sites of two adjacent receptors. In the most extended configuration, **3<sub>6</sub>** can at most span 3 nm.

The poor inhibition of fertilization by polymer **3<sub>6</sub>** implied that the potency of **3<sub>10</sub>** was due to the ligation of the terminal ECD moieties and that the internal ECD peptides did not engage receptor-binding sites. The living nature of the ROMP polymerization allows the sequential addition of monomer building blocks to the growing polymer chain. We took advantage of this polymer property to construct **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** which contained on average two ECD ligands at either terminus separated by a 7-mer spacer comprised of biologically inactive ESA peptides. The block nature of the polymer was verified by gel permeation chromatography (GPC) analysis of **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** as well as the monoblock, and diblock precursors (Figure 2). There were no low molecular weight shoulders on the **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** peak, confirming that the polymerization was living and that the intact triblock polymer had been prepared.

The inhibition potency of polymer **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** was equal to polymer **3<sub>10</sub>** in polymer concentration, and slightly better when compared on the basis of peptide concentration. We conclude that the internal ECD ligands in polymer **3<sub>10</sub>** are not required for binding or inhibition (Figure 3). The distance between ECD ligands on an extended 9-mer is approximately 4.7 nm, and on an extended 10-mer is approximately 5.3 nm, indicating that the distance between receptor binding sites can be no greater than 5 nm (Figure 4). Typically membrane protein receptors are 2-3 nm in diameter. Based on the ineffective inhibition observed with polymer **3<sub>6</sub>**, fertilin $\beta$  receptor binding sites are more than 3 nm apart. Taken together, our polymers appear to engage the egg surface receptor in a bivalent fashion.

Our results are analogous to other systems in which bivalent inhibitors show high selectivity and affinity. For example, Portoghese and coworkers designed bivalent ligands for the opioid receptors [35]. Their results are consistent with a model in which two receptors are recruited into van der Waals contact, approximately 3 nm apart. Potent proteasome inhibitors have been designed using a similar strategy. Moroder and coworkers [36,37] synthesized inhibitors containing two arg-leu-arg peptides spaced by a 6.5 nm polyethylene glycol linker. The bivalent inhibitor was 124-fold more potent than the monomeric peptide. Potent inhibition required a linker that was flexible and long enough to span the distance between the active  $\beta$ -subunits of the proteasome.

Our polymer assay results are consistent with a bivalent inhibition model like that of the opioid receptor or the proteasome. Using block synthesis of copolymers, we conclude that inhibition of fertilization by fertilin $\beta$  mimics requires recruitment of two receptors on the egg surface into an inhibitory complex. The recruitment of additional receptors with longer polymers does not enhance inhibition potency. These experiments establish the parameters for future polymer design and testing. The structural requirements defined here will be incorporated into future polymers that include tagging elements for probing the identity of egg surface fertilin $\beta$  receptors.

## Significance

The mechanisms by which spermatozoa bind and fuse to the oocyte plasma membrane during mammalian fertilization are still unknown. In this work, we utilized the fertilin $\beta$  binding site peptide ECD to define the molecular structure required for optimal inhibition of *in vitro*

fertilization, a measure of sperm-egg binding. Using two simple monomeric building blocks, a diverse series of polymers was synthesized by ring-opening polymerization (ROMP) of norbornene. We introduce block copolymer synthesis as a method to optimize polymer potency and to rapidly identify required spacer lengths. In our polymer series, length of spacer between ECD pharmacophores, and numbers of ECD pharmacophores presented to the egg were varied. Polymers tested contained as few as 3 ECD ligands and as many as 70 ECD ligands. The most potent fertilin $\beta$ -derived inhibitor of fertilization to date is an 11-mer polymer that contains two ECD ligands at either terminus separated by seven spacer monomers. These results indicate that two ECD ligands bound to the egg surface separated by 4-5 nm are sufficient for maximal inhibition. Definition of the optimal polymer structure for inhibition now allows the design of molecular probes to identify the egg cell surface members of the inhibitory complex.

## Experimental Procedures

*Materials.* Amino acids and coupling agents used were purchased from Advanced Chem Tech. (Louisville, KY) or PerSeptive Biosystems (Framingham, MA). Solvents and chemical reagents were obtained from Fisher Scientific Inc (Springfield, NJ), or Sigma-Aldrich (Milwaukee, WI). Pregnant Mare's Serum Gonadotropin (PMSG, #367222), hyaluronidase (#H3506), and Hoechst-33342, were purchased from Sigma-Aldrich and human Chorionic Gonadotropin (hCG, #230734) was obtained through NHPP, NIDDK and Dr. A. F. Parlow.  $(\text{H}_2\text{IMes})(\text{PCy}_3)\text{Cl}_2\text{Ru}=\text{CHPh}$  was purchased from Sigma-Aldrich (Milwaukee, WI).  $(\text{H}_2\text{IMes})(\text{BrPyr})_2\text{Cl}_2\text{Ru}=\text{CHPh}$  was prepared according to the literature [38].  $\text{CH}_2\text{Cl}_2$  was freshly distilled from CaH;  $\text{CH}_3\text{OH}$ , and  $\text{Et}_2\text{O}$ , were used without further purification. LiCl was oven-dried and stored over  $\text{P}_2\text{O}_5$  before use. All reactions were carried out under an Ar atmosphere in oven-dried glassware unless otherwise specified. Moisture and oxygen-sensitive reagents were handled in an  $\text{N}_2$ -filled drybox. 5-Norbornene-*exo*-carboxylic acid was synthesized according to the literature [19].

*General Methods.* Analytical thin layer chromatography (TLC) was performed on pre-coated silica gel plates (60F254), and flash chromatography on silica gel-60 (230-400 mesh). TLC spots were detected by UV light and by staining with phosphomolybdic acid (PMA). Peptides were purified by flash column chromatography on silica gel-60. The purities of all peptide monomers were assessed by RPHPLC using a Vydac C18 column. Gradient elution was performed at 1 mL/min with acetonitrile and water (both containing TFA, 0.1%). The purity of the polymers was assessed by aqueous phase gel permeation chromatography (BioSep-SEC-S2000) using 50 mM potassium phosphate, pH 7. Inova400, Inova500, and Inova600 MHz NMR spectrometers were used to perform NMR analysis, and spectra were recorded in  $\text{CDCl}_3$  unless otherwise noted.  $^1\text{H}$  NMR spectra are reported as chemical shift in parts per million (multiplicity, coupling constant in Hz, integration).  $^1\text{H}$  NMR data are assumed to be first order. The usual workup for peptide coupling reactions was three washes of the  $\text{CH}_2\text{Cl}_2$  solution with 5%  $\text{NaHCO}_3$  followed by three washes with 1N HCl and drying of the  $\text{CH}_2\text{Cl}_2$  over  $\text{Na}_2\text{SO}_4$ . After evaporation of solvent, the peptide product was purified by flash silica chromatography.

PMSG and hCG were resuspended in sterile PBS to 10 IU/100  $\mu\text{L}$ . These solutions were stored at  $-20^\circ\text{C}$ . Hyaluronidase was re-suspended in sterile water to a final concentration of 30 mg/mL and stored at  $-20^\circ\text{C}$ . Hoechst-33342 was stored in the dark at  $4^\circ\text{C}$ . All the steps involving the use of Hoechst dye were performed with minimum exposure to light.

*Fmoc-Cys(trt)Asp(OtBu)-OMe.* H-Asp(OtBu)-OMe·HCl (16.69 mmol, 4.00 g), Fmoc-Cys(trt)-OMe (18.36 mmol, 10.75 g), EDC·HCl (20.03 mmol, 3.83 g), HOBt-hydrate (20.03 mmol, 3.07 g) were dissolved in 40 mL of dry  $\text{CH}_2\text{Cl}_2$  and cooled to  $0^\circ\text{C}$ . DIEA (18.36 mmol, 3.25 mL) was added, and the reaction was stirred for 4 h at rt. The usual workup and chromatography

(acetone:CH<sub>2</sub>Cl<sub>2</sub>/1:20) yielded Fmoc-Cys(trt)Asp(OtBu)-OMe (12.7 g, 98%) as a white powder. <sup>1</sup>H-NMR (400 MHz) δ 7.74 (t, J = 7.6 Hz, 2), 7.57(d, J = 4.8 Hz, 2H), 7.31 (m, 19H), 6.78(d, J = 7.6 Hz, 1H), 5.01(d, J = 6.8 Hz, 1H), 4.71 (m, 1H), 4.34(m, 2H), 4.19 (t, J = 7.0 Hz, 1H), 3.76 (dd, J = 6.8, 6.8 Hz, 1H), 3.66 (s, 3H), 2.89 (dd, J = 17.0, 4.6 Hz, 1H), 2.73 (m, 1H), 2.65 (m, 2H), 1.38(s, 9H). **Fmoc-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe.** Fmoc-Cys(trt)Asp(OtBu)-OMe, (5.19 mmol, 4.00 g) was dissolved in 10 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. 1-Octanethiol (51.9 mmol, 9.04 mL), and DBU (0.52 mmol, 78 μL) were added, and stirred for 15 h at rt under Ar. After evaporation of the solvent, the resulting product was purified by flash chromatography eluting with a step gradient ranging from 2% to 50% EtOAc/CH<sub>2</sub>Cl<sub>2</sub>. H-Cys(trt)Asp(OtBu)-OMe (2.09 g, 73%) was obtained as a fine white powder. H-Cys(trt)Asp(OtBu)-OMe, (3.65 mmol, 2.00 g), Fmoc-Glu(OtBu)-OH (4.02 mmol, 1.71 g), EDC·HCl (4.38 mmol, 0.84 g), HOBT-hydrate (4.38 mmol, 0.67 g) were dissolved in 10 mL of dry CH<sub>2</sub>Cl<sub>2</sub> and cooled to 0 °C. DIEA (4.02 mmol, 0.71 mL) was added, and the reaction was stirred for 5 h at rt. The usual workup and chromatography (acetone: CH<sub>2</sub>Cl<sub>2</sub>/1:20) yielded Fmoc-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe (3.04 g, 87%) as a white powder. <sup>1</sup>H-NMR (400 MHz) δ 7.76 (d, J = 7.6 Hz, 2H) 7.56 (t, J = 7.2 Hz, 2H), 7.40 (m, 8H), 7.28 (m, 8H), 7.18 (m, 3H), 6.90 (d, J = 8.4 Hz, 1H), 6.61(d, J = 7.6 Hz, 1H), 5.84 (d, J = 6.4 Hz, 1H), 4.72 (m, 2H), 4.35 (d, J = 7.2 Hz, 2H), 4.15 (m, 2H), 4.04 (m, 1H), 3.67 (s, 3H), 2.82 (m, 2H), 2.67 (dd, J = 17.0 and 4.8 Hz, 1H), 2.57 (dd, J = 13.2 and 5.2 Hz, 1H), 2.40 (m, 1H), 2.30 (m, 1H), 2.03 (m, 1H), 1.88 (m, 1H), 1.44 (s, 9H), 1.40 (s, 9H).

**Ac-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe.** Fmoc-Glu(OtBu)Cys(trt)Asp-(OtBu)-OMe, (0.31 mmol, 300 mg) was dissolved in 1 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. 1-Octanethiol (3.14 mmol, 546 μL), and DBU (0.031 mmol, 30 μL) were added, and stirred for 16 h at rt. The resulting product was purified by flash chromatography eluting with a step gradient ranging from 2% to 50% EtOAc/CH<sub>2</sub>Cl<sub>2</sub>. H-Glu(OtBu)Cys(trt)Asp-(OtBu)-OMe (163 mg, 72%) was obtained as a fine white powder. H-Glu(OtBu)Cys(trt)Asp-(OtBu)-OMe (0.27 mmol, 198 mg) was dissolved in 3 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. Acetic anhydride (2.70 mol, 257 μL) was added to the reaction, and followed by DIEA (0.82 mmol, 114 μL) and the reaction stirred for 1 h at rt under Ar. The usual workup and recrystallization of the crude product from CH<sub>2</sub>Cl<sub>2</sub> yielded Ac-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe (163 mg, 78 %) as a white powder. <sup>1</sup>H-NMR (400 MHz) δ 7.42 (m, 6H) 7.30 (m, 6H), 7.21 (m, 3H), 6.86 (d, J = 8.4 Hz, 1H), 6.70 (d, J = 7.6 Hz, 1H), 5.53 (d, J = 6.8 Hz, 1H), 4.70 (m, 1H), 4.33 (m, 1H), 4.01 (m, 1H), 3.67 (s, 3H), 2.84 (dd, J = 16.8 and 5.2 Hz, 1H), 2.77 (dd, J = 13.2 and 8.0 Hz, 1H), 2.67 (dd, J = 16.8 and 4.8 Hz, 1H), 2.58 (dd, J = 12.8 and 5.2 Hz, 1H), 2.40 (m, 1H), 2.28 (m, 1H), 2.03 (m, 1H), 1.97 (s, 3H), 1.88 (m, 1H) 1.43 (s, 9H), 1.40 (s, 9H). ESI mass spectrum: Calcd (MNa<sup>+</sup>) 798.35; Found 798.52.

**Ac-GluCysAsp-OMe, 1.** Ac-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe, was deprotected in a cocktail of H<sub>2</sub>O, TIPS, and TFA (2.5:2.5:95) for 5 h. The reaction mixture was concentrated with N<sub>2</sub> and precipitated with cold Et<sub>2</sub>O. The precipitate was dissolved in 10% Acetonitrile in H<sub>2</sub>O, and reduced with DTT (10 mM) for 1 h with stirring at 37 °C. Pure deprotected product was isolated as a white solid by reversed-phase C18 HPLC (1 cm × 30 cm, 5 μ) using 0.1% TFA/H<sub>2</sub>O and a linear gradient of CH<sub>3</sub>CN. ESI mass spectrum: Calcd (MH<sup>+</sup>) 420.12, Found 420.16.

**NB-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe, 3.** H-Glu(OtBu)Cys(trt)Asp-(OtBu)-OMe (0.22 mmol, 163 mg), 5-norbornene-*exo*-carboxylic acid (0.24 mmol, 34 mg), TBTU (0.24 mmol, 78 mg), HOBT-hydrate (0.08 mmol, 13 mg) were dissolved in 2 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. DIEA (0.24 mmol, 43 μL) was added, and the reaction was stirred for 1 h at rt. The usual workup and chromatography (acetone:CH<sub>2</sub>Cl<sub>2</sub>/1:20) yielded **3** (144 mg, 76%) as a white powder. <sup>1</sup>H-NMR (400 MHz) δ 7.41 (m, 6H) 7.29 (m, 6H), 7.21 (m, 3H), 6.94 (d, J = 8.4 Hz, 1H), 6.73 (m, 1H), 6.67 (m, 1H), 6.11 (m, 1H), 6.02 (m, 1H), 4.72 (m, 1H), 4.29 (m, 1H), 4.04 (m, 1H), 3.67 (s, 3H), 2.92 (m, 1H), 2.83 (m, 3H), 2.70 (m, 1H), 2.48 (m, 2H), 2.29 (m, 1H), 2.02 (m, 2H), 1.88 (m, 4H), 1.64 (d, J = 7.2 Hz, 1H), 1.44 (d, J = 5.6 Hz, 9H), 1.41 (s, 9H), 1.27 (m, 2H). <sup>13</sup>C-

NMR (400 MHz)  $\delta$  27.15, 27.21, 28.23, 28.28, 30.72, 30.77, 32.19, 32.24, 33.70, 33.77, 37.48, 41.78, 41.81, 44.77, 46.57, 46.63, 47.24, 47.39, 49.10, 52.39, 52.44, 52.69, 53.54, 53.64, 67.40, 81.43, 81.76, 127.11, 128.28, 128.29, 129.79, 136.08, 136.24, 138.34, 138.47, 144.58, 169.67, 169.94, 171.05, 171.36, 171.37, 173.68, 173.69, 176.58.

**Cbz-Ser(tBu)Ala-OMe.** H-Ala-OMe-HCl (27.20 mmol, 3.80 g), Cbz-Ser(tBu)-OMe (29.91 mmol, 8.84 g), EDC-HCl (32.64 mmol, 6.26 g), HOBt-hydrate (32.64 mmol, 5.00 g) were dissolved in 25 mL of dry CH<sub>2</sub>Cl<sub>2</sub> and cooled to 0 °C. DIEA (29.91 mmol, 5.30 mL) was added, and the reaction was stirred for 12 h at rt. The usual workup and chromatography (acetone:CH<sub>2</sub>Cl<sub>2</sub>/1:20) yielded Cbz-Ser(tBu)Ala-OMe (7.81 g, 76%) as a white powder. <sup>1</sup>H-NMR (400 MHz)  $\delta$  7.34 (m, 5H), 5.73 (m, 1H), 5.12 (m, 2H), 4.57 (m, 1H), 4.23 (m, 1H), 3.81 (m, 1H), 3.74 (s, 3H), 3.38 (t, J = 8.4 Hz, 1H), 1.40 (d, J = 7.2 Hz, 3H), 1.21 (s, 9H).

**Cbz-Glu(OtBu)Ser(tBu)Ala-OMe.** Cbz-Ser(tBu)Ala-OMe (1.62 mmol, 616 mg) was dissolved in 3 mL of MeOH. 10% Pd/C (0.16 mmol, 17 mg) was added, and stirred for 3 h at rt under H<sub>2</sub>. After filtration of the catalyst with celite, the resulting amine product was used without further purification. H-Ser(tBu)Ala-OMe (1.62 mmol, 400 mg), Cbz-Glu(OtBu)-OH (1.79 mmol, 604 mg), TBTU (1.34 mmol, 430 mg), HOBt-hydrate (0.45 mmol, 69 mg) were dissolved in 3 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. DIEA (2.60 mmol, 460  $\mu$ L) was added, and the reaction was stirred for 12 h at rt. The usual workup and chromatography (acetone:CH<sub>2</sub>Cl<sub>2</sub>/1:20) yielded **8** (627 mg, 68%) as a white powder. <sup>1</sup>H-NMR (400 MHz)  $\delta$  7.33 (m, 6H) 7.01 (d, J = 6.8, 1H), 5.72 (d, J = 6.4 Hz, 1H), 5.10 (m, 2H), 4.56 (q, 1H), 4.43 (m, 1H), 4.23 (m, 1H), 3.81 (m, 1H), 3.73 (s, 3H), 3.36 (t, J = 7.6 Hz, 1H), 2.38 (m, 2H), 2.12 (m, 1H), 1.95 (m, 1H), 1.43 (s, 9H), 1.39 (d, J = 7.2 Hz, 3H), 1.21 (s, 9H).

**NB-Glu(OtBu)Ser(tBu)Ala-OMe, 4.** Cbz-Glu(OtBu)Ser(tBu)Ala-OMe (0.53 mmol, 300 mg) was dissolved in 3 mL of MeOH. 10% Pd/C (0.053 mmol, 6 mg) was added, and stirred for 2 h at rt under H<sub>2</sub>. After filtration of the catalyst with celite, the resulting amine product was used without further purification. H-Glu(OtBu)Ser(tBu)Ala-OMe (0.51 mmol, 220.0 g), 5-norbornene-*exo*-carboxylic acid (0.56 mmol, 78 mg), TBTU (0.56 mmol, 180 mg), HOBt-hydrate (0.19 mmol, 29 mg) were dissolved in 3 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. DIEA (0.61 mmol, 108  $\mu$ L) was added, and the reaction was stirred for 3 h at rt. The usual workup and chromatography (acetone:CH<sub>2</sub>Cl<sub>2</sub>/2:10) yielded **4** (226 mg, 74%) as a white powder. <sup>1</sup>H-NMR (400 MHz)  $\delta$  7.38 (t, J = 8.0 Hz, 1H) 7.02 (t, J = 7.4 Hz, 1H), 6.82 (d, J = 5.6 Hz, 1H), 6.12 (m, 2H), 4.56 (q, 1H), 4.44 (m, 1H), 4.38 (m, 1H), 3.86 (m, 1H), 3.73 (s, 3H), 3.37 (m, 1H), 2.96 (m, 1H), 2.91 (m, 1H), 2.47 (m, 1H), 2.37 (m, 1H), 2.13 (m, 1H), 2.07 (m, 1H), 2.00 (m, 1H), 1.90 (m, 1H), 1.65 (m, 1H), 1.45 (d, J = 4.0 Hz, 9H), 1.41 (dd, J = 7.2 and 0.8 Hz, 3H), 1.35 (m, 2H), 1.19 (d, J = 4.8 Hz, 9H) <sup>13</sup>C-NMR (400 MHz)  $\delta$  18.30, 27.47, 27.52, 28.23, 30.62, 30.79, 32.25, 41.74, 41.76, 44.62, 44.67, 46.51, 46.57, 47.22, 47.37, 48.38, 48.41, 52.45, 52.47, 53.24, 53.87, 53.89, 61.48, 74.13, 74.18, 81.32, 81.33, 136.12, 138.40, 169.86, 169.88, 171.35, 171.38, 173.07, 173.62, 176.54, 176.55.

**ROMP.** The procedure detailed below for polymer **310** is representative of the procedure followed for synthesis of all of the homopolymers and the random copolymer. Scale, yield, and spectra are presented for each of the individual polymers. Number of ligands (n) is based on the monomer:catalyst ratio used in the synthesis and confirmed by integration in the <sup>1</sup>H-NMR spectra.

**310.** NB-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe **3** (82  $\mu$ mol, 70 mg) was dissolved in 100  $\mu$ L of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1). To the reaction was added oven-dried LiCl (1.2 mmol, 51 mg) and (H<sub>2</sub>IMes)(PCy<sub>3</sub>)Cl<sub>2</sub>Ru=CHPh (8  $\mu$ mol, 7 mg) dissolved in 50  $\mu$ L of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) and an additional 250  $\mu$ L of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1). The reaction was stirred for 3 h. Ethyl vinyl ether (1 mL) was added to quench the reaction and the mixture stirred for an additional 40 min.



After removing the solvent, the residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub>. The solution was washed three times with H<sub>2</sub>O, dried with Na<sub>2</sub>SO<sub>4</sub>, concentrated by rotary evaporation, and precipitated with cold Et<sub>2</sub>O. Product was isolated by centrifugation and dried. Crude protected polymer was deprotected with TFA/TIPS/H<sub>2</sub>O (95/2.5/2.5) for 5 h. The reaction mixture was concentrated with N<sub>2</sub> and precipitated with cold Et<sub>2</sub>O. The precipitate was collected by centrifugation. Polymer was dissolved in H<sub>2</sub>O (1 mL) at pH 6 and reduced with 10-20 mM tris (2-carboxyethyl)phosphine (TCEP) for 2 h at 37 °C. Reduced polymer was isolated by precipitation with 1N HCl (200 µL). Residual TCEP was removed by repeated washing of the precipitate with H<sub>2</sub>O (3 × 1 mL). **3<sub>10</sub>**, a yellowish white solid, was collected (41 mg, 90%), dried, and stored at -20 °C. <sup>1</sup>H-NMR (400 MHz, D<sub>2</sub>O) δ 7.24 (m) 5.34 (m), 4.65-4.05 (with max at 4.61, 4.42, 4.23), 3.61 (br s), 2.90-2.29 (with max at 2.80, 2.58, 2.45, 2.25), 2.22-1.45 (with max at 2.142, 1.86, 1.78), 1.18 (br s).

**3<sub>3</sub>**. NB-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe **3** (82 µmol, 70 mg) and (H<sub>2</sub>IMes)(BrPyr)<sub>2</sub>Cl<sub>2</sub>Ru=CHPh (27 µmol, 24 mg) in a total volume of 400 µL yielded **3<sub>3</sub>** as a brownish white solid (30 mg, 82%). <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O) δ 7.26 (m) 5.35 (m), 4.65-4.01 (with max at 4.55, 4.26, 4.04), 3.24 (br s), 2.90-2.29 (with max at 2.81, 2.51), 2.22-1.45 (with max at 2.12, 1.93, 1.78), 1.19 (m).

**3<sub>6</sub>**. NB-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe **3** (82 µmol, 70 mg) and (H<sub>2</sub>IMes)(BrPyr)<sub>2</sub>Cl<sub>2</sub>Ru=CHPh (14 µmol, 12 mg) in a total volume of 400 µL yielded **3<sub>6</sub>** as a brownish white solid (45 mg, 95%). <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O) δ 7.26 (m) 5.45-5.10 (with max at 5.36, 5.29, 5.26), 4.65-4.05 (with max at 4.59, 4.45, 4.27), 3.64 (br s), 2.90-2.45 (with max at 2.83, 2.63), 2.22-1.45 (with max at 2.21, 1.94, 1.84, 1.61), 1.21 (m).

**3<sub>70</sub>**. NB-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe **3** (17 µmol, 15 mg) and (H<sub>2</sub>IMes)(PCy<sub>3</sub>)Cl<sub>2</sub>Ru=CHPh (0.17 µmol, 0.15 mg) in a total volume of 150 µL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) yielded a yellowish white solid (8 mg, 80%). <sup>1</sup>H-NMR (400 MHz, D<sub>2</sub>O) δ 7.20 (m), 5.23 (m), 4.44-4.09 (with max at 4.39, 4.08), 3.67 (br s), 3.22 (s), 3.15-1.45 (with max at 2.95, 2.80, 2.10, 1.91, 1.81, 1.60), 1.38-1.79 (with max at 1.23, 1.15, 0.90).

**4<sub>10</sub>**. NB-Glu(OtBu)Ser(OtBu)Ala-OMe **4** (38 µmol, 21 mg) and (H<sub>2</sub>IMes)(PCy<sub>3</sub>)Cl<sub>2</sub>Ru=CHPh (13.8 µmol, 3.3 mg) in a total volume of 200 µL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) yielded **4<sub>10</sub>** as a brownish white solid (10 mg, 67%). <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O) δ 4.29 (m), 5.23 (br, s), 4.29 (m), 4.00 (m), 3.65 (br, s), 3.20 (s), 3.10-2.30 (with max at 2.80, 2.45), 2.25-1.40 (with max at 2.10, 1.90, 1.79, 1.45), 1.20 (m), 1.00 (m).

**4<sub>60</sub>**. NB-Glu(OtBu)Ser(OtBu)Ala-OMe **4** (47 µmol, 26 mg) and (H<sub>2</sub>IMes)(PCy<sub>3</sub>)Cl<sub>2</sub>Ru=CHPh (0.47 µmol, 0.40 mg) in a total volume of 200 µL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) yielded **4<sub>60</sub>** as a brownish white solid (12 mg, 63%). <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O) δ 7.20 (m), 5.80-5.20 (m), 3.45 (br, s), 2.2-1.4 (with max at 2.19, 1.99, 1.68), 1.38-0.80 (with max at 1.23, 1.15).

**3<sub>8</sub>/4<sub>72</sub>**. NB-Glu(OtBu)Cys(trt)Asp(OtBu)-OMe **3** (12 µmol, 10 mg), NB-Glu(OtBu)Ser(OtBu)Ala-OMe **4** (106 µmol, 58 mg) and (H<sub>2</sub>IMes)(PCy<sub>3</sub>)Cl<sub>2</sub>Ru=CHPh (1.17 µmol, 0.99 mg) in a total volume of 400 µL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) yielded **3<sub>8</sub>/4<sub>72</sub>** as a yellowish white solid (36 mg, 73%). <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O) δ 7.22 (m) 5.45-5.10 (with max at 5.34, 5.23, 5.16), 4.65 (br s), 4.39-4.05 (with max at 4.31, 4.04), 3.75 (br s), 3.63 (br s), 2.85 (m), 2.48 (m), 2.22-1.41 (with max at 2.13, 1.90, 1.81, 1.60), 1.40-1.00 (with max at 1.31, 1.23, 1.12), 0.92 (br s).

**3<sub>2</sub>'**, **3<sub>2</sub>'-4<sub>7</sub>'**, **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'**, **3<sub>2</sub>-4<sub>7</sub>-3<sub>2</sub>**. In three separate flasks, A, B, and C, peptide **3** (23 µmol, 20 mg) was dissolved in 100 µL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1). Then oven-dried LiCl (1.2 mmol, 51 mg) and (H<sub>2</sub>IMes)(BrPyr)<sub>2</sub>Cl<sub>2</sub>Ru=CHPh (12 µmol, 10 mg) dissolved in 50 µL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) were added to each flask. The reactions were stirred for 1.5 h. To flask A, ethyl vinyl ether (2 mL) was added and the mixture was stirred for 40 min to quench the reaction

yielding **3<sub>2</sub>'**. To flasks B and C, peptide **4** (82 μmol, 45 mg) dissolved in 250 μL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) was added, and the reactions were stirred for 1.5 h. Ethyl vinyl ether (2 mL) was added to flask B, and the mixture was stirred for 40 min to yield **3<sub>2</sub>'-4<sub>7</sub>'**. Then peptide **3** (23 μmol, 20 mg) dissolved in 250 μL of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (3/1) was added to flask C and the reaction was stirred for 2 h. Ethyl vinyl ether (2 mL) was added to quench the reaction and the mixture was stirred for an additional 40 min to yield **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'**. For each reaction, TLC was used to confirm complete disappearance of monomer before addition of the next monomer. After removing the solvent from each of the flasks, the residues were dissolved separately in CH<sub>2</sub>Cl<sub>2</sub>. The solutions were washed three times with H<sub>2</sub>O, dried with Na<sub>2</sub>SO<sub>4</sub>, and the products **3<sub>2</sub>'**, **3<sub>2</sub>'-4<sub>7</sub>'**, and **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** were precipitated with cold Et<sub>2</sub>O. The precipitates were isolated by centrifugation and dried. Each of the polymers was analyzed by gel permeation chromatography using a Phenogel column (5 μ, 300 × 4.60 mm, linear mixed bed, 100-10<sup>6</sup> MW range). Elution was performed at 0.3 mL/min with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>. Narrowly dispersed polystyrene standards from Aldrich were used as molecular weight calibrants. The number average and weighted average molecular weights were calculated from the chromatogram to determine the polydispersity index (PDI).

**3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'**. Crude protected polymer **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'** was deprotected with TFA/TIPS/H<sub>2</sub>O (95/2.5/2.5) for 5 h. The reaction mixture was concentrated with N<sub>2</sub> and the polymer precipitated with cold Et<sub>2</sub>O and isolated by centrifugation. The polymer was dissolved in H<sub>2</sub>O (1 mL) at pH 6 and reduced with 10-20 mM TCEP for 2 h at 37 °C. Deprotected polymer was isolated by precipitation with 1N HCl (200 μL). Residual TCEP was removed by repeated washing with H<sub>2</sub>O (3 × 1 mL). **3<sub>2</sub>'-4<sub>7</sub>'-3<sub>2</sub>'**, a yellowish white solid (40 mg, 88%), was collected, dried, and stored at -20 °C. <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O) δ 7.26 (m), 5.35 (m), 4.40-4.01 (with max at 4.35, 4.27, 4.22, 4.06, 4.04), 3.77 (br s), 3.65 (br s), 3.25 (br s), 2.90-2.29 (with max at 2.85, 2.51), 2.22-1.45 (with max at 2.16, 1.93, 1.84, 1.62), 1.34 (br s), 1.26-1.02 (with max at 1.25, 1.15), 0.94 (br s).

*Assay for thiol oxidation.* 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was prepared as a 5 mM stock solution in 100 mM potassium phosphate buffer solution, pH 7.2 containing 0.1 mM EDTA and stored in the dark at 4 °C. All polymers were fully reduced with TCEP, washed three times with deionized water, and dissolved in aqueous ammonium hydroxide solution pH 7.2. Aliquots of polymer (< 10 μL) were added to M16 (without BSA or phenol red) to a final volume of 100 μL and incubated at 37 °C. The final concentrations of polymer were 100, 75, and 50 μM and nine aliquots were prepared at each concentration. At five min intervals for 45 min, 100 μL of stock DTNB was added to a polymer aliquot, the solution mixed, and the absorbance at 412 nm measured. The concentration of free sulfhydryl remaining was determined using  $\epsilon_{412} = 14,150 \text{ M}^{-1}\text{cm}^{-1}$  [34]. A standard curve for sulfhydryl concentration was prepared using cysteine under the same conditions.

*Isolation of spermatozoa and oocytes for IVF assay.* All experiments performed using mice were in accordance with National Institute of Health and USDA guidelines and the specific procedures performed were approved by the Stony Brook University IACUC (protocol #0616). Sperm for the in vitro adhesion and fusion assay were isolated from the cauda epididymis and vas deferens of 8 month old ICR retired male breeders ICR (Taconic, NJ). Sperm were released from dissected cauda and vas deferens into 3% BSA M16 modified Krebs-Ringer medium. Released sperm were incubated at 37 °C, 5% CO<sub>2</sub> for 3 h in the same medium to allow them to capacitate and acrosome react. Eggs were collected from the oviducts of 8-10 week-old super-ovulated female ICR mice (Taconic, NJ). Mice were super-ovulated by injecting 5 IU of PMSG followed 48-52 hours later by an injection of 10 IU of hCG. 14-16 h after hCG injection, oviducts were removed from euthanized mice and incubated in pre-warmed M16 medium with 0.5% BSA. Cumulus-egg complexes were collected and transferred to 500 μL drops of medium containing 30 μg/mL hyaluronidase surrounded by mineral oil. After 5 min

incubation at 37 °C, 5% CO<sub>2</sub>, cumulus free metaphase II eggs (eggs with one polar body) were collected, transferred first to a 80 µL drop of medium and then washed through six, 40 µL drops of medium. Eggs were recovered for 1 h before treating with acid Tyrode's. Zona pellucida of metaphase II eggs were removed by treating eggs with 100 µL Tyrode's acid drop for 1 min at RT. Zona free eggs were washed six times with 0.5% BSA medium and recovered for 2 h, then preloaded with HOECHST dye at 10 µg/mL for 30 min. at 37 °C, 5% CO<sub>2</sub>.

*Inhibitor assay.* Before use, the polymers were fully reduced with 10 mM TCEP for 1 to 2 h, precipitated with 1N HCl and washed with water, and then redissolved in water adjusted to pH 7 with NH<sub>4</sub>OH.

Zona free eggs that had been loaded with Hoechst dye were washed and placed in 100 µL drops of 3% BSA M16 medium. Polymer solution was added (no more than 5 µL of stock solution) and incubated with eggs at for 45 min prior to sperm addition. Capacitated and acrosome reacted sperm were added to eggs at a final concentration of 1-5 × 10<sup>5</sup> sperm/mL. After 45 min at 37 °C, 5% CO<sub>2</sub>, eggs were gently washed through six 40 µL drops of the M16 medium with 3% BSA. Eggs were mounted onto glass microscope slides and sperm binding and fusion were scored by epi-fluorescence microscopy and DIC microscopy (NIKON Eclipse 400, 40x, 0.75 NA objective). Fusion was scored as the fluorescent labeling of sperm nuclei with HOECHST dye present in the preloaded eggs. Two measures of fusion were used: fertilization index (FI, mean number of fused sperm per egg) and fertilization rate (FR, percentage of eggs fused with at least one sperm). IC<sub>50</sub>'s were calculated by a 3 parameter fit (GRAFIT software) by the equation:

$$y = (100 - b) / \{1 + ([I] / IC_{50})^s\} \quad (1)$$

Where y is the percent FR or FI, b is the remaining percent fertilization after saturation with inhibitor, and s is the slope of the fit. Errors were reported as s.e.m.

*Sperm Susceptibility to Polymer Assay.* Sperm in 3% BSA M16 modified Krebs-Ringer medium were allowed to capacitate and acrosome react in a 37 °C humidified incubator with 5% CO<sub>2</sub> for 2 h 30 min, then treated with 500 µM **3<sub>10</sub>**, **3<sub>70</sub>**, or **3<sub>2-47-3<sub>2</sub></sub>** for 45 min. Control sperm were incubated in the same buffer. Samples were transferred to glass slides and sperm motility and viability were checked by light microscope (NIKON TS-100, 10x, 0.25 NA objective). Only sperm that were still swimming actively were counted as viable and motile. In a second experiment, sperm were incubated for 45 min with 125 µM **3<sub>2-47-3<sub>2</sub></sub>** in 3 % BSA M-16 buffer, or in medium alone. 2 µL of the sperm incubation (1-5 × 10<sup>5</sup> sperm/mL) was transferred to a 100 µL M-16 drop containing zona free eggs. After 45 min the eggs were analyzed for number of sperm bound and fused as previously described in the assay.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

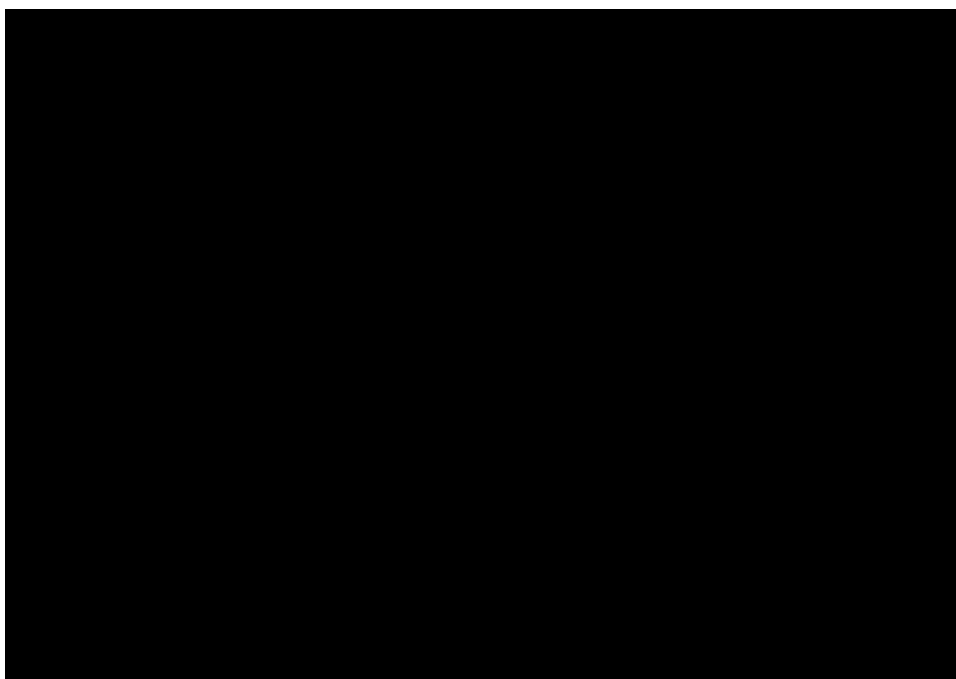
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## References

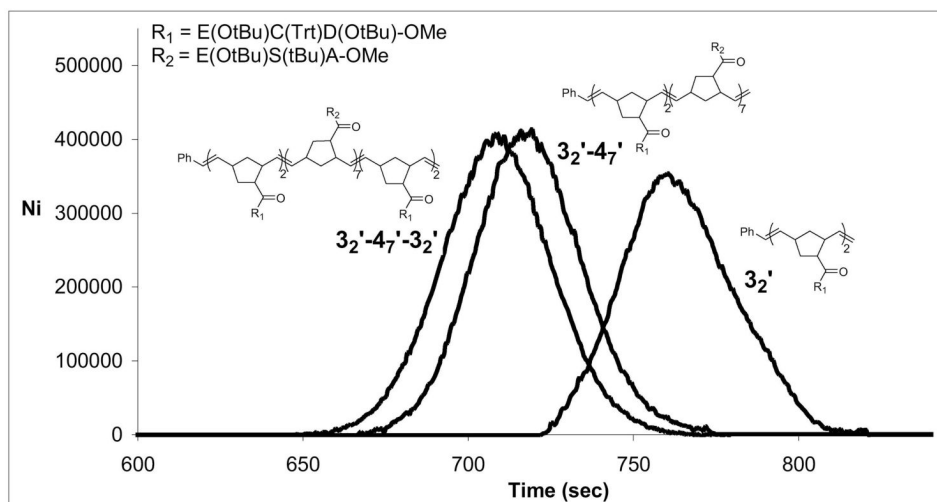
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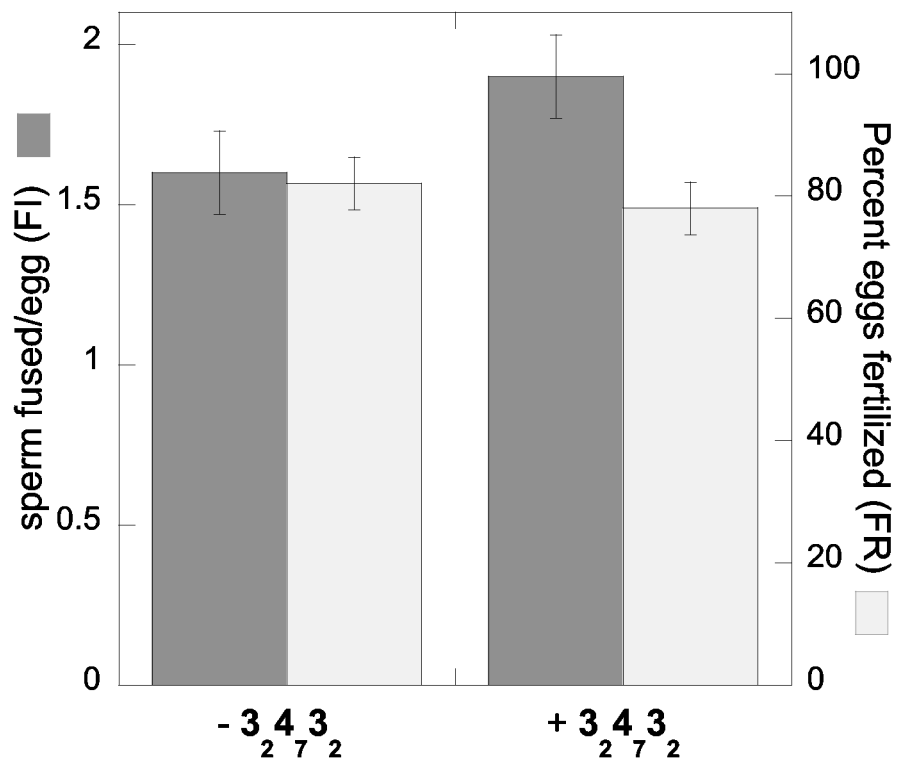
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**Figure 1:** Polymers synthesized by ROMP and tested as fertilization inhibitors. The compound number refers to the structure of the ligand. The subscript to the compound number refers to the number of that specific monomer in the polymer. Refer to Figure 2 for a cartoon representation of the polymer structures.

**Figure 2:**

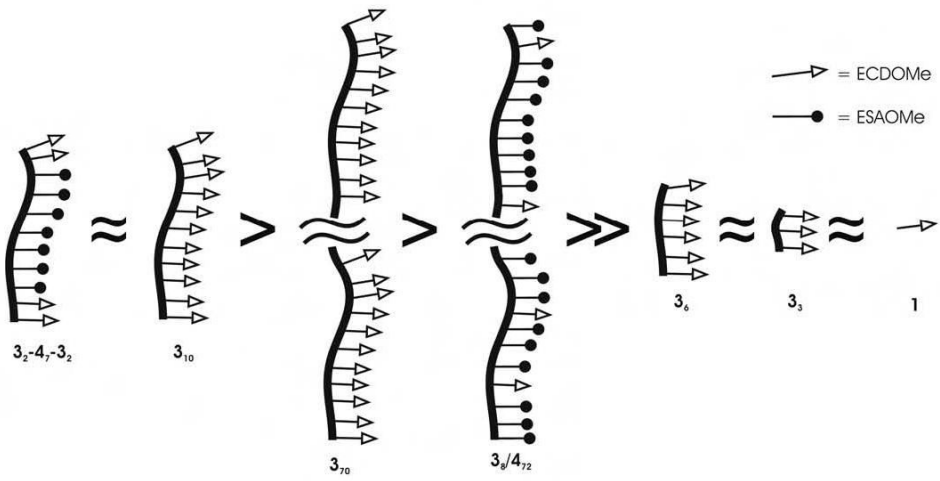
Comparison of GPC eluted peaks of  $3_2'$ ,  $3_2'-4_7'$ , and  $3_2'-4_7'-3_2'$  using a Phenogel column ( $5\mu$ ,  $300 \times 4.6$  mm, linear mixed bed,  $100\text{-}10^6$  MW range) and eluting with  $0.3$  mL/min  $10\%$  MeOH in  $\text{CH}_2\text{Cl}_2$ . Narrowly dispersed polystyrene standards were used to calibrate number-averaged molecular weight. Polymer  $3_2'$  eluted at an apparent  $M_n = 3,372$  (PDI: 1.18; expected MW: 3,974); polymer  $3_2'-4_7'$  eluted at apparent  $M_n = 10,840$  (PDI: 1.18; expected MW: 12,780) and the triblock copolymer  $3_2'-4_7'-3_2'$  has an apparent  $M_n = 15,013$  (PDI: 1.21; expected MW: 18,198).



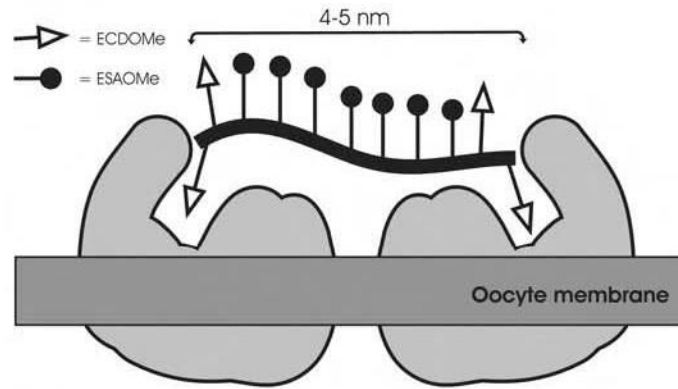
**Figure 3:**

Assay of sperm susceptibility to polymers. Sperm were incubated with  $3_2-4_7-3_2$  (125  $\mu\text{M}$  in peptide) or M16 buffer only for 45 minutes prior to egg insemination (final  $3_2-4_7-3_2$  concentration was approximately 2  $\mu\text{M}$  in peptide). The  $3_2-4_7-3_2$  pretreated sperm and control sperm fertilized eggs equally efficiently as measured by both fertilization rate (FR) and fertilization index (FI) demonstrating that the polymer does not have an adverse affect on the sperm. A total of 60 eggs were used in three independent experiments. Errors are reported as standard error of measurement.





**Figure 4:**  
Relative potencies of fertilin $\beta$  polymers when compared on the basis of peptide ligand concentration.



**Figure 5:**  
Proposed model for receptor-polymer binding.

**Table 1:**  
Polymer IC<sub>50</sub>'s for inhibition of *in vitro* fertilization.<sup>a</sup>

Polymer	IC50 (μM) in peptide by F.I.	IC50 (μM) in peptide by F.R.	IC50 (μM) in polymer by F.I.	IC50 (μM) in polymer by FR
1 (monomer)	>500 <sup>b</sup>	>500 <sup>b</sup>	n.a. <sup>c</sup>	n.a.
2 <sub>10</sub>	5.1 ± 1.4	5.8 ± 0.3	0.51 ± 0.14	0.58 ± 0.03
3 <sub>10</sub>	3.4 ± 0.3	3.2 ± 0.2	0.34 ± 0.03	0.32 ± 0.02
3 <sub>70</sub>	68 ± 11.1	99 ± 31	0.97 ± 0.16	1.4 ± 0.44
3 <sub>8</sub> /4 <sub>72</sub>	23 ± 5	80 ± 24	2.9 ± 0.7	10 ± 4
4 <sub>10</sub>	n.i. <sup>d</sup>	n.i.	n.i.	n.i.
4 <sub>70</sub>	n.i. <sup>d</sup>	n.i.	n.i.	n.i.
3 <sub>3</sub>	~500 <sup>e</sup>	~500 <sup>e</sup>	~167	~167
3 <sub>6</sub>	~500 <sup>e</sup>	~500 <sup>e</sup>	~83	~83
3 <sub>2</sub> -4 <sub>7</sub> -3 <sub>2</sub>	1.1 ± 0.3	5.5 ± 2.3	0.28 ± 0.08	1.4 ± 0.6

<sup>a</sup>F.I. (fertilization index) is the average number of fused sperm per egg. The average FI of the control was 1.93. F.R. (fertilization rate) is the percent of eggs fertilized. The average FR of the control was 85.7 %. 200-300 eggs were assayed for each polymer in 8-10 independent experiments. IC<sub>50</sub>'s were calculated by a 3 parameter fit (GRAFIT software) using the equation: percent fertilization = (100-b) / {1 + ([I]/IC<sub>50</sub>)}<sup>s</sup>. Where b is the remaining percent fertilization after saturation with inhibitor, and s is the slope of the fit. Errors are reported as s.e.m.

<sup>b</sup>At 500 μM 1, 29% (FI) and 32% (FR) was observed.

<sup>c</sup>n.a.: not applicable.

<sup>d</sup>n.i.: no inhibition. Negative control polymers 4<sub>10</sub> and 4<sub>70</sub> did not inhibit fertilization at 500 μM (peptide).

<sup>e</sup>At 500 μM 3<sub>3</sub> (peptide), the percent inhibition observed was 66 ± 9 (FI) and 58 ± 2 (FR).

<sup>f</sup>At 500 μM 3<sub>6</sub> (peptide), the percent inhibition observed was 57 ± 5 (FI) and 52 ± 4 (FR).