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Polymeric ADAM Protein Mimics Interrogate Mammalian Sperm–Egg Binding

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The sperm proteins ADAM2 and ADAM3, members of the ADAM family of proteins, have been implicated in mammalian sperm–egg binding. However, elucidating their roles is complex because of the interdependence of ADAM protein expression in the testis. Hence, multivalent probes containing the three-amino acid binding sequence of ADAM2, glutamate-cysteine-aspartate (ECD), and ADAM3, glutamine-cysteine-aspartate (QCD), were designed, synthesized, and tested to investigate gamete interactions. In this work, ECD polymer mimics were synthesized by ring-opening metathesis polymerization with a faster initiating ruthenium catalyst than previously used. Polymers containing 100 copies of the ECD peptide mimic were found to be the best inhibitors of fertilization. The multivalent QCD polymers were also tested as inhibitors of fertilization. The structure-activity profile was the same as ECD polymers, but the overall potency was lower. Both ECD and QCD polymers require the presence of β_1 integrin to inhibit fertilization. Next, triblock ABA and ABC copolymers containing both ECD and QCD ligands were synthesized with 96 monomer spacers as their B blocks. Although these polymers had lower densities of ECD and QCD peptides, their potencies correlated with the potencies of their corresponding homopolymers. In addition, no synergy between ECD and QCD mimics was observed. All the data suggest that QCD and ECD bind to the same complex of proteins that includes β_1 integrin.

Introduction

Mammalian fertilization is a multistep process whereby two gametes (egg and sperm) bind and fuse together to form a zygote. The egg activates the sperm metabolism, and the sperm reciprocates by activation of the egg metabolism to initiate fertilization.[1] The ovulated oocyte is surrounded by the cumulus layer and the zona pellucida. A capacitated and acrosome-intact sperm can pass through the cumulus complex and bind to the zona pellucida. The acrosome reaction results in the release of the contents of the acrosomal vesicle including proteolytic enzymes, and allows the sperm to penetrate the zona pellucida. Finally, the sperm adheres to and fuses with the egg plasma membrane, at which point egg activation is triggered. However, the precise mechanisms of sperm binding and fusion are not yet understood. Therefore, an understanding of the molecular interaction between sperm and egg proteins is essential for the study of infertility and the development of new contraception strategies.

We undertook a chemical biology approach to elucidate the molecular mechanism of sperm–egg binding. The ADAM family of proteins[2–5] is widely expressed with a large subset present in mouse testis.[3,6] About half of ADAMs found in testis including ADAM2 (fertilin β) and ADAM3 (cyritestin) are exclusively or predominantly expressed there.[6,7] These testis specific proteins, ADAM2 and ADAM3, have roles in sperm–egg adhesion, and their disintegrin domains are the primary binding domains.[8,9]

ADAM2 and ADAM3 are located in the equatorial region of the sperm head, and during sperm maturation, their disintegrin domains are exposed on the sperm head.[10–16] The short peptide sequences glutamate-cysteine-aspartate (ECD) and glutamine-cysteine-aspartate (QCD) are highly conserved across species in the ADAM2 and ADAM3 disintegrin loops, respectively.[15] These tripeptides, ECD[17–20] and QCD, [18,21] are the minimal recognition element necessary for binding to the egg.

Knockout of the ADAM2 or ADAM3 gene in mice reduces binding of sperm to the egg plasma membrane.[22,23] However, interpretation of the genetic studies is complicated by the unexpected interdependence of protein expression amongst ADAM proteins in the testis. Knockout of the ADAM2 gene results in a significant reduction of other ADAM protein levels on the sperm surface, including ADAM1a, ADAM1b, ADAM3, ADAM5 and ADAM7, whereas others are unchanged, for example, ADAM27 and ADAM32.[24–28] Knockout of the ADAM3 gene shows a similar, but not identical codependence of protein expression and maturation. The dependence of non-ADAM protein expression levels on the presence of ADAM genes is unknown. In addition, at least nine testis-expressed ADAM proteins contain the ECD motif.[16,29–31] The array of ADAM proteins expressed in sperm with potentially overlapping functions makes a genetic approach problematic. Here, we utilize an ECD mimic and a QCD mimic to explore the interrelationship between the two mimics and the consequences of blocking ADAM-dependent adhesion.

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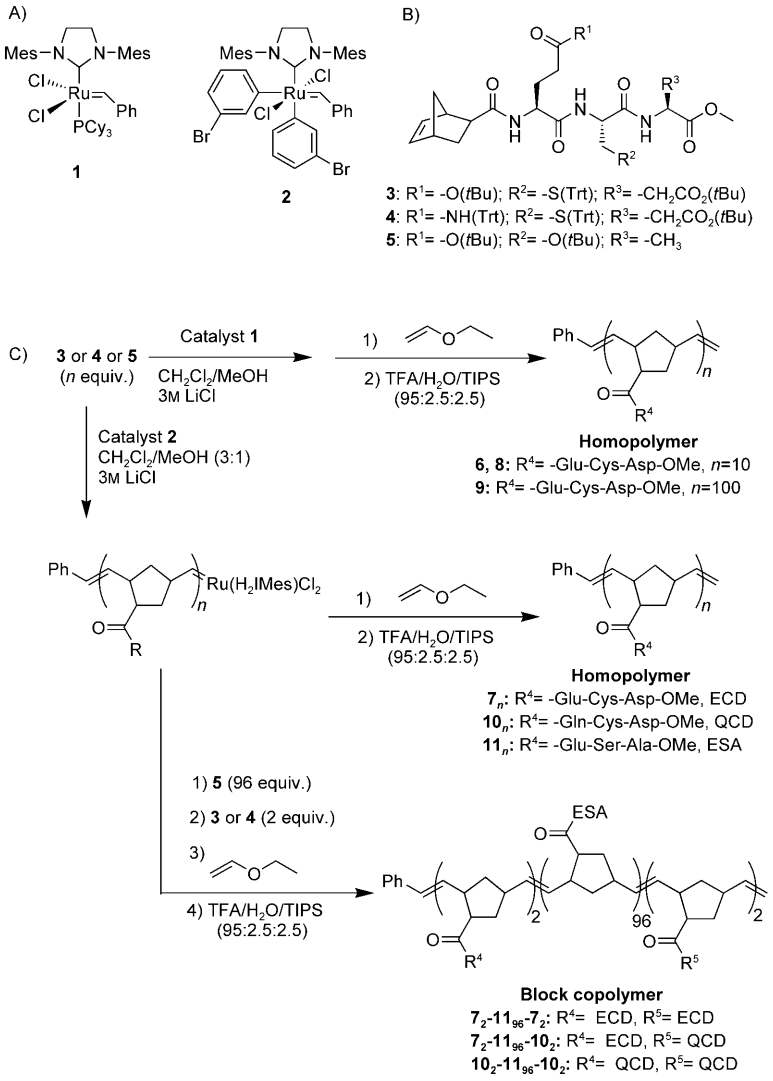
Short peptides incorporating the ECD or QCD sequence inhibit sperm binding at high micromolar concentrations.[16–18,32–35] To improve the binding affinity of monomeric peptide ligands for the egg surface, we developed synthetic multivalent polymers to mimic the multivalent display of ADAM2 on the sperm surface.[36–39] We employed ruthenium-catalyzed ring opening metathesis polymerization (ROMP) to prepare these mimics.[39] In the series of linear ECD polymers tested, **6**, which was designed to have ten copies of the ECD ligand, was the best inhibitor. Moreover, the mechanism of inhibition is direct competition with sperm binding to the β_1 integrin rather than activation of an egg signalling pathway that alters egg fertilizability.[40]

In the present work, we addressed the following questions about the contributions of the ECD and QCD motifs to binding at the egg plasma membrane. First, we determined whether their structure activity profiles are identical. We synthesized and tested a series of linear QCD polymers, and we compared their inhibition potencies and patterns with ECD mimics. As a result of increased initiation rates with catalyst **2**, we obtained much narrower molecular weight distributions for these polymers than in previous work. Therefore, we resynthesized the ECD polymers to compare their potencies to the newly synthesized QCD polymers. We found that both longer ECD and QCD polymers were better inhibitors.



Scheme 1. Polymers synthesized by ROMP and tested as inhibitors.

Next, the interdependence of these two polymers was tested. Inhibition by a copolymer of ECD and QCD was compared to inhibition by individual polymers. Lastly, we examined the importance of β_1 integrin for inhibition by both binding motifs and found that both inhibit fertilization through the β_1 integrin receptor.

Results

Synthesis of oligopeptides polymers

The norbornenyl tripeptide monomers were synthesized in the solution phase with Fmoc, Cbz α-amino protection and *tert*-butyl or trityl side-chain protection. Polymers were produced by ROMP using the fully protected monomers in dichloromethane/methanol (3:1) with 3 M LiCl (Scheme 1).[39,41] The polymerization of the norbornenyl monomers was catalyzed with $(H_2IMes)-(PCy_3)Cl_2Ru=CHPh$, **1**, or $(H_2IMes)(3-BrPyr)_2Cl_2Ru=CHPh$, **2**, to form homopolymers, and the polymerizations were terminated by adding ethylvinyl ether.

The polymers were treated with a trifluoroacetic acid/water/triisopropylsilane cocktail mixture to deprotect the side chains and were precipitated with diethyl ether. Polymers were reduced with tris(2-carboxyethyl)phosphine, were precipitated with dilute acid, 1 N HCl, and were resuspended in aqueous ammonium hydroxide to a final pH of 7 before use in assays. Isolated yields were 67–90%. Polymers were characterized by NMR spectroscopy, gel permeation chromatography (GPC), and laser light scattering. ^{1}H NMR spectra confirmed that no monomer was retained in the polymer precipitation. The integration of the phenyl end group against the ligand side chains in the ^{1}H NMR spectra agreed with the expected degree of polymerization. The number-average molecular weights (M_n), the weight-average molecular weights (M_w), and the polydispersity

index (PDI) were determined for the protected polymers by gel permeation chromatography utilizing a differential refractometer and a multiangle light scattering detector. The size distribution profiles of deprotected polymers in the assay buffer were monitored by dynamic light scattering to determine whether aggregates of polymers formed under the assay conditions.

ADAM2 mimic polymers, **6** and **8** were synthesized with catalyst **1** and monomer **3** at 25 °C or 55 °C, respectively, whereas polymer $\mathbf{7_{10}}$ was synthesized with catalyst **2** at 25 °C (Table 1).

Table 1. Analytical data for ECD mimic homopolymers.

Polymer[a]	C[b]	$[M]_0/[C]_0$	T [°C][c]	Theo. M_n [$\times 10^3$][d]	M_n [$\times 10^3$][e]	PDI[e]
6	**1**	10	25	8.6	82.1	1.46
$\mathbf{7_{10}}$	**2**	10	25	8.6	14.9	1.15
8	**1**	10	55	8.6	12.8	1.37
9	**1**	100	25	85.5	130.7	1.54
$\mathbf{7_{100}}$	**2**	100	25	85.5	133.7	1.21

[a] Side-chain protection was intact and norbornenyl ECD monomer **3** was used. [b] Catalyst **1** or **2** was used. [c] Temperature at which polymerizations were performed. [d] Theoretical molecular weights were calculated based on the ratio of monomer/catalyst. [e] Determined by GPC in THF utilizing a differential refractometer and a multiangle light scattering detector.

The M_n values of $\mathbf{7_{10}}$ (14900) and **8** (12800) were similar to the expected degree of polymerization. Despite initiation at 55 °C, **8** had a higher polydispersity than $\mathbf{7_{10}}$. In addition to a high polydispersity, **6** had an M_n of 82100, which was ten-fold greater than the theoretical value, and six-fold greater than that of $\mathbf{7_{10}}$.

ADAM3 mimic polymers, $\mathbf{10_1}$, $\mathbf{10_2}$, $\mathbf{10_3}$, $\mathbf{10_6}$, $\mathbf{10_{10}}$, and $\mathbf{10_{100}}$, and mutant polymers, $\mathbf{11_{10}}$ and $\mathbf{11_{100}}$, were prepared with catalyst **2** and monomers **4** and **5**, respectively, as described above (Table 2). The ABA and ABC block copolymers $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{7_2}$, $\mathbf{10_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$, and $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$ were synthesized with monomers **3**, **4**, and **5** with catalyst **2**. Two ECD or QCD ligands were placed at each terminus of the bivalent block copolymers, $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{7_2}$, $\mathbf{10_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$, and $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$ polymers, so that statistically at least one ligand was present on both ends of the polymers. Block copolymers were produced by sequential addition of monomers. After complete disappearance of the first norbornenyl monomer, the second monomer was added to the reaction followed by the third. The consumption of monomers was monitored by TLC before addition of the subsequent monomers. They were deprotected and purified as described for homopolymers.

Table 2. Inhibition of fertilization by QCD mimic homopolymers and mutant control polymers.

Polymer[a]	IC_{50} [μM] in polymer by FI	by FR	M_n [$\times 10^3$][c]	PDI[c]
$\mathbf{10_2}$	469±29	492±65	1.1[d]	1.00[d]
$\mathbf{10_3}$	11±5	26±19	3.6	1.15
$\mathbf{10_6}$	28±17	24±6	4.9	1.21
$\mathbf{10_{10}}$	3.0±1.5	14.7±8.8	20.8	1.06
$\mathbf{10_{100}}$	4.1±0.9	5.7±2.5	149.9	1.45
$\mathbf{11_{10}}$	n.i.[b]	n.i.[b]	10.1	1.07
$\mathbf{11_{100}}$	n.i.[b]	n.i.[b]	–	–

[a] Subscript represents monomer/catalyst ratio. [b] n.i., no inhibition. Negative control polymers, $\mathbf{11_{10}}$ and $\mathbf{11_{100}}$ inhibited fertilization less than 15% at 500 μM in peptide concentration. At least 10–15 independent experiments with 250–350 eggs were performed at each concentration. In the untreated controls, 71±2% eggs were fertilized. The average number of sperm fused per egg was 1.3±0.2. Errors are the S.E.M. [c] Determined by GPC in THF utilizing a differential refractometer and a multiangle light scattering detector. [d] Determined by ESI mass spectrometry after deprotection.

Assay of polymers

In vitro fertilization assays were executed with zona pellucida-free mouse eggs. Eggs were preincubated in Hoechst 33442, and polymers were incubated with eggs prior to inseminating with sperm. Sperm fusion was used as an endpoint to measure inhibition of sperm binding. Sperm were scored as fused if their chromatin stained with Hoechst 33442 that was loaded into eggs. Fertilization was scored two ways.[32] The fertilization rate, FR, reports the ratio of fertilized eggs to the total number of eggs. The fertilization index, FI, reports the total number of sperm fused divided by the total number of eggs.

The concentrations of inhibitors are reported as concentration of polymer because the density and type of ligands varied. The concentrations of ADAM3 mimic homopolymers used covered at least a 1000-fold range in the determination of their IC_{50}s by both FR and FI. For other polymers, comparisons of inhibition were performed at fixed concentrations of polymer.

Inhibition of fertilization by **6**, $\mathbf{7_{10}}$ and $\mathbf{7_{100}}$ was tested (Figures 1 and S1). Polymers **6** and $\mathbf{7_{100}}$ were nearly equipotent

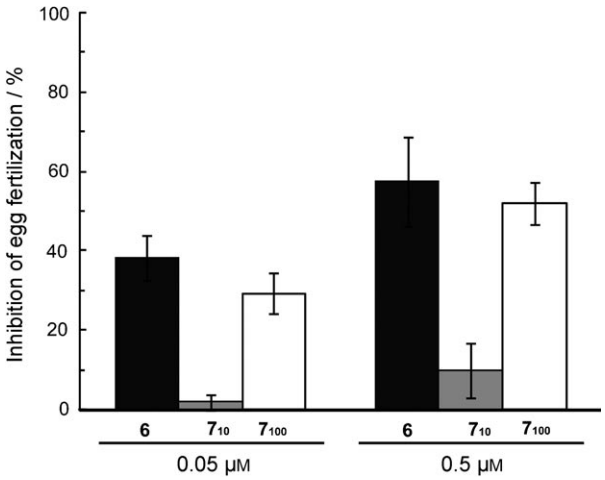


Figure 1. Inhibition of fertilization by ECD mimic polymers at 0.05 μM and 0.5 μM in polymer concentration by FR (percentage of eggs fertilized). At least five independent experiments with 100–120 eggs were performed at each concentration. In the untreated controls, 79±3% eggs were fertilized. The average number of sperm fused per egg was 1.5±0.1. Errors are the S.E.M.

and inhibited fertilization more than 50% at 0.5 μM in polymer concentration. $\mathbf{7}_{10}$ was a poor inhibitor and its potency was ten-fold lower than that of **6** or $\mathbf{7}_{100}$.

Next, the inhibition potencies of QCD polymers were measured (Table 2). Polymer $\mathbf{10}_1$, a monomer, did not inhibit consistently at 500 μM. $\mathbf{10}_2$, isolated as a side product from the preparation of $\mathbf{10}_1$, inhibited fertilization 50% at 500 μM in polymer concentration. $\mathbf{10}_{100}$ was 100 times more potent with an IC_{50} of 5.7 μM by FR. The IC_{50} of $\mathbf{10}_{10}$ was similar to that of $\mathbf{10}_{100}$ but the reproducibility was not as robust. Control polymers $\mathbf{11}_{10}$ and $\mathbf{11}_{100}$ inhibited less than 15% at 500 μM in peptide concentration.

The codependency of inhibition of fertilization was investigated with a mixture of ECD polymer $\mathbf{7}_{100}$ and QCD polymer $\mathbf{10}_{100}$ (Figures 2A and S2A). These positive controls were tested at two independent polymer concentrations, 0.5 μM and 1 μM. The mixture of $\mathbf{7}_{100}$ at 0.5 μM and $\mathbf{10}_{100}$ at 0.5 μM in polymer concentration inhibited 34% of egg fertilization, whereas $\mathbf{7}_{100}$ alone at 0.5 μM in polymer concentration inhibited 52% of egg fertilization. This difference was not statistically significant.

Inhibition of fertilization by bivalent ECD mimic block copolymer $\mathbf{7}_2$–$\mathbf{11}_{96}$–$\mathbf{7}_2$ at 0.5 μM in polymer concentration was 46% by FR (Figures 2B and S2B). This inhibition was equipotent to homopolymer $\mathbf{7}_{100}$. Likewise, bivalent QCD mimic block copolymer $\mathbf{10}_2$–$\mathbf{11}_{96}$–$\mathbf{10}_2$ was nearly equipotent to homopolymer, $\mathbf{10}_{100}$ at 0.5 μM in polymer concentration with 15% inhibition of egg fertilization.

Polymer $\mathbf{7}_2$–$\mathbf{11}_{96}$–$\mathbf{10}_2$, the heterobivalent, triblock copolymer containing both ECD and QCD ligands with a 96 monomer spacer, was tested. The inhibition potency of polymer $\mathbf{7}_2$–$\mathbf{11}_{96}$–$\mathbf{10}_2$ was lower than ECD mimic polymers, but better than QCD mimic polymers. It inhibited fertilization 30% by FR at 0.5 μM in polymer concentration.

Assay of KO eggs

The inhibition potencies of $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$ were tested with β_1 integrin knockout eggs (Figures 3 and S3). Eggs homozygous for the β_1 integrin knockout allele ($Cre^+\beta_1 f/f$, KO) and wild-type ($Cre^-\beta_1+/+$, WT) eggs were obtained as previously described.[32]

Immunofluorescence microscopy with anti-β_1 and anti-α_6 integrin antibodies confirmed that the β_1 integrin knockout eggs had no β_1 integrin or α_6 integrin on the plasma membrane.[32,40] $\mathbf{7}_{100}$ inhibited fertilization 12% by F.R. at 1 μM in polymer concentration, as compared to 52% in WT eggs. $\mathbf{10}_{100}$ inhibited egg fertilization 10%, as compared to 35% in WT eggs.

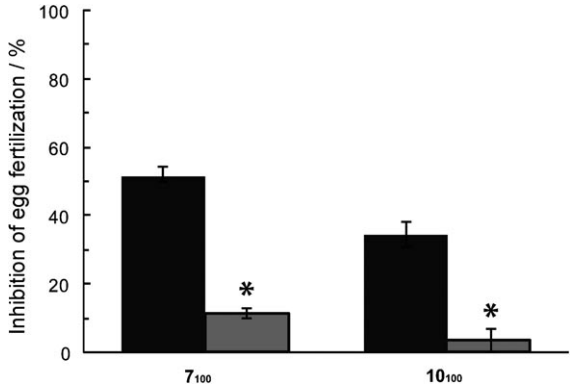


Figure 3. Inhibition by homopolymers at 1 μM in polymer concentration by FR (percentage of eggs fertilized). Black bars: wild-type (WT) eggs. At least six independent experiments with 100–120 eggs were performed at each polymer concentration. In the untreated controls, 76±2% eggs were fertilized. The average number of sperm fused per egg was 1.6±0.1. Gray bars: β_1 integrin KO eggs. Two independent experiments with 25 eggs for $\mathbf{7}_{100}$, and three independent experiments with 39 eggs for $\mathbf{10}_{100}$ were performed. In the untreated controls, 70±3% eggs were fertilized. The average number of sperm fused per egg was 1.2±0.1. *$p < 0.05$ for WT vs. β_1 KO eggs. Errors are the S.E.M.

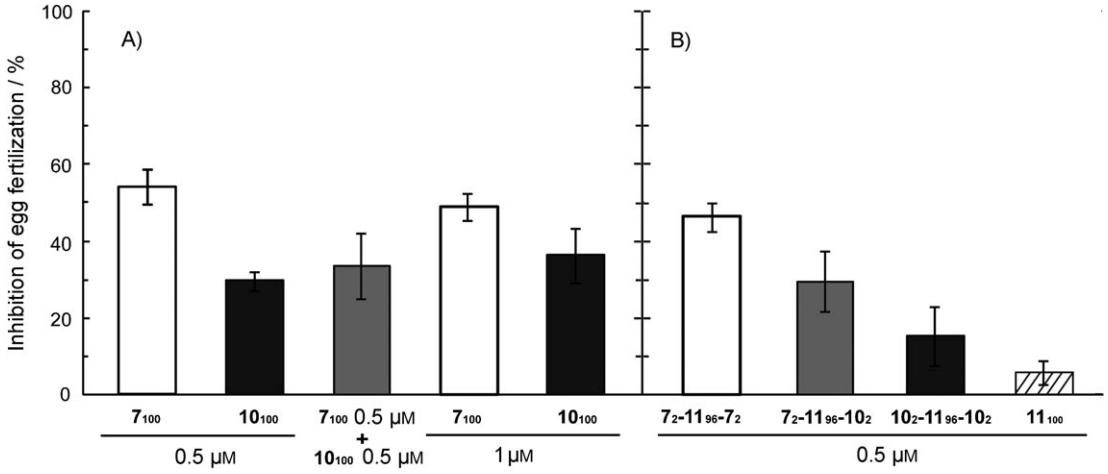


Figure 2. A) Inhibition of fertilization by a mixture of $\mathbf{7}_{100}$ at 0.5 μM and $\mathbf{10}_{100}$ at 0.5 μM in polymer concentration by FR (percentage of eggs fertilized). At least three independent experiments with 60–100 eggs were performed at each concentration. In the untreated controls, 66±8% eggs were fertilized. The average number of sperm fused per egg was 1.5±0.1. B) Inhibition by bivalent block copolymers $\mathbf{7}_2$–$\mathbf{11}_{96}$–$\mathbf{7}_2$, $\mathbf{7}_2$–$\mathbf{11}_{96}$–$\mathbf{10}_2$, and $\mathbf{10}_2$–$\mathbf{11}_{96}$–$\mathbf{10}_2$ at 0.5 μM in polymer concentration. At least three independent experiments with 60–80 eggs were performed at each concentration. In the untreated controls, 65±3% eggs were fertilized. The average number of sperm fused per egg was 1.4±0.2. Errors are S.E.M.

Discussion

Testing the functions of molecules that have been implicated in mediating mammalian sperm–egg binding and fusion is the first step for understanding the molecular mechanisms of gamete interactions. For this type of discovery, it is critical to develop specific methods for exploring the mechanisms of binding. Probe–protein interaction studies are a valuable tool in many biological systems, but they have been applied in a limited fashion to fertilization because of the limited quantities of material available to study gametes in mammalian systems and the lack of cell culture models. Most experiments in the field of fertilization biology rely on genetic and immunohistochemical methods. Here, we employed chemical synthesis, ROMP, to produce multivalent mimics of ECD and QCD to imitate sperm complexes that bind the egg with optimal binding affinity.

Comparison of polymers synthesized with catalysts 1 and 2

Previously in our laboratory, multivalent ECD mimic polymer **6**, synthesized to display ten copies of the ECD peptide with catalyst **1**, was found to be a potent inhibitor of fertilization.[39] However, extensive analysis revealed that the average length of **6** by catalyst **1** was not ten monomer units as originally determined. Next, we synthesized a series of ECD mimic polymers with catalyst **2** and undertook a comparison of the molecular weight distributions of the protected polymers by GPC using static light scattering.

ECD mimic polymers, **6** and **9** synthesized with catalyst **1** at 25 °C had uncontrolled molecular weights and broad polydispersities. M_n values were at least six-fold greater than intended based on initial monomer:catalyst feed ratios. The lack of control resulted from slow initiation rates, fast propagation rates,[42,43] and competing chain-transfer reactions.[44–46] Because chain transfer is minimized with catalyst **1** at 55 °C,[47] **8** was synthesized with catalyst **1** at 55 °C. ROMP with catalyst **1** at 55 °C yielded **8** with the desired M_n. However, its polydispersity was still broad (PDI of 1.37). Because the electron-deficient 3-bromopyridine ligand is dissociated rapidly and rebinding of the ligand is slow, catalyst **2** initiates ROMP least six orders of magnitude faster than catalyst **1**.[48–50] ROMP of **7** with catalyst **2** resulted in polymers $\mathbf{7}_{10}$ and $\mathbf{7}_{100}$ with lower PDIs ranging from 1.15 to 1.21 and better molecular weight control. Thus, catalyst **2** promotes living ROMP of norbornenyl oligopeptides with both higher activity and better control.[51]

Assay of homopolymers

Because the molecular weight analysis revealed that the most potent inhibitor, **6** was actually a 100-mer, we tested $\mathbf{7}_{100}$ which also displays 100 copies of the ECD peptide, but is synthesized with catalyst **2** to compare their biological activities. Although their average molecular weights are the same, their weight dispersities and backbone stereochemistries were not identical. Inhibition by $\mathbf{7}_{100}$ was equipotent to inhibition by **6** and was 100-fold more potent than the corresponding monomeric peptide. These results suggested that the 100-mer ECD mimic polymers bound multivalently to the egg surface with a large tolerance for varying molecular structure.

Polymers mimicking sperm protein QCD were also prepared with catalyst **2**. These mimic polymers contained the conserved QCD tripeptide sequence thought to be required for fertilization. Their sizes ranged from 1 to 100 monomer units in length (Table 2). $\mathbf{10}_{100}$ was more potent than the monomeric peptide or the shorter mimic polymers as an inhibitor of fertilization. However, its inhibition potency (IC_{50}) was four times worse than the corresponding ECD mimics, **6** and $\mathbf{7}_{100}$; this suggests that the binding affinity is weaker.

We also mutated the ECD and QCD sequences to ESA, glutamate-serine-alanine, to synthesize control polymers $\mathbf{11}_{10}$ and $\mathbf{11}_{100}$ with catalyst **2**. We chose to mutate ECD and QCD rather than scramble them, because two of the ECD amino acids contained carboxylates.[39] ESA polymers, similar in length to the ECD and QCD polymers, were tested as inhibitors of fertilization and no inhibition was observed. Thus, the polymer backbone does not contribute to binding.

Are the ECD and QCD receptors the same?

Based on the inhibition results, we explored the identity between ECD and QCD receptors. We expected two possibilities. In the first case, different receptors or protein complexes on the egg membrane might be targeted by either mimic. If so, their inhibitory behaviors should be independent, and the combination of two mimics would inhibit more than each individual mimic. In a second scenario, ECD and QCD might bind the same receptors on the egg and no net increase in inhibition at a fixed concentration of polymer would be expected. Therefore a mixture of polymers $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$ was tested.

Inhibition by the mixture of 0.5 μM $\mathbf{7}_{100}$ and 0.5 μM $\mathbf{10}_{100}$ was not dramatically increased relative to inhibition by 1 μM of either homopolymer. Within experimental error, inhibition by a mixture of $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$ was the same as by $\mathbf{10}_{100}$ alone. The combination of $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$ decreased the potency of $\mathbf{7}_{100}$ 20%. Thus, these two mimic polymers $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$ appear to compete for the same receptor rather than bind to independent receptors.

This comparison was further tested with a series of triblock copolymers. First homobivalent polymers 100 units in length, $\mathbf{7}_2$–$\mathbf{11}_{96}$–$\mathbf{7}_2$ and $\mathbf{10}_2$–$\mathbf{11}_{96}$–$\mathbf{10}_2$, were synthesized. Two ECD or QCD ligands were at each terminus of the polymers to ensure that statistically at least one ligand would be present on the end of every polymer. The inactive ESA peptides were incorporated in the center of the triblock copolymers as spacers. These polymers are the same average length as $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$, but they have a lower density of ECD and QCD peptides. Interestingly, they were only 10–15% less potent as inhibitors of fertilization compared to their corresponding homopolymers, $\mathbf{7}_{100}$ and $\mathbf{10}_{100}$ (Figures 2 and S2). This result suggested that of the 100 ligands present in the homopolymers only a small percentage are required for binding to the egg surface and that it is the overall size of the polymer that is most important for inhibition, rather than the ligand density.

Because both the ECD and QCD bivalent mimics were effective inhibitors, we replaced the norbornenyl ESA spacer with a flexible linker, polyethylene glycol (PEG), between the ligands. PEG is resistant to the nonspecific adsorption of proteins,[52,53] and would be better suited for covalent cross-linking experiments to identify receptors and coreceptors. PEG linkers comprised of twelve, 67, and 178 ethylene glycol units were introduced between ECD ligands. However, inhibition by all of three of the PEG-linked polymers was no more effective than with the monomeric peptides. The increased degrees of freedom in the PEG backbone compared to norbornenyl polymers most likely diminished their binding affinities.[54] Thus, the conformational constraints inherent to the norbornenyl backbone dictate the shape of the polymers and consequently, make a positive contribution to the binding affinity.

Next, $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$, a heterobivalent triblock copolymer containing both ECD and QCD ligands separated by a 96-mer spacer was tested to explore the relationship and degree of identity between ECD and QCD receptors. The inhibition potency of polymer $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$ was lower than ECD mimic polymers, $\mathbf{7_2}$–$\mathbf{11_{96}}$–$\mathbf{7_2}$ and $\mathbf{7_{100}}$. However its potency was better than QCD mimic polymers, $\mathbf{10_2}$–$\mathbf{11_{96}}$–$\mathbf{10_2}$ and $\mathbf{10_{100}}$. Thus, synergy between ECD and QCD mimics was not observed. In agreement with the results of the mixed inhibition experiments, these data suggest that these two mimics engage the same egg-surface receptors or protein complex and that the QCD ligand binds more weakly than the ECD ligand to the receptor.

The $\alpha_6\beta_1$ integrin on the mouse egg plasma membrane has been identified as the ECD binding partner on the egg through a multitude of different experiments.[10,40,55,56] We tested whether $\mathbf{7_{100}}$ and $\mathbf{10_{100}}$ inhibit fertilization of β_1 integrin knockout eggs (Figures 3 and S3). As expected from related studies,[40] polymer $\mathbf{7_{100}}$ did not significantly inhibit fertilization of β_1 integrin knockout eggs. Consistent with QCD binding to the same receptor, polymer $\mathbf{10_{100}}$ also did not inhibit fertilization of knockout eggs.

We conclude that both the ADAM ECD and QCD binding motifs adhere to the β_1 integrin in sperm–egg binding that leads to fertilization. However, the affinity of QCD is weaker than the affinity of ECD. This difference suggests that ADAM2 and other ECD-presenting ADAMs are the primary adhesion proteins for the β_1 integrin on the egg.

Experimental Section

Materials: Amino acids and coupling agents used were purchased from Advanced Chem Tech. (Louisville, KY) or Novabiochem (Gibbstown, NJ). Solvents and chemical reagents were obtained from Fisher Scientific, Inc. or Sigma–Aldrich. $(H_2IMes)(3\text{-}BrPyr)_2Cl_2Ru{=}CHPh$, **2**, was prepared according to the literature.[48] CH_2Cl_2, CH_3OH and Et_2O were dried and purified by pushstill solvent-dispensing system (SG Water USA LLC, Nashua, NH). LiCl was oven-dried and stored over P_2O_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 N_2-filled drybox. 5-Norbornene-*exo*-carboxylic acid was synthesized according to the literature.[57]

Eight- to ten-week-old virgin female mice (ICR or CD-1) were purchased from Taconic Inc.(Hudson, NY, USA) or Charles River Laboratories, (Troy, NY, USA). Eight-month-old ICR retired male breeders were purchased from Taconic Inc. Mice containing the floxed β_1 integrin gene were provided by Ruth Globus (NASA Ames Research Center, Mountain View, CA, USA) with permission from Reinhardt Fässler (MPI, Martinsried, Germany). Transgenic mice expressing the Cre recombinase under the control of the ZP3 promoter were obtained from Paul Primakoff (University of California, Davis) with permission from Jamie Marth (University of California, San Diego). Pregnant mare's serum gonadotropin (PMSG, #367 222), hyaluronidase (#H3506), and Hoechst 33342, were purchased from Sigma–Aldrich, and human chorionic gonadotropin (hCG, #230734) was obtained through the National Hormone and Peptide Program, the National Institute of Diabetes and Digestive Kidney Diseases, and Dr. A.F. Parlow. Chemicals for assay buffers were purchased from Sigma–Aldrich and Fisher Scientific.

General methods: Analytical thin layer chromatography (TLC) was performed on precoated silica gel plates ($60F_{254}$) and flash chromatography on silica gel-60 (230–400 mesh). TLC spots were detected by UV and by staining with 10% phosphomolybdic acid (PMA) in ethanol. The molecular weight of the polymers was assessed by gel permeation chromatography (Phenogel 5 μ Linear(2) GPC column, Phenomenex) eluting with 10% CH_3OH in CH_2Cl_2 or THF. Inova400, Inova500, and Inova600 MHz NMR spectrometers were used to perform NMR analysis, and spectra were recorded in $CDCl_3$ unless otherwise noted. ^{1}H NMR spectra are reported as chemical shift in parts per million (multiplicity, coupling constant in Hz, integration). ^{1}H NMR data are assumed to be first order. Copies of polymer spectra are available in the Supporting Information with this article online. The purities of all peptide monomers were assessed by RP-HPLC on a Vydac C_{18} column (Atlanta, GA, USA). Linear gradient elution was performed at 1 mL min^{-1} with CH_3CN and H_2O (both containing TFA, 0.1%).

PMSG and hCG were resuspended in sterile PBS to 7.5 IU per 100 μL. These solutions were stored at −20 °C. Hyaluronidase was resuspended in sterile water to a final concentration of 30 mg mL^{-1} and was stored at −20 °C. Tyrode's acid was prepared with NaCl (0.8 g), KCl (0.02 g), $CaCl_2$ (0.02 g), $MgCl_2$ (0.01 g), Na_2HPO_4 (0.005 g), glucose (0.1 g), and polyvinylpyrrolidone (0.4 g) dissolved in 100 mL sterile water. After filtration through a 0.2 μm filter, diluted 1 N HCl was added to adjust the pH to 2.0. Tyrode's acid was stored at −20 °C. Hoechst 33342 (2′-[4 ethoxyphenol]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benz-imidazole trihydrochloride) was stored in the dark at 4 °C. All of the steps involving the use of Hoechst 33342 were performed with minimum exposure to light.

ROMP: The procedure detailed below for polymer **6** is representative of the procedure followed for synthesis of all of the homopolymers and the block copolymers. Scale, yield, and spectra are presented for each of the individual polymers. The number of ligands (*n*) is based on the monomer:catalyst ratio used in the synthesis. Monomers **3** and **5** were prepared as described.[39]

Fmoc-Gln(Trt)-Cys(Trt)-Asp(O*t*Bu)-OMe: Fmoc-Cys(Trt)-Asp(O*t*Bu)-OMe,[39] (5.19 mmol, 4.0 g) was dissolved in dry CH_2Cl_2 (10 mL). 1-Octanethiol (51.9 mmol, 9.0 mL) and DBU (0.52 mmol, 78 μL) were added, and the reaction was 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_2Cl_2. H-Cys(Trt)-Asp(O*t*Bu)-OMe (2.1 g, 73%) was obtained as a fine white powder. H-Cys(Trt)-Asp(O*t*Bu)-OMe, (3.47 mmol, 1.9 g), Fmoc-Gln(Trt)-OH (3.82 mmol, 2.3 g), EDC·HCl

(4.16 mmol, 0.8 g), and HOBt·hydrate (4.16 mmol, 0.6 g) were dissolved in dry CH_2Cl_2 (13 mL) and cooled to 0 °C. DIEA (3.82 mmol, 0.5 mL) was added to the mixture, and the reaction was stirred for 5 h at RT under Ar. The crude mixture was diluted into CH_2Cl_2 and was washed three times with 5% $NaHCO_3$, followed by three washes with 1 N HCl, and was dried over Na_2SO_4. After evaporation of solvent, the peptide product was purified by flash silica chromatography (acetone/CH_2Cl_2 1:20) to yield Fmoc-Gln(Trt)-Cys(Trt)Asp-(O*t*Bu)-OMe (3.4 g, 87%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (d, J = 7.6 Hz, 2H) 7.55 (d, J = 8.0 Hz, 2H), 7.38 (m, 8H), 7.21 (m, 26H), 6.87 (d, J = 8.0 Hz, 1H), 6.61(d, J=6.8 Hz, 1H), 5.91 (d, J = 5.6 Hz, 1H), 4.62 (m, 1H), 4.33 (d, J = 7.6 Hz, 2H), 4.16 (t, d = 7.2, 1H), 4.00 (m, 1H), 3.93 (m, 1H), 3.60 (s, 3H), 2.70 (m, 2H), 2.57 (m, 2H), 2.45 (m, 1H), 2.40 (m, 1H), 2.02 (m, 1H), 1.92 (m, 1H), 1.38 (s, 9H).

NB-Gln(Trt)-Cys(Trt)-Asp(O*t*Bu)-OMe, 4: H-Gln(Trt)-Cys(Trt)-Asp-(O*t*Bu)-OMe (0.33 mmol, 300 mg) deprotected with DBU and 1-octanethiol as described above, 5-norbornene-*exo*-carboxylic acid (0.36 mmol, 50 mg), EDC·HCl (0.40 mmol, 76 mg), and HOBt·hydrate (0.40 mmol, 61 mg) were dissolved in dry CH_2Cl_2 (2 mL). DIEA (0.40 mmol, 70 μL) was added to the mixture, and the reaction was stirred for 1 h at RT under Ar. The crude mixture was diluted into CH_2Cl_2 and was washed three times with 5% $NaHCO_3$, followed by three washes with 1 N HCl, and was dried over Na_2SO_4. After evaporation of solvent, the peptide product was purified by flash silica chromatography (acetone/CH_2Cl_2 1:20) to yield **4** (220 mg, 64%) as a white powder. ^{1}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); ^{13}C NMR (100 MHz): δ = 26.92, 27.31, 28.24, 28.28, 30.61, 30.88, 33.72, 37.50, 41.79, 44.42, 46.51, 46.71, 46.96, 47.53, 49.22, 52.49, 52.59, 52.61, 54.21, 54.52, 67.39, 67.42, 70.90, 81.60, 127.12, 127.27, 128.16, 128.31, 128.94, 129.78, 136.24, 136.39, 138.06, 138.21, 144.55, 144.69, 144.72, 169.71, 169.87, 171.14, 171.43, 172.73, 172.82, 177.03, 177.14.

6: Monomer **3** (117 μmol, 100 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 1 mL). Oven-dried LiCl (2.4 mmol, 102 mg), **1** (11.7 μmol, 10 mg) dissolved in CH_2Cl_2/MeOH (3:1, 500 μL) and additional CH_2Cl_2/MeOH (3:1, 200 μL) at 0 °C were added to the reaction mixture under Ar. The reaction was stirred for 1 h at RT under Ar. Ethylvinyl ether (1 mL) was added to quench the reaction, and the mixture was stirred for an additional 40 min. After removing the solvent, the residue was dissolved in CH_2Cl_2. The solution was washed three times with H_2O, dried with Na_2SO_4, concentrated by rotary evaporation, and precipitated with cold Et_2O. The product was isolated by centrifugation and dried. Crude protected polymer was deprotected with TFA/TIPS/H_2O (95/2.5/2.5) for 5 h. The reaction mixture was concentrated with N_2 and was precipitated with cold Et_2O. The precipitate was collected by centrifugation. Polymer was dissolved in H_2O (1 mL) at pH 6–7 and reduced with 10–20 mM tris(2-carboxyethyl)-phosphine (TCEP) for 2 h at RT. Reduced polymer was isolated by precipitation with 1 N HCl (200 μL). Residual TCEP was removed by repeated washing of the precipitate with H_2O (3×1 mL). **6**, a yellowish-white solid, was collected (40 mg, 67%), dried, and stored at −20 °C. ^{1}H NMR (500 MHz, D_2O): δ = 7.32 (m) 5.39 (m), 4.56–3.93 (with max at 4.68, 4.42, 4.25), 3.66 (brs), 3.02–2.40 (with max at 2.87, 2.63, 2.48), 2.34–1.44 (with max at 2.19, 1.95, 1.83, 1.60), 1.24 (brs).

7_{10}: Monomer **3** (117.1 μmol, 100 mg) and **2** (11.7 μmol, 10.4 mg) in CH_2Cl_2/MeOH (3:1, 1 mL) yielded a yellowish-white solid (48 mg, 80%). ^{1}H NMR (500 MHz, D_2O): δ = 7.28 (m) 5.36 (m), 4.06–4.03 (with max at 4.58, 4.44, 4.27), 3.64 (brs), 3.09–2.36 (with max at 2.83, 2.65, 2.48), 2.37–1.38 (with max at 2.22, 1.93, 1.82, 1.59), 1.20 (brs), 0.93 (brs).

8: Monomer **3** (47 μmol, 40 mg) and **1** (4.7 μmol, 4 mg) in CH_2Cl_2/MeOH (3:1, 1 mL) at 55 °C yielded a yellowish-white solid of a side-chain protected polymer (34 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.66–7.08 with max at 7.40, 7.27, 7.19) 5.14 (m), 4.84–4.22 (with max at 4.64, 4.39), 3.62 (brs), 3.20–2.46 (with max at 2.81, 2.68), 2.46–1.49 (with max at 2.25, 1.94, 1.76), 1.40 (brs).

9: Monomer **3** (58.5 μmol, 50 mg) and **1** (0.59 μmol, 0.5 mg) in CH_2Cl_2/MeOH (3:1, 1 mL) yielded a yellowish-white solid of a side-chain protected polymer (44 mg, 87%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.48–7.10 (with max at 7.40, 7.27, 7.19) 5.19 (m), 4.93–4.14 (with max at 4.64, 4.37), 3.63 (brs), 3.23–2.49 (with max at 2.81, 2.68, 2.56), 2.45–1.51 (with max at 2.24, 1.97, 1.65), 1.40 (brs).

7_{100}: Monomer **3** (117 μmol, 100 mg) and **2** (1.17 μmol, 1.0 mg) in CH_2Cl_2/MeOH (3:1, 1.5 mL) yielded a yellowish-white solid (47 mg, 80%). ^{1}H NMR (500 MHz, D_2O): δ = 7.34 (m) 5.34 (m), 4.65–4.14 (with max at 4.64, 4.39), 3.67 (brs), 3.15–2.44 (with max at 2.86, 2.74), 2.43–1.41 (with max at 2.34, 1.99, 1.87), 1.22 (brs), 0.95 (brs).

10_1 and 10_2: Monomer **4** (96.9 μmol, 100 mg) and **2** (101.7 μmol, 90 mg) in CH_2Cl_2/MeOH (3:1, 2 mL) yielded a mixture of **10_1** and **10_2** as a brownish white solid (62 mg, 56%). After deprotection with a mixture of H_2O, TFA and TIPS, the polymer was purified by RP-HPLC with a prep C_{18} column. MALDI mass spectrum: **10_1**; calcd: 625.23 $[M+Na]^+$, found 625.20; **10_2**; calcd (MNa^+) 1123.41, found 1123.59.

10_3: Monomer **4** (145.3 μmol, 150 mg) and **2** (48.4 μmol, 43 mg) in CH_2Cl_2/MeOH (3:1, 2 mL) yielded **10_3** as a brownish white solid (68 mg, 84%). ^{1}H NMR (500 MHz, D_2O): δ = 7.28 (m) 5.33 (m), 4.65–4.01 (with max at 4.18), 3.63 (brs), 3.29–2.40 (with max at 3.13, 2.59), 2.35–1.80 (with max at 2.21, 2.00), 1.21 (m), 0.93 (brs).

10_6: Monomer **4** (145.3 μmol, 150 mg) and **2** (24.22 μmol, 22 mg) in a total volume of 2 mL yielded **10_6** as a brownish white solid (65 mg, 81%). ^{1}H NMR (500 MHz, D_2O): δ = 7.28 (m) 5.36 (m, br), 4.54–4.13 (with max at 4.44, 4.33), 3.63 (brs), 3.30–2.40 (with max at 2.84, 2.65, 2.59), 2.38–1.43 (with max at 2.23, 1.98, 1.86), 1.21 (m), 0.93 (brs).

10_{10}: Monomer **4** (145.3 μmol, 150 mg) and **2** (14.53 μmol, 12.90 mg) in CH_2Cl_2/MeOH (3:1, 2 mL) yielded **10_{10}** as a brownish white solid (58.9 mg, 79%). ^{1}H NMR (500 MHz, D_2O): δ = 7.28 (m) 5.35 (m), 4.58–4.13 (with max at 4.47, 4.33), 3.65 (brs), 3.10–2.42 (with max at 2.85, 2.74), 2.41–1.41 (with max at 2.25, 1.99, 1.88, 1.62), 1.21 (m), 0.93 (brs).

10_{100}: Monomer **4** (112.67 μmol, 117 mg) and **2** (1.12 μmol, 1.0 mg) in CH_2Cl_2/MeOH (3:1, 2 mL) yielded a yellowish white solid (45 mg, 79%). ^{1}H NMR (500 MHz, D_2O): δ = 7.26 (m) 5.36 (m), 4.56–4.09 (with max at 4.48, 4.33), 3.64 (brs), 3.04–2.38 (with max at 2.85, 2.66, 2.61), 2.38–1.32 (with max at 2.24, 1.98, 1.88, 1.60), 1.21 (m), 0.93 (brs).

11_{10}: Monomer **5** (38 μmol, 21 mg) and **2** (13.8 μmol, 3.3 mg) in CH_2Cl_2/MeOH (3:1, 200 μL) yielded **11_{10}** as a brownish-white solid (10 mg, 67%). ^{1}H NMR (500 MHz, D_2O): δ = 7.29 (m) 5.36 (m), 4.56–3.96 (with max at 4.34, 4.07), 3.77 (brs), 3.65 (brs), 3.07–2.34 (with max at 2.90, 2.51), 2.36–1.50 (with max at 2.20, 1.95, 1.86, 1.62), 1.44–1.12 (with max at 1.34, 1.27).

11_{100}: Monomer **5** (225 μmol, 124 mg) and **2** (2.25 μmol, 2.0 mg) in CH_2Cl_2/MeOH (3:1, 2 mL) yielded **11_{100}** as a brownish-white solid (89 mg, 90%). ^{1}H NMR (500 MHz, D_2O): $\delta = 7.30$ (m) 5.38 (m), 4.44–3.93 (with max at 4.35, 4.07), 3.77 (br s), 3.65 (br s), 3.10–2.30 (with max at 2.91, 2.51), 2.32–1.47 (with max at 2.15, 1.93, 1.82, 1.61), 1.43–1.03 (with max at 1.34, 1.25), 0.95 (br s).

7_2–11_{96}–7_2: Monomer **3** (4.5 μmol, 3.9 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 300 μL). Oven-dried LiCl (1.2 mmol, 51 mg) and **2** (2.25 μmol, 2.0 mg) dissolved in CH_2Cl_2/MeOH (3:1, 100 μL) were added and additional CH_2Cl_2/MeOH (3:1, 100 μL) was added to the reaction at 0 °C under Ar. The reaction was stirred for 20 min at RT under Ar. Monomer **5** (216.3 μmol, 119 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 500 μL) and was added to the reaction at 0 °C under Ar. The reaction was stirred for 1 h at RT under Ar. Monomer **3** (4.5 μmol, 3.9 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 300 μL) at 0 °C under Ar. The reaction was stirred for 1 h at RT under Ar. After quenching the reaction, crude polymer was precipitated, and deprotected to yield **7_2–11_{96}–7_2** as a yellowish-white solid (77 mg, 77%). ^{1}H NMR (500 MHz, D_2O): $\delta = 7.24$ (m) 5.37 (m), 4.53–3.97 (with max at 4.33, 4.07), 3.77 (br s), 3.65 (br s), 3.14–2.34 (with max at 2.90, 2.50), 2.35–1.45 (with max at 2.19, 1.94, 1.85, 1.61), 1.33 (br s), 1.27–0.90 (with max at 1.15, 0.95).

10_2-11_{96}-10_2: Monomer **4** (4.5 μmol, 4.7 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 300 μL). To the reaction was added oven-dried LiCl (1.2 mmol, 51 mg) and **2** (2.25 μmol, 2.0 mg) dissolved in CH_2Cl_2/MeOH (3:1, 100 μL) and additional CH_2Cl_2/MeOH (3:1, 100 μL) was added at 0 °C under Ar. The reaction was stirred for 20 min at RT under Ar. Monomer **5** (216.3 μmol, 119 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 500 μL) and was added to the reaction at 0 °C under Ar. The reaction was stirred for 4 h at RT under Ar. Monomer **4** (4.5 μmol, 4.7 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 300 μL) and was added at 0 °C under Ar. The reaction was stirred for 5 h at RT under Ar. After quenching the reaction, crude polymer was precipitated and deprotected to yield **10_2-11_{96}-10_2** as a yellowish-white solid (82 mg, 82%). ^{1}H NMR (500 MHz, D_2O): $\delta = 7.36$ (m) 5.37 (m), 4.52–3.94 (with max at 4.34, 4.07), 3.77 (br s), 3.65 (br s), 3.16–2.31 (with max at 2.90, 2.49), 2.34–1.50 (with max at 2.16, 1.93, 1.84, 1.60), 1.34 (br s), 1.29–1.04 (with max at 1.25, 1.14).

7_2-11_{96}-10_2: Monomer **3** (4.5 μmol, 3.9 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 300 μL). To the reaction was added oven-dried LiCl (1.2 mmol, 51 mg) and **2** (2.25 μmol, 2.0 mg) dissolved in CH_2Cl_2/MeOH (3:1, 100 μL) and additional CH_2Cl_2/MeOH (3:1, 100 μL) was added at 0 °C under Ar. The reaction was stirred for 20 min at RT under Ar. Monomer **5** (216.3 μmol, 119 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 500 μL) and was added to the reaction at 0 °C under Ar. The reaction was stirred for 4 h at RT under Ar. Monomer **4** (4.5 μmol, 4.7 mg) was dissolved in CH_2Cl_2/MeOH (3:1, 300 μL) and was added at 0 °C under Ar. The reaction was stirred for 5 h at RT under Ar. After quenching the reaction, crude polymer was precipitated, and deprotected to yield **7_2–11_{96}–10_2** as a yellowish-white solid (73 mg, 73%). ^{1}H NMR (500 MHz, D_2O): $\delta = 7.32$ (m) 5.38 (m), 4.45–3.96 (with max at 4.36, 4.07), 3.77 (br s), 3.18–2.31 (with max at 2.90, 2.50), 2.32–1.42 (with max at 2.14, 1.93, 1.78, 1.60), 1.25 (br s), 1.12 (m).

In vitro fertilization assay

Isolation of oocytes and spermatozoa for IVF assay: All experiments performed with mice were in accordance with the National Institute of Health and United States Department of Agriculture guidelines, and the specific procedures performed were approved by the Stony Brook University IACUC (protocol #0616). Eggs were collected from the oviducts of 8- to 10-week-old superovulated female ICR/CD-1 mice or C57 mutant progeny that were wild type (Cre^- β_1 +/+) or knockouts (Cre^+ β_1 f/f) for the β_1 allele. Mice were superovulated by injecting PMSG (7.5 IU), followed 48–52 h later by an injection of hCG (7.5 IU). 12–14 h after hCG injection, mice were sacrificed. All manipulations and incubations of eggs were performed at 37 °C, 5% CO_2 unless otherwise noted. The oviducts were removed from euthanized mice and were incubated in prewarmed 0.5% BSA/M16 (medium). Cumulus-egg complexes were collected and transferred to 500 μL drops of medium containing hyaluronidase (30 μg mL^{-1}) surrounded by mineral oil. After a 10 min incubation at 37 °C, 5% CO_2, 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 80 μL drops of medium. Eggs were recovered for 1 h before treating with Tyrode's acid. Zona pellucidae of metaphase II eggs were removed by treating eggs with a Tyrode's acid drop (100 μL) for 1 min at RT. Zona free eggs were washed six times with medium and were recovered for 2 h, then preloaded with Hoechst 33342 dye (10 μg mL^{-1}) for 30 min at 37 °C, 5% CO_2. Meanwhile, 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. 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_2 for 2.5 h to 3 h in the same medium to allow them to capacitate and acrosome react.

Inhibitor assay: The polymers were dissolved in aqueous NH_4OH and fully reduced with 10 mM TCEP for 1–2 h. The pH of reducing solutions was between 6 and 7 to prevent formation of precipitates. The solutions were centrifuged at 16000 *g* for 3 min, and the supernatants were collected. The polymers were precipitated by adding 1 N HCl to the supernatants, centrifuged at 2000 *g* for 3 min until the polymers formed a thin film on the Eppendorf tubes, washed with water three times, and residual water was removed by lyophilization. Polymers of high molecular weight were handled carefully to prevent aggregate formation upon centrifugation. Polymers were redissolved in water adjusted to pH 7 with NH_4OH immediately before assays to a final concentration of 0.1 mM. Zona free eggs that had been loaded with Hoechst 33342 were washed six times with 3% BSA/M16, and placed in 100 μL drops of polymer solution in 3% BSA/M16, and incubated with eggs for 45 min prior to sperm addition. No more than 6 μL of stock solution was diluted into an egg drop. Capacitated and acrosome reacted sperm were added to eggs at a final concentration of $1–5 \times 10^4$ sperm per mL. After 45 min at 37 °C, 5% CO_2, eggs were gently washed through six 60 μL drops of 3% BSA/M16. Eggs were mounted onto glass microscope slides, covered with glass cover slips, and sperm binding and fusion were scored by epi-fluorescence microscopy and DIC microscopy (NIKON Eclipse 400, 40×, 0.75 NA objective). Fusion was scored as the fluorescent labeling of sperm nuclei with Hoechst 33342 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_{50}s were calculated by a three parameter fit (GRAFIT software) by Equation (1):

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

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.

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