Comparison of Fertilinβ-Peptide-Substituted Polymers and Liposomes as Inhibitors of In Vitro Fertilization

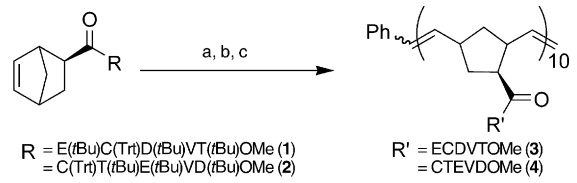
Kenny S. Roberts,[a] Samidha Konkar,[a] and Nicole S. Sampson*[a]

Mammalian fertilization depends upon successful binding and fusion of the membranes of the spermatozoon and the oocyte. These processes are thought to be mediated by a series of protein – protein interactions in which sperm proteins known as ADAMs (A disintegrin and metalloproteinases) play a key role.[1, 2] Fertilinβ and cyritestin are members of the ADAM family and their disintegrin domains mediate sperm – egg binding.[3–6] Two different integrins have been implicated as fertilinβ receptors: $\alpha_6\beta_1$ and $\alpha_9\beta_1$[5, 7–11] The proposed identity of the receptor depends on the experimental approach utilized. However, integrin knockout experiments in the mouse egg call into question whether either $\alpha_6\beta_1$ or $\alpha_9\beta_1$ integrin is the only fertilinβ receptor.[12] These genetic experiments suggest that a member of a nonintegrin receptor family may be sufficient for sperm – egg binding and fusion. The cyritestin receptor has not yet been identified. Thus, there is much uncertainty about the identity and number of sperm receptors on the egg.

Peptides derived from the fertilinβ and cyritestin disintegrin binding loops inhibit sperm – egg binding and subsequent fusion in vitro. Mimics of these peptides may be used to probe biological function, for example, to establish the identity of the receptor(s) and to elucidate the role of individual ligand proteins. However, for the probes to be useful, they must have high affinity for their target. Both linear peptides[13–16] and existing peptide mimics[17] of fertilinβ and cyritestin are modest inhibitors of sperm – egg binding, with IC_{50} values of around 500 μM. We report herein that norbornyl homopolymers containing 10 pendent fertilinβ-derived oligopeptides are significantly improved inhibitors of adhesion and fertilization in vitro and work in a multivalent fashion.

We hypothesized that the modest inhibition observed with linear peptides might be due to a monovalent binding interaction that is of low affinity. We previously determined that liposomes bearing 80 copies of fertilinβ peptide per outer membrane are 100-fold improved inhibitors of fertilization compared to linear peptides,[18] which demonstrates that polyvalency does effect adhesion in this system. In this work, we further investigated the role of polyvalency in the sperm – egg adhesion process by synthesizing multivalent peptide mimics that not only allow control of the effective concentration of ligands presented, but are also different macromolecular sizes.

The approach adopted was to synthesize peptide polymers by ring-opening metathesis polymerization (ROMP) of norbornyl oligopeptides with Grubbs' ruthenium carbene catalyst, $Cl_2(PCy_3)_2Ru{=}CHPh$ (**5**). In addition to its functional group tolerance, **5** catalyzes living polymerizations that allow the preparation of polymers of defined molecular weight and of block copolymers.[19] Several strategies have been employed to prepare polynorbornenes substituted with bioactive, water-soluble molecules by using Grubbs' catalyst. We used homogenous polymerization conditions and *t*butyl-protected peptide.[20, 21] We found that it was necessary to include 6 M LiCl to prevent aggregation and solubilize the growing fertilinβ polyvalent mimics (Scheme 1).[22] After polymerization, the peptide polymer was deprotected and fully reduced. We employed this methodology because the purity and quality of the fertilinβ polymers obtained were superior to those of fertilinβ polymers obtained by other methods.



Scheme 1. *Polymer synthetic scheme. Yields:* ***3****, 80%;* ***4****, 77%. a)* $Cl_2(PCy_3)_2$-*Ru=CHPh (***5***),* CH_2Cl_2*/MeOH (3:1), LiCl (6 M); b)* $CH_2{=}CHOEt$*; c) TFA/*H_2O*/TIS (95:2.5:2.5). Ph, phenyl; TFA, trifluoroacetic acid; TIS, triisopropyl silane.*

To prepare the monomers for ROMP, we synthesized short peptides with the sequences norbornene-Glu-Cys-Asp-Val-Thr-OMe (**1**) and norbornene-Cys-Thr-Glu-Val-Asp-OMe (**2**). Monomer **1** corresponds to the fertilinβ disintegrin binding loop; linear peptides with this sequence are inhibitors of in vitro fertilization. Monomer **2** is a scrambled control peptide; linear peptides with this sequence do not inhibit in vitro fertilization. We chose to synthesize the monomers by solution phase peptide chemistry so that purified monomers could be prepared in large quantities in their protected form. The monomers were protected at the side chains with *t*butyl groups that could readily be removed after polymerization. The purities of the protected monomer peptides were assessed by C18 reversed-phase HPLC and found to be greater than 98%.

The monomer peptides were then subjected to ROMP. The ratio of the starting concentration of monomer to that of the catalyst ($[M]_o/[C]_o$) was 10/1 and corresponded to the degree of polymerization determined by ^1H NMR spectroscopy. Side-chain-protecting groups on the oligopeptide polymers were removed by treatment with trifluoroacetic acid mixed with water and triisopropylsilane as scavengers. The presence of free cysteine in the peptide sequence required that care be taken to use fully reduced polymer in the in vitro fertilization (IVF) assay. As a result of the acidic nature of the fertilinβ peptide, the polymers were

[a] *Prof N. S. Sampson, K. S. Roberts, S. Konkar*
Department of Chemistry
State University of New York
Stony Brook, NY 11794-3400 (USA)
Fax: (+1) 631-6325731
E-mail: nicole.sampson@stonybrook.edu

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insoluble in water at low pH values. Immediately before use, the polymers were fully reduced by the addition of tris(2-carboxyethyl)phosphine (TCEP) at pH 6 for 3 – 4 h, precipitated, washed with 1 N HCl, and then redissolved in water adjusted to pH 7. The degree of oxidation was assessed by gel-filtration chromatography before each assay.

The polymers were tested as inhibitors in a mouse IVF assay to characterize their ability to block sperm – egg adhesion.[7, 15] Two measures of inhibition were assessed: the average number of sperm fused per egg (fertilization index), and the percentage of eggs fertilized (fertilization rate). The monovalent ligand, Ac-Asp-Glu-Cys-Asp-Ala-Tyr-NH_2 (**6**) was used as a monomeric positive control.[13]

The fertilinβ polymer is a significantly better inhibitor of sperm – egg adhesion than its monomeric counterpart (Table 1) and has an IC_{50} that is 50 to 70-fold improved, as measured by fertilization rate and index and based on the concentration of fertilinβ peptide needed to inhibit fertilization rather than on molar concentrations of polymer. The polymer with scrambled peptide **4** showed no inhibition at 400 μM. This result confirms that multivalent interaction of the polymer is important for inhibition. However, there is a significant difference in inhibition behavior between the liposome and polymer polyvalent systems. Despite the improved potency of the fertilinβ polymer, maximal inhibition reaches a plateau at 72 ± 6 %, as with the monomer (Figure 1, Table 1). This incomplete inhibition has been observed for all fertilinβ monomers assayed to date. One postulated explanation is that the monomers are of such low affinity that it is not possible to add a sufficiently high concentration of peptide to saturate its receptor. The improved potency of the fertilinβ polymer demonstrates that this is not the case. We now postulate that there is more than one type of egg receptor whose interaction with sperm ligands leads to sperm – egg binding and that the peptide sequences of these fertilinβ-derived monomers and polymers only block one type of receptor. This incomplete blocking is consistent with the reduced, but not eliminated, binding and fusion of fertilinβ −/− sperm to eggs,[23] and with egg integrin knockout experiments that implicate the presence of a second nonintegrin sperm receptor on the egg.[12]

Interestingly, liposome inhibitors containing 1 mol % dimyristoylated fertilinβ peptide,[18] which corresponds to 80 peptides presented on the outer leaflet, are approximately equipotent

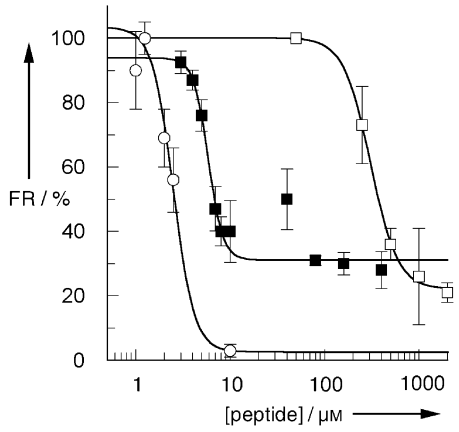

Figure 1. *Concentration dependence of inhibition measured as number of eggs fertilized (FR). Solid squares, fertilinβ polymer **3**; open squares, fertilinβ monomer **6**; open circles, 1 mol % fertilinβ liposome. The fertilization rate shown is relative to a buffer control in which 76 % of the eggs were fertilized and an average of 1.6 sperm were fused per egg. Error bars are the SEM values, where* n = *2 – 6, 30 – 75 eggs.*

with the 10-mer fertilinβ polymer (Table 1, Figure 1). However, the maximal inhibition obtained with liposomes is 97 ± 2 %. The liposomes are 40 nm in diameter as compared to the 6-nm theoretical span of the extended 10-mer polymer backbone. When the IC_{50} values were compared by calculating the number of molecular complexes (liposome or polymer) per milliliter at half-maximal inhibition, we found that 30-fold more polymers are required than liposomes to achieve the same level of inhibition. This comparison demonstrates that a small number of multivalent contacts are required for efficient attachment of the inhibitor (either polymer or liposome) to the receptor on the target membrane, but the physical size of the inhibitory complex contributes to steric blocking of sperm binding to the egg plasma membrane. Steric stabilization has also been reported for the hemagglutination inhibition activities of polyacrylamides, much larger polymers than our fertilinβ-containing molecules.[24] The difference between liposome and polymer behavior suggests that binding of the liposome to the surface of the egg blocks sperm access to an unidentified sperm receptor or receptors by creating a physical barrier that prevents the sperm from approaching the egg surface. That is, attachment of the

Table 1. *IC_{50} values for inhibition of fertilization by fertilinβ mimics.*[a]

Mimic	IC_{50} [μM peptide] by FI	IC_{50} [μM peptide] by FR	Maximal Inhibition Observed [%]	IC_{50} [complexes mL^{-1}] by FR
6[13]	360 ± 30	306 ± 10	79 ± 3	not applicable
3	5.1 ± 1.4	5.8 ± 0.3	72 ± 6	3×10^{14}
4	no inhibition[b]	no inhibition[b]	no inhibition[b]	no inhibition[b]
1 mol % fertilinβ liposome	2.4 ± 1.9	2.5 ± 0.3	97 ± 2	1×10^{13}

[a] Fertilization in the presence of peptide as measured by number of zona-free eggs fertilized (FR, fertilization rate) or average number of sperm fused per egg (FI, fertilization index) relative to a buffer control. The average FR for the buffer control was 76 % eggs fertilized and the average FI was 1.6 sperm fused per egg. Error bars are the standard error in the mean (SEM) for a fit at 5 – 10 different concentrations, with 30 – 75 eggs per concentration. [b] Tested at 400 μM.

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liposome to the egg is a result of direct binding between the fertilinβ peptide and its receptor. This liposome – egg complex is sufficiently large that sperm access to a second type of receptor on the egg surface is blocked by the steric bulk of the liposome rather than the fertilinβ peptide binding to the receptor binding site. In contrast, the short oligopeptide polymers appear to competitively block the fertilinβ receptor by direct blocking of the receptor binding site. These experiments highlight the value of comparing two different types of polyvalent inhibitor to elucidate the importance of individual receptor – ligand interactions. We are currently testing polymers and liposomes of varying size, as well as investigating their biological mechanisms of action.

Materials and Methods

Synthesis of inhibitors: Inhibitors were synthesized as previously described[18, 22] by solution phase peptide methods with Carbobenz-(yl)oxy (Cbz) or 9-fluorenylmethoxycarbonyl (Fmoc) α-amino protection and *t*butyl-based side-chain protection. The catalyst $Cl_2(PCy_3)_2$ Ru=CHPh was dissolved in CH_2Cl_2/CH_3OH (3:1) to give a typical concentration of 0.03 M. Monomers were each dissolved in a minimum amount of CH_2Cl_2/CH_3OH (3:1), and LiCl (6 M) was added to the mixture. A typical reaction was carried out at an initial monomer concentration of 0.2 – 0.3 M. The reaction was stirred at RT for 3 – 4 h before quenching with ethyl vinyl ether and stirring for an additional 30 min. The solvent was removed and the product was washed with H_2O. Polymers were dissolved in CH_2Cl_2 and precipitated with cold Et_2O. The product was isolated by centrifugation and dried under vacuum in the presence of P_2O_5. Polymers were deprotected in a cocktail containing H_2O, TIS, and TFA (2.5:2.5:95) for 5 h. The reaction mixtures were concentrated under N_2, precipitated in cold Et_2O, and centrifuged. Polymers were dissolved in H_2O at pH 6 and reduced by treatment with excess tris(2-carboxyethyl)phosphine (TCEP) for 5 h with stirring at 37 °C. Pure deprotected product was isolated by precipitation with 1 N HCl. Excess TCEP was removed by repeated washing with H_2O. A gray white solid was collected, dried, and stored at −20 °C.

In vitro fertilization assay: Eggs and sperm were isolated from ICR (Taconic farms) or CD-1 (Charles River Laboratories) mice as described in Yuan et al.[15] Zona pellucida were removed by treatment with acid Tyrode's solution for 30 sec and recovered at 37 °C, 5% CO_2 for 1 h. Zona-free eggs were loaded with Hoechst 33342 (1 μg mL^{-1}) for 30 min and washed with six drops of M16 medium (0.5% bovine serum albumin (BSA)). Eggs (20 – 30/100 μL) were incubated with peptide in M16 medium (3% BSA) for 45 min and then sperm were added (5×10^4 mL^{-1}). After 45 min, the eggs were washed in M16 medium (3% BSA) and mounted onto microscope slides. Fusion was scored by fluorescent labeling of sperm nuclei with Hoechst 33342 present in preloaded eggs. 3 – 7 experiments were performed per peptide and 25 – 100 eggs per peptide were used. A 4′,6-diamidino-2-phenylindole (DAPI; 465 nm) cutoff filter was used for fluorescence microscopy. IC_{50} values were determined by fitting to the equation $y = (y\text{-range})/\{1+([I]/IC_{50})\}^s$. s, cooperativity; y-range = (y-max) − (y-min); [I], inhibitor concentration.

Supporting Information: Complete experimental procedures for the preparation of compounds **1** – **4** and the in vitro fertilization assay. Spectra of compounds **1** – **4**.

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Keywords: cell recognition · peptidomimetics · polyvalency · protein models · vesicles

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