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A Facile Synthetic Method to Prepare Fluorescently Labeled ROMP Polymers

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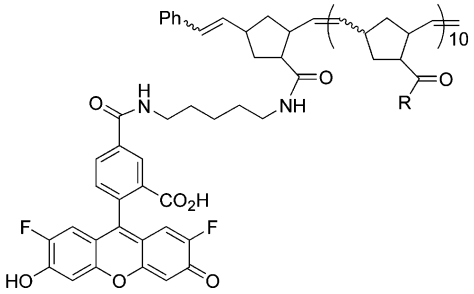
ABSTRACT



To probe the activities of sperm ADAM protein (fertilinβ), we devised a general synthetic strategy to generate fluorescently labeled fertilinβ oligopeptide polymers. Immunofluorescence studies with these polymers demonstrated that fertilinβ polymers bind specifically to a protein receptor on the mouse egg plasma membrane.

The use of fluorescence microscopy is an extremely powerful tool for studying cellular localization of biomolecules in cell biology. The development of a wide array of dyes has provided detailed views of cellular exteriors and interiors and allowed monitoring of biological activities. A wide variety of processes involving protein−protein interactions, signal transduction, or downstream intracellular responses can be investigated selectively in vitro and in vivo using fluorescence techniques.[1] The application of fluorescent probes in studying membrane proteins and subcellular compartments is rapidly expanding as a result. The types of probes available cover a wide range that includes noncovalent reagents, e.g., fluorescent antibodies, and covalent reagents, e.g., GFP fusion proteins or fluorophore-tagged small molecules.

We previously reported that norbornyl oligopeptide polymers such as **1a** are 50- to 70-fold improved inhibitors of in vitro fertilization in mouse compared to their monomeric counterpart.[2] The IC_{50} for inhibition of in vitro fertilization by **1a** is 5.8 ± 0.3 μM. The sequence of the oligopeptides was derived from the binding loop of fertilinβ. Fertilinβ is a sperm transmembrane protein implicated in sperm−egg binding.[3,4] Its putative receptor is $\alpha 6\beta 1$ integrin,[5,6] although genetic experiments suggest that its biological activity results from binding another unidentified cell surface protein.[7,8] We required a fluorescently labeled polymer derivative to determine the cellular localization of the inhibitor. Here we report a facile and versatile approach to synthesizing end-labeled block copolymers that will be useful in many polymer systems.

Our norbornyl monomers are polymerized using fully protected peptides, e.g., **3** (Scheme 1). After polymerization, side-chain protection is removed using trifluoroacetic acid. We were concerned that commonly used fluorophores would not survive the acidic deprotection conditions and chose a

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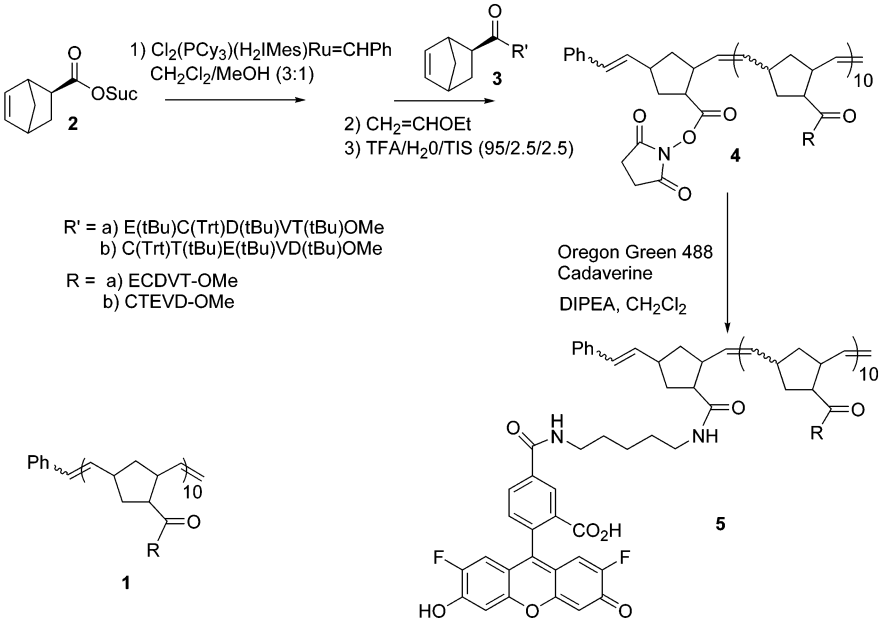
Scheme 1. Synthesis of Labeled Fertilinβ Oligopeptide-Substituted Polymer



strategy to introduce the fluorophore after deprotection of the polymer. We incorporated *N*-hydroxysuccinimide norbornyl carboxylate monomer **2** into the oligopeptide polymer in a sequential fashion. The polymerization is initiated with a 1:1 ratio of **2** and catalyst. After ring-opening metathesis is complete, 10 equiv of **3** is added to synthesize the oligopolymer block. The average DP determined by ^{1}H NMR was consistent with the $[M]_0/[C]_0$ of 11/1 in the reaction mixture. The *N*-hydroxysuccinimide ester withstands treatment with trifluoroacetic acid. After deprotection, treatment with the appropriate nucleophilic fluorophore and base yields the end-labeled fluorescent polymers. The fluorophore was incorporated into the polymer as determined by gel filtration chromatography with detection at 220 nm (peptide backbone) and 495 nm (fluorophore). Moreover, the polymers show the expected fluorescence excitation and emission characteristics (Figure 1).

This method is efficient because a single preparation of polymer may be conjugated with a range of fluorophores to tune the fluorescence properties of the polymer for individual applications. Alternative strategies have been reported for end-labeling polymers synthesized by ROMP, for example, terminating the polymerization with an ethylene glycol based trimethylsilyl ester, hydrolysis of the ester, electrophilic activation of the unmasked carboxylate, and conjugation to a nucleophilic fluorophore.[9] Like ours, this method also allows substitution of a single preparation of polymer with a variety of fluorophores or other molecular tags. However, it was not suitable for use with carboxylic oligopeptide norbornyl polymers such as **5.** Electrophilic activation of the terminal carboxylic acid in the presence of many peptide side chain carboxylates is not synthetically feasible. In addition, our synthetic route is convergent; the monomer is activated before polymerization and no synthetic elaboration of the polymer is required before conjugation with the desired fluorophore. This reduces the number of synthetic manipula-

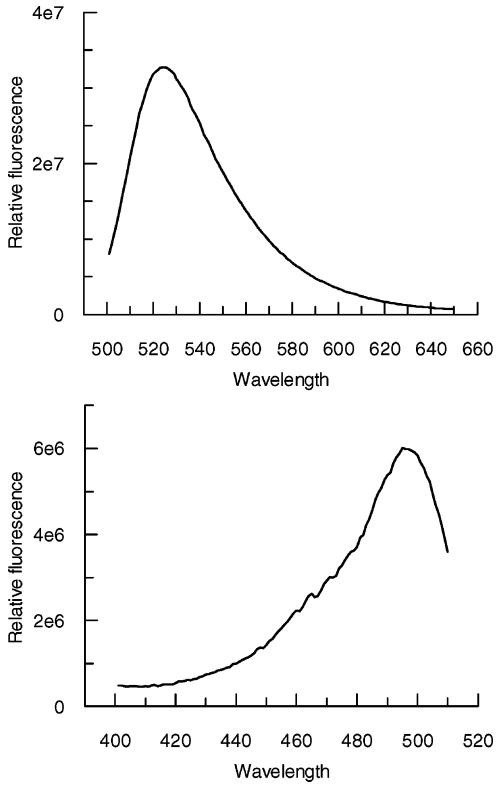


Figure 1. (Top) Representative emission spectrum of **5a** (0.5 μM) with excitation at 495 nm. (Bottom) Representative excitation spectrum of **5a** (0.5 μM) with emission at 520 nm.

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tions of polymer required for preparation. Our method has the additional advantage of amplification of signal. The number of fluorophores or other reporter groups such as affinity handles incorporated into a single polymer may be increased by increasing the ratio of *N*-hydroxysuccinimide monomer **2** to catalyst.

We chose to conjugate Oregon green 488 cadaverine to our polymer because it is pH-insensitive in the physiological pH range and contains a primary amine linker. We prepared a fluorescent polymer with fertilinβ oligopeptide **5a** and a control with a scrambled sequence **5b**. The fluorescent polymers **5a** and **5b** were purified by precipitation with 1 N HCl and extensive washing. We stained live zona-free oocytes with **5a** and **5b** to determine whether the polymer **5a** specifically interacts with the cell surface; 10% goat serum was used to block non specific binding. Following incubation, oocytes were washed in 3 mL of M16 buffer and fixed using 3.7% paraformaldehyde. The oocytes were washed and mounted with Vecta Shield.

We imaged the stained oocytes using epi-fluorescence and DIC microscopy (Figure 2). Our staining experiments demonstrated that binding of the polymers to the cell surface is dependent on the oligopeptide sequence. Polymer **5a** bearing the fertilinβ binding sequence stains the egg plasma membrane (Figure 2A and B), and **5b** bearing the scrambled sequence does not even if the image is overexposed (Figure 2C and D). Control eggs with no polymer show that all of the fluorescence observed with **5b** is due to autofluorescence of the egg (Figure 2E and F). Moreover, **5a** did not stain the oocyte if the cell surface proteins were fixed with paraformaldehyde before staining. This experiment implies that native receptor is required for oligopeptide polymer binding. These studies demonstrate that inhibition of in vitro fertilization by polymer **1a** is due to specific binding at the egg plasma membrane surface. Future experiments will employ **5a** as a tool to probe polymer−receptor interactions on the egg surface.

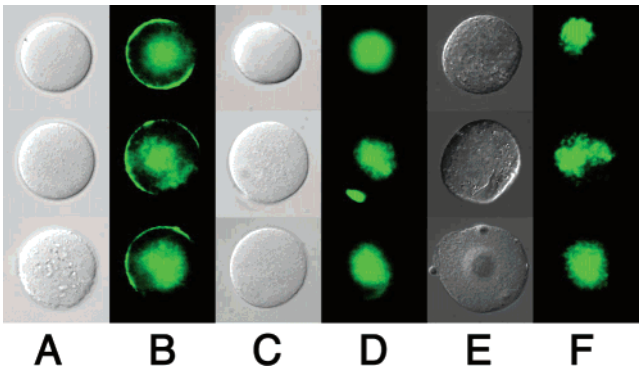

Figure 2. DIC (A, C, and E) and epi-fluorescence images (B, D, and F) of zona-free oocytes stained with **5a** (A, B), zona-free oocytes with **5b** (C, D), and no polymer (E, F) with a 40×/0.75 NA objective. Eggs were incubated with 50 μm (in peptide) **5a** or **5b** for 35 min, washed, fixed, and mounted. Exposure times for D and F were 4 times that of B.

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Supporting Information Available: Detailed descriptions of experimental procedures and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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