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# Conjugation of Proteins to Polymer Chains to Create Multivalent Molecules

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## Abstract

The activation of cellular signaling cascades, critical for regulating cell function and fate, often involves changes in the organization of receptors in the cell membrane. Using *synthetic* multivalent ligands to control the nanoscale organization of cellular receptors into clusters is an attractive approach to elicit desired downstream cellular responses, since multivalent ligands can be significantly more potent than their corresponding monovalent ligands. Synthetic multivalent ligands can serve as both versatile biological tools and potent nanoscale therapeutics, for example in applications to harness them to control stem cell fate in vitro and in vivo. Here we describe the use of recombinant protein expression and bioconjugate chemistry to synthesize multivalent ligands that have the potential to regulate cell signaling in a variety of cell types.

**Keywords:** Multivalency, Stem cells, Conjugation, Biopolymers

## 1 Introduction

Multivalent ligand interactions, characterized by multiple copies of a ligand in close proximity simultaneously interacting with multiple binding partners, are ubiquitous in cellular signal transduction (1, 2). Membrane-bound ligand and receptor pairs—such as Delta/Jagged and Notch (3), ephrins and Ephs (4), and receptors in the immune system—are involved in juxtacrine signaling between neighboring cells and often form multimeric assemblies upon binding. Also, some soluble signaling molecules can oligomerize due to posttranslational modifications, including Sonic hedgehog (Shh) (5). Additionally, many growth factors—such as fibroblast growth factors (FGF) (6) and vascular endothelial growth factor (VEGF) (7)—bind heparin and/or extracellular matrix (ECM) proteins and may thus be tethered in close proximity upon secretion from a cell. Such multivalent ligand–receptor interactions are sometimes required for downstream signaling initiation (8), and the degree of multivalency may dictate the potency of

numerous elicited responses (9). Therefore, the generation of multivalent versions of synthetic or recombinant forms of these ligands may aid in both basic investigation of signaling mechanisms and the development of high potency signaling molecules for therapeutic application.

The current established method for oligomerizing proteins involves antibody-induced clustering (8); however, this method is not well controlled, resulting in a range of oligomer sizes which can undergo dissociation. Here, we describe a protocol for synthesizing multivalent ligands in a modular fashion via chemical conjugation of recombinant protein to long hyaluronic acid (HyA) polymer chains. We have successfully utilized this technique to develop multivalent Sonic hedgehog (Shh) and demonstrate that higher valency Shh increases potency (10). HyA polymers are flexible, allowing for rotation of bound ligands, and by varying the conjugation ratio of protein to HyA a range of valencies can be achieved. These multivalent ligands can be employed to activate pathways that benefit from receptor clustering, study the basic role of ligand/receptor clustering in signaling, and generate potent signaling agonists for therapeutic application.

## 2 Materials

### 2.1 Recombinant Protein Production and Purification

#### 2.1.1 Recombinant Protein Production

1. LB media: 1 % (w/v) tryptone peptone, 0.5 % (w/v) yeast extract, and 0.5 % (w/v) NaCl in water. Adjust pH to 7. Autoclave and store at room temperature.
2. LB agar plate with antibiotic: Add agar at 1.5 % (w/v) to LB media. Autoclave and allow to cool to ~50 °C (able to hold in hands). Add antibiotic at appropriate concentration (e.g., 0.1 mg/mL ampicillin). Pour plates using sterile technique and store at 4 °C.
3. TB media: 1.2 % (w/v) tryptone peptone, 2.4 % (w/v) yeast extract, and 0.4 % glycerol in water. Adjust pH to 7. Autoclave and store at room temperature.
4. L-arabinose (Sigma-Aldrich) or alternative protein induction reagent.
5. Lysis buffer: 5 mM imidazole (Sigma-Aldrich), 50 mM $KH_2PO_4$, and 300 mM KCl in water. Adjust pH to 8. Filter sterilize, and store at 4 °C. Degas before use.
6. Phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich): Make 10 mg/mL stock in isopropanol. Store at 4 °C. Note: PMSF is highly toxic, so follow correct safety protocols when handling.
7. β-Mercaptoethanol (β-ME).
8. Lysozyme, from chicken egg white (Sigma-Aldrich).

#### 2.1.2 Protein Purification

1. Purification materials, e.g., columns, affinity media, and wash and elution buffers.
2. Dialysis tubing of appropriate molecular weight cut off (MWCO) for protein and dialysis accessories (Spectrum Labs).
3. Dialysis buffer 1: 2 mM EDTA and 150 mM NaCl in PBS. Adjust pH to 6.5. Filter sterilize and store at room temperature. Degas before use.
4. Dialysis buffer 2: 2 mM EDTA, 10 % glycerol in PBS. Adjust pH to 6.5. Filter sterilize and store at room temperature. Degas before use.
5. BCA Protein Assay (Pierce).
6. SDS-PAGE materials.

### 2.2 Bioconjugation and Multivalent Molecule Characterization

#### 2.2.1 Bioconjugation

1. MES Buffer: 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma-Aldrich) in water. Adjust pH to 6.5. Store at room temperature. Degas before use.
2. Sodium hyaluronic acid (HyA), MW ~800 kDa (Genzyme, Lifecore, etc.).
3. 3,3′-*N*-(e-Maleimidocaproic acid) hydrazide, trifluoroacetic acid salt (EMCH, Pierce), 1.2 mg/mL working solution in MES buffer.
4. *N*-Hydroxysulfosuccinimide (Sulfo-NHS, Pierce), 2.8 mg/mL working solution in MES buffer.
5. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce), 10 mg/mL working solution in MES buffer.
6. Dialysis tubing of appropriate molecular weight cut off (MWCO) for HyA (e.g., 100 kDa MWCO for 800 kDa HyA) and dialysis accessories (Spectrum Labs).
7. Dialysis buffer 3: 2 mM EDTA, 10 % glycerol in PBS. Adjust pH to 7. Filter sterilize and store at room temperature. Degas before use.
8. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, Pierce).
9. Dialysis buffer 4: 2 mM EDTA in PBS. Adjust pH to 7. Filter sterilize and store at room temperature. Degas before use.
10. Sodium azide.
11. Penicillin Streptomycin (Life Technologies).
12. Spectra Gel Absorbent (Spectrum Labs).

## 3 Methods

### 3.1 Recombinant Protein Production and Purification

To create multivalent bioconjugates, one must first design and produce recombinant proteins that present functional groups that can be used for tethering to polymer chains. Care should be taken determining the appropriate site to attach a chemically labile functional group to assure that the bioactivity of the protein is maintained. In this protocol, we will be using a protein that has been recombinantly modified to display a cysteine at its C-terminus. The protein must also be purified, and a protein purification tag can facilitate this process. We commonly use a hexahistidine tag and purify using immobilized metal (such as $Ni^{2+}$) affinity chromatography (IMAC). The modified protein must be subcloned into a protein expression vector that is amenable to producing large amount of protein (e.g., pBAD, Life Technologies). Finally, transformation of this plasmid into a bacterial cell strain optimized for protein production (e.g., BL21, Life Technologies) should be performed.

#### 3.1.1 Recombinant Protein Production

1. Using a frozen aliquot of BL21 *E. coli* transformed (11) with pBAD containing the recombinant protein, streak a bacterial LB agar plate with the appropriate antibiotic (e.g., 0.1 mg/mL ampicillin for BL21 *E. coli*) to ensure that only transformed colonies grow (see Note 1). Culture overnight (14–16 h) at 37 °C.
2. Pick one colony and inoculate 100 mL of liquid LB media with appropriate antibiotic added. Culture overnight (16 h) shaking at 37 °C.
3. Inoculate 1 L of liquid TB media, containing the appropriate antibiotic, with 25 mL of overnight LB culture, and split into two aliquots of 500 mL in Erlenmeyer flasks of at least 1 L in volume each. Shake at 37 °C until the $OD_{600}$ of the culture media is ~0.6 (2–4 h).
4. Add appropriate protein induction reagent to the appropriate concentration (e.g., 0.1 % (w/v) L-arabinose for the pBAD vector). Shake at 30 °C for 5 h.
5. Pellet cells in 250 mL polypropylene bottles, 5,000 × *g* for 20 min at 4 °C.
6. Pour off supernatant and freeze cell pellet at −80 °C until use (see Note 2).

*From this point on, all steps should be performed at 4 °C unless otherwise specified.*

7. Add PMSF stock to cold lysis buffer to a final concentration of 200 μg/mL, β-ME to 20 mM, and lysozyme to 1 mg/mL.

Thaw cells in lysis buffer (30 mL for 1 L culture) by swirling buffer constantly on ice (see Note 3).

8. Incubate at 4 °C for 30 min.
9. Sonicate cell suspension using sonicator (Sonicator 3000, Giltron) on power setting 7. For 30 mL cell suspension in 50 mL Falcon tubes, the power output reading on the sonicator should read ~57 W. Pulse 10 s on, 10 s off, for a total of 240 s.
10. Centrifuge sonicated suspension at 28,000 × *g*, 60 min, 4 °C.
11. Carefully pipet or decant supernatant into a 50 mL Falcon tube (see Note 4).

#### *3.1.2 Protein Purification*

1. Purify supernatant using IMAC chromatography or an analogous method (12). Add β-ME to appropriate solutions directly before purification to reduce disulfide bond formation (see Note 5).
2. Determine concentration of protein of interest on a NanoDrop spectrophotometer (Thermo Scientific) at $OD_{280}$ using the protein's extinction coefficient if known. Bovine serum albumin (BSA) is often used as a standard if the extinction coefficient of the protein of interest is unavailable.
3. Pool protein-containing fractions into dialysis membranes of the appropriate molecular weight cut off (MWCO) size and dialyze at 4 °C in 1,000 mL dialysis buffer 1 for 4 h while constantly stirring (see Note 6).
4. Discard dialysis buffer 1, then add 1,000 mL of dialysis buffer 2, and dialyze at 4 °C overnight while stirring (see Note 7).
5. Validate protein concentration using a BCA assay and protein purity by running an SDS-PAGE gel (13).

### *3.2 Bioconjugation and Multivalent Molecule Characterization*

Protein bioconjugation is a useful technique for a variety of applications. For instance, it can allow for display of biological ligands in a tethered orientation from a material for prolonged signaling compared to soluble ligands. Conjugation of ligands to long polymer chains also allows for multivalent display of ligands, which often have more potent signaling properties compared to monovalent or divalent ligands. In the following protocol, we will discuss how to achieve such multivalent molecules and their subsequent characterization.

#### *3.2.1 Bioconjugation*

1. Prepare a 3 mg/mL solution of HyA in degassed MES buffer by stirring very slowly at 4 °C for at least 4 h (see Note 8).
2. Equilibrate EMCH, Sulfo-NHS, and EDC stocks to room temperature.
3. Make working solution (refer to Section 2.2.1, steps 3–5) containing EMCH, Sulfo-NHS, and EDC (see Note 9).

4. Add working solution to fully dissolved HyA solution. Allow to react for 4 h while stirring slowly at 4 °C to create activated HyA-EMCH.
5. Remove the reactants from the product by dialyzing using appropriate MWCO dialysis tubing in 1,000 mL dialysis buffer 3 for 4 h at 4 °C while stirring (see Note 10).
6. Discard dialysis buffer and repeat step 5 two more times (see Note 11).
7. Add TCEP-HCl to thawed purified recombinant protein in 200-fold molar excess, and allow to react for 5 min at 4 °C while stirring.
8. Add activated HyA-EMCH solution in desired molar conjugation ratios (10), and bring all reactions to equal volumes using dialysis buffer 3 (see Note 12).
9. Purge reaction vial headspace with nitrogen, cover vial with aluminum foil to reduce light exposure, and react at 4 °C overnight.
10. Dialyze with dialysis buffer 4 for 4 h at 4 °C while stirring (see Note 13).
11. Discard dialysis buffer and repeat step 11 two more times (see Note 10).
12. If necessary, reduce reaction volume using Spectra Gel Absorbent after the last dialysis step (see Note 14).

*3.2.2 Multivalent Molecule Characterization*

1. Quantify protein concentration of purified conjugates using a BCA assay.
2. Characterize true conjugation ratios using size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) (14) as described (15) on an HPLC with the appropriate column (e.g., PolySep-GFC-P 6000, Phenomenex).

## 4 Notes

1. Make sure to streak a new LB agar plate before every protein production, since using old plates may cause issues with protein folding and solubility during bacterial growth.
2. Freezing the cell pellet can help disrupt the cell membrane to increase protein yields.
3. Make sure to avoid excess bubbles during pellet resuspension in lysis buffer, as the surface tension from bubbles tends to denature proteins.

4. Avoid saving hazy supernatant, as this is indicative of insoluble proteins and lipids that may overload the IMAC column and thus decrease the purity of the final protein sample, while increasing the overall amount of protein in the final eluate.
5. Save 5–10 mL of dialysis buffer 2 for BCA assay and ~1 mL elution buffer for spectrophotometry measurements.
6. For most proteins, 3–5 kDa MWCO dialysis tubing is recommended.
7. Glycerol in the dialysis buffer will concentrate the protein sample as well as increase stability of the protein in solution.
8. Stirring slowly with a stir bar is necessary to fully solubilize high molecular weight HyA. Too vigorous stirring will, however, physically shear the long polymer chains and result in decreased average molecular weight, which is to be avoided.
9. It is advised to dissolve these reagents in a minimal volume of MES buffer to increase their concentration during the reaction with HyA. Sterile filter the working solution after fully dissolved.
10. For 800 kDa HyA, it is recommended to use 100 kDa MWCO tubing, to remove any lower molecular weight impurities.
11. Save approximately 50 mL of fresh dialysis buffer 3 for use in the subsequent conjugation reactions.
12. Conjugation reaction volumes should be 5–10 mL.
13. To sterilize conjugates, add 0.02 % (w/v) sodium azide and 1 % (v/v) Pen-Strep to the first dialysis volume.
14. Using aluminum foil as a wrapper, pack absorbent gel around the dialysis tubing, and place upright in cold room *for no more than 2 h*. Extended incubation will precipitate samples and render them inactive. Physically remove absorbent powder from exterior of dialysis tubing, followed by dipping the tubing into cold sterile water to completely remove powder.

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## References


1. Mammen M, Choi S-K, Whitesides GM (1998) Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. Angew Chem Int Ed 37:2754–2794
2. Kiessling LL, Gestwicki JE, Strong LE (2000) Synthetic multivalent ligands in the exploration of cell-surface interactions. Curr Opin Chem Biol 4:696–703
3. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770–776
4. Ashton RS, Conway A, Pangarkar C, Bergen J, Lim KI, Shah P, Bissell M, Schaffer DV (2012) Astrocytes regulate adult hippocampal neurogenesis through ephrin-B signaling. Nat Neurosci 15:1399–1406
5. Vyas N, Goswami D, Manonmani A, Sharma P, Ranganath HA, VijayRaghavan K, Shashidhara LS, Sowdhamini R, Mayor S (2008) Nanoscale organization of hedgehog is essential for long-range signaling. Cell 133:1214–1227
6. Ye S, Luo Y, Lu W, Jones RB, Linhardt RJ, Capila I, Toida T, Kan M, Pelletier H, McKeehan WL (2001) Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs. Biochemistry 40:14429–14439
7. Krilleke D, Ng YS, Shima DT (2009) The heparin-binding domain confers diverse functions of VEGF-A in development and disease: a structure-function study. Biochem Soc Trans 37:1201–1206
8. Davis S, Gale NW, Aldrich TH, Maisonpierre PC, Lhotak V, Pawson T, Goldfarb M, Yancopoulos GD (1994) Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. Science 266:816–819
9. Arlaud GJ, Colomb MG, Gagnon J (1987) A functional-model of the human C1 complex—emergence of a functional-model. Immunol Today 8:106–111
10. Wall ST, Saha K, Ashton RS, Kam KR, Schaffer DV, Healy KE (2008) Multivalency of Sonic hedgehog conjugated to linear polymer chains modulates protein potency. Bioconjug Chem 19:806–812
11. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Zachariou M (2008) Affinity chromatography: methods and protocols. Humana, Totowa, NJ
13. Hames BD (1998) Gel electrophoresis of proteins : a practical approach. Oxford University Press, Oxford
14. Yu Y, DesLauriers PJ, Rohlfing DC (2005) SEC-MALS method for the determination of long-chain branching and long-chain branching distribution in polyethylene. Polymer 46:5165–5182
15. Pollock JF, Ashton RS, Rode NA, Schaffer DV, Healy KE (2012) Molecular characterization of multivalent bioconjugates by size-exclusion chromatography with multiangle laser light scattering. Bioconjug Chem 23:1794–1801