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# Cysteine-Containing Fusion Tag for Site-Specific Conjugation of Therapeutic and Imaging Agents to Targeting Proteins

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## Summary

Targeted delivery of therapeutic and imaging agents requires conjugation of a corresponding payload to a targeting peptide or protein. The ideal procedure should yield a uniform preparation of functionally active conjugates and be translatable for development of clinical products. We describe here our experience with site-specific protein modification via a novel cysteine-containing fusion tag (Cys-tag), which is a 15-amino-acid (aa) N-terminal fragment of human ribonuclease I with the R4C substitution. Several Cys-tagged proteins and peptides with different numbers of native cysteines were expressed and refolded into functionally active conformation, indicating that the tag does not interfere with the formation of internal disulfide bonds. We also describe standardized procedures for site-specific conjugation of very different payloads, such as functionalized lipids and liposomes, radionuclide chelators and radionuclides, fluorescent dyes, drug-derivatized dendrimers, scaffold proteins, biotin, and polyethyleneglycol to Cys-tagged peptides and proteins, as well as present examples of functional activity of targeted conjugates in vitro and in vivo. We expect that Cys-tag would provide new opportunities for development of targeted therapeutic and imaging agents for research and clinical use.

**Key Words:** Cysteine-containing tag; site-specific conjugation; targeted imaging; targeted drug delivery; liposomes; dendrimers.

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## 1. Introduction

### *1.1. Fusion Cys-tag for Site-Specific Modification of Targeting Proteins*

Coupling therapeutic or imaging agents to a protein or a peptide for targeted "smart" delivery of a payload to a given site within the body is one of the fundamental goals of molecular medicine. There are a variety of tumor-specific antigens and receptors that can be assayed in vitro with recombinant proteins or peptides. However, the number of targeted drugs and contrast agents used for in vivo therapy or imaging is very small. This is due primarily to a lack of efficient technologies for coupling drugs and contrast agents to targeting proteins. Any technology based on random conjugation of "payloads" to carriers, usually to ε-amino groups of lysine residues, is bound to generate highly heterogeneous products with unknown distribution of functional characteristics **(Fig. 1A)**. This is particularly true for growth factors or cytokines, whose lysine residues are not readily dispensable. There are several site-specific labeling approaches that are being developed to avoid these problems, including ***(1)*** insertion of a reactive cysteine in a rationally selected region, where this cysteine will not interfere with refolding or activity, ***(2)*** insertion of terminal reactive cysteine, where such interference is expected to be less significant, and ***(3)*** development of fusion tags for site-specific enzymatic modification (e.g., Avi-tag from Rosche Applied Sciences, SNAP-tag from Covalys Biosciences AG). However, the success of the cysteine insertion approach depends on the structure of a protein or peptide of interest, while fusion tags for enzymatic modification are not readily translatable to making clinical products.

We have recently developed a cysteine-containing fusion tag for site-specific conjugation (**Fig. 1B**, also in **ref. *1***). This tag, named Cys-tag, is an α-helical

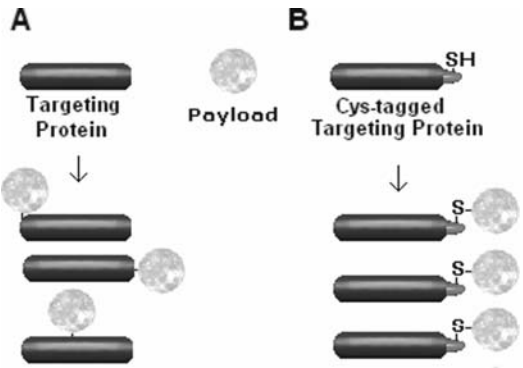


Fig. 1. Conjugation of the payload to a protein for targeted delivery. (A) Random conjugation results in a heterogeneous mixture of products. (B) Site-specific conjugation to Cys-tagged proteins leads to functionally active uniform products.

15-aa-long N-terminal fragment of human ribonuclease I, in which arginine in position 4 is substituted for cysteine. In our experience, Cys-tag is compatible with polypeptides varying in length from 53 to 358 aa **(Table 1)**, and can be used for site-specific conjugation of very different payloads **(Table 2)** without affecting protein or peptide functionality. We therefore expect that Cys-tag would provide a standardized approach to coupling therapeutic and contrast agents to various bioactive polyamides and will be translatable into the development of clinical products.

We will briefly review accumulated experience in making and using Cys-tagged peptides and proteins, focusing on Cys-tagged human vascular endothelial growth factor (VEGF). We will discuss technical problems that might arise in using our platform for development of new conjugates for targeted delivery of therapeutic and diagnostic agents.

### *1.2. Cys-Tagged Proteins*

Plasmids for bacterial expression of proteins with N- or C-terminal Cys-tag have been described previously ***(1)***. They were made by inserting the coding sequence for Cys-tag (Lys-Glu-Ser-Cys-Ala-Lys-Lys-Phe-Gln-Arg-Gln-His-Met-Asp-Ser) and a Ser-Gly linker in the appropriate sites in the commercially available pET29 plasmid for bacterial expression of proteins under control of T7 promoter (Novagen, WI). So far, expression of Cys-tagged proteins in insect, yeast, or mammalian hosts has not been tested.

**Table 1**
**Peptide and Proteins Expressed with Cys-tag**

| Proteins and peptide | Total number of amino acids | Number of native cysteines | Ref. |
|---|---|---|---|
| VEGF[a] | 110–222 | 16–18 | ***(1,2,3,4,5,6,7,15)*** |
| EGF[b] | 53 | 6 | ***(6)*** |
| EGF-SLT[c] | 358 | 8 | Unpublished |
| Soluble FLT3 ligand (FLex) | 154 | 6 | Unpublished |
| Annexin V | 330 | 1 | Unpublished |
| Catalytically inactive fragment of anthrax lethal factor (LFn) | 254 | 0 | ***(5,7)*** |

[a]Several human $VEGF_{121}$-based Cys-tagged proteins were constructed and tested, including $VEGF_{121}$ dimer, truncated $VEGF_{110}$ dimer, and single-chain (sc) VEGF comprising two 3-110 fragments of $VEGF_{121}$ fused head-to-tail.

[b]Cys-tag was fused to human EGF via $(G_4S)_3$ linker.

[c]EGF-SLT comprises Cys-tagged EGF fused to catalytic subunit of *E. coli* Shiga-like toxin.

**Table 2**
**Payloads Conjugated to Cys-Tagged Proteins**

| Payloads | Applications | Ref. |
|---|---|---|
| Lipid/Liposome | Drug delivery | ***(3,5,7)*** |
| Dendrimers | Drug delivery | ***(1)*** |
| DOTA | Targeted PET imaging | ***(7)*** |
| PEG-DOTA | Targeted PET imaging | ***(7,15)*** |
| | Targeted SPECT imaging | Unpublished |
| | Targeted radiotherapeutics | Unpublished |
| HYNIC | Targeted SPECT imaging | ***(2,7)*** |
| "Self-chelation" of $^{99m}$Tc | Targeted SPECT imaging | ***(15)*** |
| PEG | Changing protein's pharmacokinetics and immunogenicity | ***(7)*** |
| Fluorescent dyes | Targeted imaging in whole animals | ***(1,5,7)*** |
| | Tagging cells with active and accessible receptors in vivo | ***(7)*** |
| | Receptor-mediated endocytosis in vitro | ***(1,6,7)*** |
| Biotin | Targeted imaging with quantum dots and ultrasound microbubbles | Unpublished |
| Adapter protein | Flexibility in constructing conjugates | ***(5)*** |
| Fibronectin | Tissue culture scaffolds | ***(6)*** |

To date, all Cys-tagged proteins and peptides were expressed in *Escherichia coli* BL21(DE3) strain. As usual, growth temperature, bacterial density for protein induction, concentration of inducer (IPTG), and duration of induction should be optimized for every protein. Almost all our Cys-tagged proteins were found in inclusion bodies. In our experience, recovery of proteins from inclusion bodies might be greatly improved by a combination of sequential washings and mild sonication prior to solubilization of inclusion bodies.

Expression of proteins with Cys-tag adds an additional cysteine to the set of native cysteines, and therefore it might complicate the formation of native disulfide bonds. In general, protein refolding from a denatured state is achieved by slow removal of denaturing components, while maintaining Red-Ox environment using a mixture of reduced and oxidized forms of glutathione. While peptide is denatured, each cysteine can form a reversible disulfide bond either with other cysteine or with glutathione. Over time, as denaturing components are slowly removed, this process selects stable cysteine–cysteine disulfide bonds that correspond to thermodynamically favored properly folded peptide conformations. Importantly, since cysteine in Cys-tag does not have a "natural" partner, successful refolding leaves the C4 thiol group in a mixed disulfide with glutathione. The key point in refolding is to maintain a reasonable pace of

disulfide bond shuffling, so that the native set of disulfide bonds is established before denaturing components are completely removed and protein conformational flexibility is greatly decreased. It should be also noted that recently EMD Biosciences/Novagen (La Jolla, CA) introduced iFOLD™ Protein Refolding System that allows rapid optimization of refolding conditions.

Examples of optimized protocols for recovery from inclusion bodies and refolding of Cys-tagged proteins are presented in **Subheadings 3.1** and **3.2**, respectively. The protocols were worked out for recovery and refolding of single-chain (sc) VEGF that combines two 3- to 112-aa fragments of human $VEGF_{121}$ cloned "head-to-tail" and is fused to a single N-terminal Cys-tag ***(4,6)***.

### *1.3. Site-Specific Conjugation to Cys-Tagged Proteins*

After refolding of Cys-tagged proteins in Red-Ox buffer, the thiol group in Cys-tag is not available for conjugation because it is "protected" by glutathione in a form of a mixed disulfide. Deprotection involves a mild dithiothreitol (DTT) treatment (*see* **Subheading 3.3.**) that can be followed by thiol-directed modifications without removing residual DTT from the solution. The critical point in DTT treatment is to obtain protein with a free thiol group in Cys-tag without affecting native disulfide bonds.

Thiol-reactive derivatives of all major payloads, such as maleimide derivatives of fluorescent dyes, PEGs, lipids, biotin, and chelators, are available from commercial vendors. Maleimide derivatives form stable thioester bond with thiols. Alternatively, the thiol group in Cys-tagged protein can be activated with thiol-disulfide exchange reagent DPDS (2,2'-dipyridyl disulfide) and then reacted with thiol-containing payloads. Finally, bifunctional cross-linking agents with one functionality for reaction with a thiol group and another for reaction with another group (e.g., amino, carboxy) can be used for designing more complex constructs, such as dendrimers, nanoparticles, etc. Reaction conditions should be optimized for every specific conjugate with a general caveat that in order to decrease the risk of nonspecific conjugation it is better to use low ratios of payload to protein and the shortest possible incubation time at room temperature. Purification schemes for conjugates are as different as conjugates themselves and should be optimized for a specific purpose.

Site-specific conjugation to Cys-tag can be validated using either enzymatic digestion with Asp-N endopeptidase or CNBr cleavage of the conjugate. Both methods result in the cleavage of Cys-tag between Met-13 and Asp-14 and the release of a labeled N-terminal fragment. Protein digest is separated by RP-HPLC, and all labeled fragments are isolated and subjected to N-terminal sequencing or to mass-spec analysis ***(6)***. If conjugation is site-specific, only N-terminal fragments contain payload.

Functional validation of conjugates requires appropriate tissue culture or biochemical assay, where activity of the conjugate can be measured against parental unmodified protein.

## 1.4. The Use of Cys-Tagged Proteins—Specific Examples

### 1.4.1. Making Targeted Liposomes

Encapsulation of drugs into liposomes protects drugs from degradation in bodily fluids, delays their clearance, increases the amount of drugs delivered into cells via a single act of endocytosis, and decreases systemic toxicity ***(8)***. For example, encapsulation of doxorubicin, one of the most effective chemotherapeutic drugs, within sterically stabilized liposomes improves its stability while decreasing systemic toxicity ***(9)***. To enhance selectivity of drug-loaded liposomes, several groups decorated liposomes with peptides that recognize unique cell surface markers on targeted cells. Both in vitro and in vivo studies demonstrated that significant increase in efficacy of liposome-encapsulated drugs is achieved by molecular targeting to specific tumor biomarkers ***(10,11)***.

In order to decorate liposomes with proteins, a targeting peptide or protein is derivatized with a synthetic phospholipid that carries a chemically active group, usually *N*-hydroxysuccinimide, for conjugation to readily available ε-amino groups of lysine residues. At the next step, lipidated protein is either mixed with lipids at the stage of liposome preparation or inserted into preformed liposomes. The functional activity of proteins associated with liposomal membrane depends on their ability to tolerate lipidation and liposome coupling. In turn, efficacy of targeted liposomes depends on the proportion of functionally active proteins on their surface. As we show here, site-specifically lipidated scVEGF inserted into commercially available doxorubicin-loaded liposomes (Doxil®) retains the ability to interact with its receptor VEGFR-2 and efficiently delivers doxorubicin in cells overexpressing this receptor **(Fig. 2)**. To date, we have lipidated several Cys-tagged proteins and used them to construct targeted liposomes ***(3,7)***. In this work we used a protocol that was optimized for lipidation of scVEGF and construction of scVEGF-driven liposomes (*see* **Subheadings 3.3.** and **3.4.**).

### 1.4.2. Making Targeted Radiolabeled Tracers

Radionuclide chelators, such as DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or HYNIC (6-hydrazinopyridine-3-carboxylic acid), can be conjugated to Cys-tag and then "loaded" with radionuclides at the point of use ***(2,6)***. Furthermore, chelators can be conjugated via a PEGylated linker, which might improve the tracer's biodistribution ***(6)***. Interestingly, Cys-tag apparently has "self-chelating" activity that was described recently for a

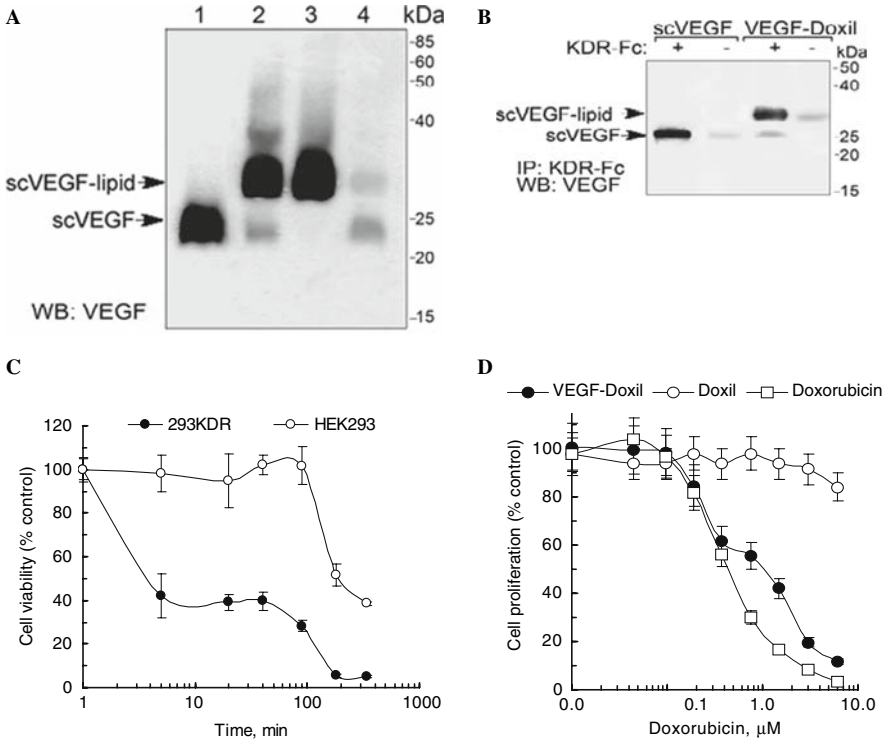


Fig. 2. Using Cys-tagged scVEGF for targeting Doxil® to cells expressing VEGF receptors. **(A)** Western blot analysis of scVEGF-decorated Doxil® (VEGF-Doxil). Lanes: 1, Unmodified scVEGF; 2, lipidation reaction mixture; 3, VEGF-Doxil collected from Sepharose CL-4B column immediately after column void volume; 4, free scVEGF and scVEGF-lipid conjugate eluted from column (pooled fractions). Samples were loaded on a 17.5% gel, separated by SDS-PAGE and analyzed by Western blotting with anti-VEGF antibody. **(B)** VEGF-Doxil or free scVEGF, at equal concentrations of 1 n*M*, were collected from solution with Protein A Sepharose beads with or without preadsorbed soluble VEGF receptor KDR-Fc and analyzed by Western blotting with anti-VEGF antibody. **(C)** 293/KDR cells expressing ~2.5 × $10^6$ VEGF receptor VEGFR-2 per cell, and HEK293 control cells without VEGF receptors were plated on 96-well plates, 1000 cells/well, 20 h before the experiment. Cells in triplicate wells were exposed to VEGF-Doxil at a final doxorubicin concentration of 1 μ*M* for times indicated, then shifted to fresh culture medium and quantitated 96 h postexposure by an MTT-based assay (Promega). **(D)** VEGFR-2 expressing 293/KDR cells were plated on 96-well plates, 1000 cells/well. Twenty h later cells in triplicate wells were exposed to liposomal or free doxorubicin for 5 min, then shifted to fresh culture medium and quantitated 96 h postexposure as above.

mutant annexin V ***(14)***. Thus, Cys-tagged peptide can be labeled with radionuclides in several different ways in order to optimize targeting, clearance, and biodistribution of a specific tracer. As an example, we have recently compared three different protocols for radiolabeling Cys-tagged scVEGF with $^{99m}$Tc ***(15)*** and found that biodistribution of radiolabeled tracer is affected by the choice of a chelator **(Fig. 3)**. For this protein the most advantageous biodistribution with the lowest nonspecific liver and kidney uptake was achieved with DOTA chelator linked to Cys-tag via a 3.4 kDa PEGylated linker.

Optimized protocols for radiolabeling scVEGF with $^{99m}$Tc for SPECT imaging and $^{64}$Cu for PET imaging are described in **Subheadings 3.5.–3.7.** and **3.8.**, respectively.

### *1.4.3. Site-Specifically Biotinylated Cys-Tagged Proteins*

Biotin-maleimide (available from Sigma) has been conjugated to deprotected Cys-tagged proteins using a combination of methods described in **Subheading 3.3.** (C4 deprotection and modification) and **Subheading 3.5.** (separation

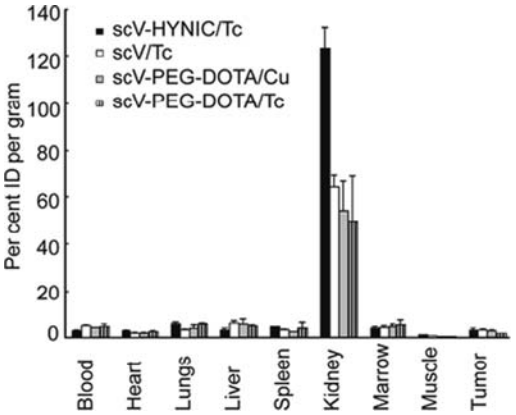


Fig. 3. Biodistribution of scVEGF-based radiotracer in 4T1 tumor-bearing mice is affected by the nature of chelating functionality. Cys-tag in scVEGF was radiolabeled either directly or via Cys-tag conjugated chelators HYNIC or PEG-DOTA, using methods described in **Subheadings 3.5–3.8**. The resulting radiotracers, named scV/Tc, scV-HYNIC/Tc, scV-PEG-DOTA/Tc, and scV/PEG-DOTA/Cu, were injected via the tail vein into 4T1-tumor bearing mice ($n$ = 5) at doses of 80 μCi of $Tc^{99m}$ or 5 μCi of $Cu^{64}$ per mouse. Mice were sacrificed 1 h after injection, blood and organs were harvested, weighed and counted in a gamma counter. Data are presented as percent of injected dose (ID) per gram, an average per group, ± SD.

of unreacted biotin by gel-filtration on PD-10). Site-specifically biotinylated bioactive polyamides can be coupled to streptavidin-coated nanoparticles, such as CdSe/ZnS core/shell quantum dots of different size and emission wavelength (available from Quantum Dot, Hayward, CA) for optical imaging or to biotin-coated microbubbles (available from Targeson, Charlottesville, VA) for ultrasound imaging.

### *1.4.4. Making Targeted Fluorescent Tracers*

Whole animal imaging with near-infrared fluorescent tracers targeted to specific receptors is a new and exciting area of basic and translational research ***(12,13)***. Such tracers can be also used for in vivo tagging cells via receptor-mediated endocytosis followed by fluorescent microscopy on histological sections prepared from harvested tissues ***(6)***. Importantly, such tagging provides information on cells with accessible and active receptors as opposed to immunohistochemical staining that visualizes the whole pool of receptors, regardless of their activity or accessibility. There are also translational efforts to develop targeted near-infrared fluorescent tracers and the corresponding hardware for various diagnostic applications.

Fluorescent dyes are relatively large molecules, whose conjugation to targeting proteins can affect interactions with cognate receptors, especially for small peptide ligands. For example, random conjugation of even a single fluorescent dye Cy5.5 to VEGF dramatically inhibits its activity ***(5)***. Thus, site-specific conjugation of fluorescent dyes provides an efficient way to generate functionally active fluorescent tracers.

Currently, maleimide derivatives of many dyes are commercially available. In our experience, incubation of deprotected (as described in **Subheading 3.3.**) scVEGF with 1.5- to 2.5-fold molar excess of dye-maleimide for 30 min in 0.1 *M* Tris-HCl pH 8.0 at room temperature yields 30–60% of conjugates carrying one dye molecule per one protein. Free dye is then readily removed by gel filtration on PD-10 as described in **Subheading 3.5.** However, removal of unmodified and excessively modified protein (two or more molecules of dye per protein) requires additional purification, which is conjugate-specific. For scVEGF-based conjugates with highly charged Cy5.5 and Cy7 dyes, we have successfully used anion-exchange choromatography on Q-Sepharose columns to separate unmodified VEGF, 1:1 conjugate, and overmodified protein. Example of in vivo imaging of mouse tumor vasculature with a combination of functionally active scVEGF site-specifically labeled with AlexaFluor-594-maleimide (Invitrogen) and inactivated scVEGF/Cy (SibTech, Inc.) to evaluate nonspecific tracer uptake is shown in **Fig. 4A.**

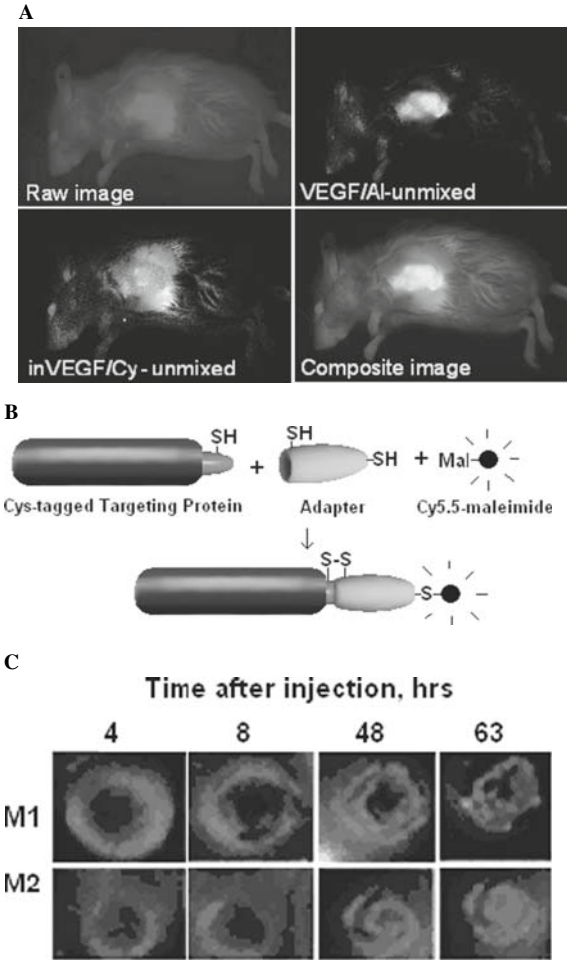


Fig. 4. In vivo fluorescent imaging of angiogenic tumor vasculature with scVEGF-based fluorescent tracers. (**A**) Balb/c mice bearing orthotopic 4T1 tumors were injected via the tail vein with an equimolar mixture of functionally active scVEGF/AlexaFluor-594 (scVEGF/Al) and inactivated inVEGF/Cy5.5 (inVEGF/Cy) fluorescent conjugates, total of 20 μg per mouse. VEGF inactivated by excessive biotinylation does not bind to VEGF receptors ***(6)***. Mice were anesthetized 5 min postinjection with ketamine (120 mg/kg) and xylazine (8 mg/kg) mixture. Tumor images were obtained using Maestro Imaging Station (CRI, Boston, MA) and processed with CRI software to obtain unmixed and composite images. **(B)** Conjugation of adapter protein to Cys-tag. Adapter can be modified with Cy5.5-maleimide before or after conjugation to Cys-tagged targeting protein. **(C)** Balb/c mice bearing subcutaneous 4T1 tumors were injected via the tail vein with $VEGF_{121}$/Adapter/Cy fluorescent conjugate, 10 μg per mouse. Mice were anesthetized at indicated times postinjection with ketamine (120 mg/kg) and xylazine (8 mg/kg) mixture. Tumor images were obtained on Kodak Image Station 2000 at indicated time after tracer injection. M1, mouse 1; M2, mouse 2.

### 1.4.5. Protein–Protein Conjugation

A special case is site-specific conjugation of bioactive Cys-tagged polyamides to other peptides or proteins. In some cases, conjugating either bulky or highly charged payloads directly to Cys-tag might interfere with the functional activity of the resulting conjugates. To minimize this interference, payloads can be linked to a recently developed adapter protein, which binds to and forms a disulfide bond with Cys-tag, a so-called "dock-and-lock" system ***(5)***. The adapter is a V118C, N88C double mutant C-terminal fragment of human ribonuclease I that naturally forms complexes with Cys-tag (an R4C mutant of the N-terminal fragment of human ribonuclease I), whereby complimentary C4 and C118 spontaneously form a disulfide bond **(Fig. 4B)**. We have recently derivatized this adapter with Cy5.5-maleimide, conjugated it to Cys-tagged $VEGF_{121}$, and then tested the resulting "dock-and-lock" conjugate for tumor imaging in mice with subcutaneous tumors. The conjugates selectively accumulated in the tumor area, and fluorescent dye remained internalized for at least several days, allowing noninvasive monitoring of tumor vasculature remodeling **(Fig. 4C)**.

A different class is conjugation to polymers that can be used as scaffolds for cell growth and/or tissue engineering. It might be beneficial to use functionally active growth factors or a combination of different growth factors for uniform derivatization of collagen, fibronectin, or other supporting proteins or polymers. Recent experiments, in which scVEGF was conjugated to fibronectin and then VEGF-fibronectin-coated tissue culture plates were used for cell growth, established the feasibility of such strategy ***(4)***.

## 2. Materials

### 2.1. Protein Expression and Purification

1. Concentrated PBS (10X), 0.5 *M* ethylendiamine tetraacetic acid (EDTA), 1 *M* Tris-HCl pH 8.0, 5 *M* NaCl, 3 *M* NaAc pH 5.2, kanamycin and 1 *M* isopropyl-β-D-thiogalactopyranoside (IPTG) from Gibco/BRL (Bethesda, MD).
2. LB Broth base bacterial growth medium (Invitrogen, Carlsbad, CA).
3. $Na_2SO_3$, $Na_2S_4O_6$, Triton X-100, and DTT from Sigma (St. Louis, MO).
4. DTT is dissolved in sterile Milli-Q water at 1 M, stored in small aliquots at −20°C and added to solubilized inclusion bodies as required.
5. Solution of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (Pierce, Rockford, IL) in Milli-Q water at 1 *M* is made and stored as described above for DTT.
6. Urea (Fisher Scientific) is dissolved in DI water at 8 *M*, sterilized by filtration through 0.45-μm filter and stored at room temperature.
7. High-salt wash buffer: 0.1 *M* Tris-HCl pH 8.0, 0.5 *M* NaCl.

8. TES buffer: 0.1 *M* Tris-HCl pH 8.0, 1 m*M* EDTA, 50 m*M* NaCl.
9. TES-T buffer: 0.1 *M* Tris-HCl pH 8.0, 1 m*M* EDTA, 50 m*M* NaCl, 2.5% (v/v) Triton X-100.
10. Solubilization buffer: 7.6 *M* urea, 20 m*M* Tris-HCl pH 8.0, 0.15 *M* NaCl .
11. Sulfonating buffer: Solubilization buffer containing $Na_2SO_3$ at 17 mg/mL and $Na_2S_4O_6$ at 5.3 mg/mL .
12. Sterilize buffers 7–11 by filtration through 0.45-μm filter.

### 2.1.1. Refolding of Cys-Tagged Proteins

1. Arginine (Sigma), reduced glutathione (GSH), and oxidized glutathione (GSSG) (Novagen, La Jolla, CA).
2. Refolding buffer: 20 m*M* Tris-HCl pH 8.0, 2 *M* urea, 0.5 *M* arginine, 1 m*M* GSH, 0.4 m*M* GSSG.
3. No salt basic dialysis buffer: 20 m*M* This-HCl pH 8.0.
4. No-salt acidic dialysis buffer: 20 m*M* NaAc pH 5.2. Prior to use, incubate refolding and both dialysis buffers at 4°C for 2–3 h with constant stirring.

## 2.2. Site-Specific Modifications of Cys-Tagged Proteins

### 2.2.1. Lipidation for Insertion of Targeting Proteins into Liposomes

1. *N*-(1-Pyrene)-maleimide (Molecular Probes, Eugene, OR). Dissolve *N*-(1-pyrene)-maleimide in dimethylsulfoxide (DMSO) at 10 mg/mL and use immediately for reaction.
2. HPLC column C4 MACROSPHERE 300 (150 × 4.6 mm) from Alltech (Deerfield, IL).
3. Poly(ethylenglycol)-α-distearoyl phosphatidylethanolamine,-ω-maleimide FW 3,400 (mPEG-DSPE-maleimide, from Shearwater Polymers, Huntsville, AL). Dissolve mPEG-DSPE-maleimide in DMSO at 10 mg/mL immediately before reaction.
4. Sepharose CL-4B (Sigma).
5. Doxil® (doxorubicin HCl liposome injection, 2 mg/mL) from Ortho Biotech.
6. HEPES buffer solution (1 *M*) of pH 7.2 (Invitrogen).
7. Running buffer: 10 m*M* HEPES pH 7.2; 0.15 *M* NaCl, 0.1 m*M* EDTA. Sterilize running buffer by filtration through 45 m*M* filter.
8. HPLC column Vydac Diphenyl 219TP5415 (250 × 4.6 mm) (Vydac, Hesperia, CA).

### 2.2.2. Radiolabeling for PET and SPEC Imaging

1. scVEGF (MW 28 kDa) protein, scVEGF-HYNIC and scVEGF-PEG-DOTA conjugates from SibTech (Newington, CT).
2. Deoxygenate 0.9% NaCl (plastic vials, from Abbott Laboratories, Abbott Park, IL) by purging nitrogen by cannula for 60 min before use.

3. PBS pH 7.4: dilute 10X PBS concentrate (Gibco/BRL) with Milli-Q deoxygenated water (*see* **Note 1**).
4. Tricine buffer (114 m*M*): dissolve 2 g *N*-[tris(hydroxymethyl)methyl]glycine (Tricine, from Sigma) in 100 mL Milli-Q deoxygenated water (*see* **Note 2**).
5. $SnCl_2$ solution: dissolve $SnCl_2 \cdot 2H_2O$ (Sigma) in 0.1 *N* HCl at 50 mg/mL.
6. Tin-Tricine reagent: dissolve 2.0 g Tricine in 97 mL of deoxygenated Milli-Q water, adjust pH to 7.1 using approximately 1.3 mL of 1 *N* NaOH. Seal the flask with a cannulated airtight septum and purge with nitrogen for 60 min. Add 1.2 mL of $SnCl_2$ solution, mix and transfer to nitrogen-filled septum-capped vials, 1 mL in each, with a final composition of 20 mg tricine and 0.6 mg of $SnCl_2$ at pH 7.1 (*see* **Note 3**).
7. Radionuclides: $^{99m}TcO4$ (5—10 mCi in a volume of approx 50 mL, GE Healthcare, Sunnyvale, CA) and $^{64}Cu$ (5–10 mCi in 2–10 μL from Washington University Medical School, St. Louis, MO).
8. PD-10 columns (GE Healthcare).
9. Indicator strips pH 4.0–7.0 (Merck, West Point, PA).
10. $NaOOCCH_3$ buffer (1 *M*) pH 6.0: Dilute 3 M NaAc, pH 5.5 (Sigma) with Milli-Q water and adjust pH to 6.0 with 0.1 *N* NaOH.
11. $NaOOCCH_3$ buffer (0.1 *M*) pH 5.5: Dilute 3 *M* NaAc, pH 5.5 with Milli-Q water.

## 3. Methods

### 3.1. Recovery of scVEGF from Inclusion Bodies

1. To obtain 1 L of induced bacterial culture, grow *E. coli* BL21(DE3) bacteria transfected with the pET/C4(G4S)/scVEGF plasmid in four 1-L flasks, each flask containing 250 mL LB medium supplemented with 30 mg/mL of kanamycin. Maintain the temperature at 37°C, shaking rate may vary from 220 to 300 rpm.
2. Once bacteria have reached an optical density of 0.4–0.6 optical units at 600 nm, induce scVEGF expression by adding 0.25 mL of 1 *M* IPTG to each flask. Continue incubating at 37°C with 300 rpm shaking for 2 h.
3. Harvest 1 L of induced bacterial culture by centrifugation at 4400 g for 30 min at 4°C (Beckman J2-21 centrifuge, Beckman Coulter, Fullerton, CA). Carefully remove and discard the supernatant. Resuspend bacterial pellet in 1X PBS using a 10-mL disposable plastic pipet. Pipet the solution up and down until no visible pellet is left in the suspension. Adjust the final volume to 25 mL with 1X PBS.
4. Pass bacterial suspension twice through high-pressure homogenizer (EmulsiFlex-C5, Avestin, Canada) at 10,000–15,000 psi.
5. Split homogenized bacteria in two 50-mL sterile centrifuge tubes and centrifuge at 12,000 g for 15 min at 4°C to separate the soluble part of bacterial lysate and inclusion body fraction. Discard the supernatants, trying not to disturb the pellet (inclusion bodies).
6. Resuspend each pellet in 2 mL of ice-cold high-salt wash buffer by pipetting up and down several times. Once the pellet is resuspended, adjust solution volume to 30 mL in each tube, using the same ice-cold buffer.

7. Sonicate briefly (20–30 s) at 40–50% of output power (Virsonic-475 sonicator VirTis, NY). This step is essential for complete homogenization of the pellet, which increases the recovery of recombinant protein (*see* **Note 4**).
8. Centrifuge at 23,500 g for 15 min at 4°C. Discard the supernatants.
9. Repeat resuspension, sonication, and centrifugation **(steps 6–8)** using ice-cold TES-T buffer. At this step, inclusion bodies are purified from detergent-soluble material.
10. Repeat resuspension and centrifugation **(steps 6–8)** using ice-cold TES buffer to wash out TritonX-100.
11. Add 5 mL of solubilization buffer to each pellet and incubate at room temperature for 10–15 min to soften inclusion bodies. Try to break the pellet apart with sterile plastic pestle or by passing it through a glass homogenizer. Some insoluble material can still remain in the solution after this step.
12. Incubate partially solubilized inclusion bodies at 4°C with constant agitation for 60 min; then combine the solutions in one 175-mL disposable centrifuge tube. Adjust the volume to 30 mL with sonication buffer and sonicate for 60 s at maximum output power (*see* **Notes 4, 5**).
13. Add 45 mL of sulfonation buffer. Final volume is now 75 mL; final concentrations of sulfonating agents are: 10 mg/mL for $Na_2SO_3$ and 3.2 mg/mL for $Na_2S_4O_6$.
14. Add 0.75 mL of 1 *M* DTT to a final concentration of 10 m*M*. Place tube with solution at 4°C on a rocking platform and incubate for 18–22 h with constant rocking. Usually, all residual pieces of inclusion body pellet are completely dissolved during 1–2 h of incubation.
15. Add 0.375 mL of 1 *M* TCEP to a final concentration of 2.5 m*M*. Continue incubation at 4°C for 16–18 h. After this incubation, scVEGF is ready for refolding.

### 3.2. Refolding of scVEGF

1. Transfer solubilized and completely reduced inclusion bodies into a dialysis bag with molecular weight cut-off pore size of 3500 and dialyze it against 10 volumes of refolding buffer for 24–36 h at 4°C. Once dialysis bag with protein is placed in dialysis beaker, stirring should be stopped to slow down the dialysis rate at this stage.
2. Transfer dialysis bag in a beaker containing 50 volumes of no-salt basic dialysis buffer and continue dialysis for 24–48 h at 4°C with constant slow stirring.
3. Transfer dialysis bag in a beaker containing 50–100 volumes of no-salt acidic dialysis buffer and continue dialysis for 12–16 h at 4°C with constant slow stirring (*see* **Note 6**).
4. After dialysis is complete, carefully transfer protein solution from dialysis bag into three sterile 50-mL centrifuge tubes. Centrifuge at 34,700 g for 30 min at 4°C.
5. Pool the supernatants together and pass the combined solution through 45-mm sterile filter. This protein solution is ready for column purification. scVEGF is

further purified by ion-exchange chromatography on SP-Sepharose Fast-Flow (1-mL prepacked columns from GE Healthcare), according to the manufacturer's instructions.

### 3.3. Deprotection and Site-Specific Lipidation of scVEGF

1. To "deprotect" C4 thiol group and make it available for SH-directed modification, incubate 0.1 m*M* scVEGF with 0.1 m*M* DTT in a buffer containing 0.1 *M* Tris-HCl pH 8.0 for 30 min at room temperature.
2. Optional: availability of free cysteines can be assayed by reacting with an SH-directed *N*-(1-pyrene)-maleimide. Add *N*-(1-pyrene)-maleimide to deprotection reaction mixture to make a molar protein-to-pyrene ratio of 1:2 and incubate for 40 min at room temperature. Load on RP HPLC C4 Alltech MACROSPHERE 300 5-mm column and elute at 0.75 mL/min with 0.1% TFA (v/v) and a linear gradient of acetonitrile (5–50% over 15 min) with detection at 216 nm for protein and 340 nm for pyrene. Calculate the extent of pyrene modification using a ratio of integral peak intensities at 216 nm and 340 nm.
3. To make scVEGF-lipid conjugate, add mPEG-DSPE-maleimide directly to deprotection reaction to a final molar protein-to-lipid ratio of 1:2. Residual DTT will not affect modification reaction. Incubate lipidation reaction mixture at room temperature for 1 h.
4. After incubation, this mixture can be used immediately for insertion into liposomes, or stored in liquid nitrogen or at -70°C in working aliquots for several weeks.

### 3.4. Insertion of scVEGF–Lipid Conjugate into Preformed Liposomes

1. For insertion into premade doxorubicin-loaded liposomes (Doxil), mix equal volumes of liposomes and lipidation reaction mixture and incubate at 37°C for 12–16 h (*see* **Note 7**). Purification of unreacted lipid and protein is not necessary at this step, because they do not interfere with insertion process and will be removed eventually by gel-filtration of decorated liposomes on Sepharose CL-4B.
2. Equilibrate Sepharose CL-4B column with 5 column volumes of running buffer.
3. After insertion of lipidated protein is complete (*see* **Note 7**), load liposomes on an equilibrated Sepharose CL-4B column. Loaded sample will be separated at the very beginning of gel filtration into two doxorubicin-containing (red-color) peaks. Collect fast-migrating red-colored peak immediately after collecting void column volume. This peak contains liposomes. Place purified liposomes on ice or to 2–8°C for storage, do not freeze. Continue collecting elution fractions until second red-colored peak (free doxorubicin leaked out from Doxil) is eluted from the column. Monitor optical density in eluting material at 280 nm to identify and collect free protein and excess lipid for further analysis **(Fig. 3A)**.
4. Analyze the efficiency of protein insertion by RP HPLC on Vydac Diphenyl column with elution at 0.75 mL/min with 50 m*M* thriethylamine phosphate pH 2.8 and 20% tetrahydrofuran with a linear gradient of acetonitrile (0–70% v/v over 25 min).

5. Alternatively, use Western blot analysis with a specific antibody to analyze and quantify protein inserted into liposomes.
6. This insertion procedure usually results in concentration of liposome-associated Cys-tagged protein in a micromolar range (2–10 μ*M*).

### 3.5. Deprotection and Direct Radiolabeling of scVEGF with $^{99m}$Tc via Cys-tag

1. To deprotect C4 thiol groups and make them available for modification, incubate scVEGF with equimolar DTT as follows: mix 0.1 mL PBS, 50 μg of scVEGF, and 1.8 μL of 1 m*M* DTT in a 1.5-mL Eppendorf tube and incubate it for 20 min at 25°C.
2. During this incubation, mix 0.25 mL PBS, add 10–15 mCi $^{99m}TcO_4$ in another glass tube, cover it with parafilm, and purge the mixture with nitrogen for 15 min.
3. Add the entire scVEGF deprotection reaction mixture to the tube with deoxygenated $^{99m}TcO_4$. There is no need to remove residual DTT, because it will not interfere with direct radiolabeling of scVEGF with $^{99m}$Tc.
4. Purge the mixture with nitrogen shortly, add 20 μL of Tin-Tricine reagent, seal the tube with parafilm and incubate it at 37°C for 60 min.
5. Equilibrate PD-10 desalting column with 5 column volumes of PBS.
6. When incubation of scVEGF with $^{99m}TcO_4$ is over, remove the unreacted technetium by gel filtration on PD-10 column. Load the reaction mixture on equilibrated PD-10 column and elute with PBS. After passing a bed volume, which is 2.4 mL for PD-10, collect 0.5-mL fractions in 1.5-mL microcentrifuge tubes. scVEGF is eluted in fractions 2 through 4–5. Combine fractions with highest activities together. This procedure usually results in incorporation of 100–200 μCi of $^{99m}$Tc per μg of protein.
7. $^{99m}$Tc-labeled scVEGF should be used for further analysis, tissue culture, or animal experiments immediately. For example, for bio-distribution studies in a mouse model, inject 40–100 μCi of $^{99m}$Tc –labeled scVEGF via the tail vein.

### 3.6. Radiolabeling of scVEGF-HYNIC Conjugate with $^{99m}$Tc

1. Add 5 mCi $^{99m}TcO_4$ to a glass 10-mL tube with 0.3 mL Tricine buffer, cover with parafilm, and purge with nitrogen for 15 min.
2. Add 50 μg of scVEGF-HYNIC conjugate and 10 μL of Tin-Tricine reagent. Purge with nitrogen shortly and seal with parafilm.
3. For loading scVEGF-HYNIC with $^{99m}$Tc, incubate the reaction mixture for 60 min at 25°C.
4. Remove free technetium by gel-filtration on PD-10 column as described in **Subheading 3.5.**
5. This procedure usually results in incorporation of 50–100 μCi of $^{99m}$Tc per μg of protein.

### 3.7. Radiolabeling of scVEGF-PEG-DOTA with $^{99m}$Tc

1. Add 10 mCi $^{99m}TcO_4$ to a glass tube with 1 mL PBS, cover with parafilm, and purge with nitrogen gas for 15 min.
2. Add 20 μg scVEGF-PEG-DOTA conjugate and 25 μL of Tin-Tricine reagent. Purge the mixture with nitrogen shortly and seal with parafilm.
3. Incubate for 60 min at 37°C
4. Remove free technetium by passing through PD-10 column as described in **Subheading 3.5.**
5. This procedure usually results in incorporation of 100–200 μCi of $^{99m}$Tc per μg of protein.

### 3.8. Radiolabeling of scVEGF/PEG-DOTA with $^{64}$Cu

1. Transfer 0.5–1 mCi $^{64}$Cu to 1.5-ml Eppendorf tube and adjust pH to ~5.5 with 10–15 μL 0.1 *M* $NaOOCCH_3$, pH 5.8–6.0. Check pH by spotting ~0.3 μL on indicator paper. Important note: do not use alkaline solution for pH adjustment as Cu ions will form insoluble hydroxides.
2. Add 50 μg scVEGF-PEG-DOTA conjugate, check its pH again, it must be within 5.0–5.5.
3. Incubate for 60 min at 55°C. Add EDTA to a 1 m*M* final concentration to chelate free $^{64}$Cu.
4. Equilibrate PD-10 column with 5 column volumes of 0.1 *M* $NaOOCCH_3$ pH 5.5.
5. For removal of unreacted $^{64}$Cu, load the reaction mixture on equilibrated PD-10 column and elute with 0.1 *M* $NaOOCCH_3$. Collect protein-containing fractions as described in **Subheading 3.5.**
6. This procedure usually results in incorporation of 0.5–5 μCi of $^{64}$Cu per μg of protein.
7. As with $^{99m}$Tc-labeled scVEGF, $^{64}$Cu -loaded scVEGF should be used for further analysis, tissue culture, or animal experiments immediately. For example, for biodistribution studies in a mouse model, inject 3–5 μCi of $^{64}$Cu-labeled scVEGF via the tail vein.

## 4. Notes

1. Prepare deoxygenated Milli-Q water by boiling fresh Milli-Q water for at least 5 min on a hot plate, followed by flushing it with argon (or nitrogen) for 10 min. Cool the flask on ice to room temperature and bubble with nitrogen for 75 min. Store deoxygenated Milli-Q water in a tightly closed flask at room temperature. If water has been stored for several days between uses, bubble more nitrogen through before use. Filter all solutions prepared with deoxygenated Milli-Q water through 0.2-μm filter and bubble nitrogen through for 15–20 min before use.
2. Check out pH of Tricine buffer, it should be $6.0 \pm 2$. If necessary, adjust pH with small amounts of 1 *M* NaOH or 1 *M* HCl until pH reaches $6.0 \pm 2$. For making this solution,

use acid-washed, thoroughly rinsed, and dried Erlenmeyer flask. Freshly made Tin-Tricine reagent can be stored frozen at -20°C in 1-mL aliquots for at least several months. Alternatively, freeze 1-mL aliquots at -80°C, then lyophilize. Reconstitute one vial with 1 mL of deoxygenated 1xPBS just before use.

To get a higher specific activity of $^{99m}$Tc-labeled scVEGF-HYNIC (for imaging purposes), the amount of $^{99m}$Tc in the reaction might be increased up to 50–60 mCi per 50 μg protein, with the respective increase of Tin-Tricine reagent in reaction mixture. This can result in ~1000 μCi/μg labeling efficiency. The increase of $^{99m}$Tc in other reactions is much less effective. For $^{64}$Cu-labeled scVEGF-PEG-DOTA, a higher specific activity can be achieved by a 2-3 fold decrease of the protein in the reaction mixture.

3. $^{99m}$Tc and $^{64}$Cu are radionuclides emitting γ-radiation at 140 keV and 511 keV, respectively. Standard shielding and radionuclide-handling procedures must be used. Typically, labeling is done in a lead brick–surrounded area, in a lead-shielded container. Individuals working with the material should monitor their radiation exposure with appropriate devices. As $^{99m}$Tc and $^{64}$Cu are short-lived isotopes ($t_{1/2}$ = 6.03 h and 12.70 h, respectively), injected animals and their waste products at the doses needed for biodistribution or imaging experiments do not represent any significant radiation hazard after 3–5 d (10 half-lives) of decay.
4. Continuous sonication of bacterial suspension or solubilized inclusion bodies will result in the significant increase of the temperature of the solution, which might be detrimental for the protein activity. To avoid overheating, place tubes with solutions for sonication on ice and start sonication only when the solutions are ice-cold. Importantly, keep every tube completely immersed in ice during the entire sonication step and do not apply ultrasound for longer than 60 s at a time. If additional sonication is needed, let the solution to cool down on ice, and then repeat sonication.
5. It may happen that inclusion bodies are not completely dissolved even after several rounds of sonication. In this case, proceed directly to the reducing step **(Subheading 2.2.1., steps 13–15)**. In our experience, all traces of insoluble material disappear after 1–2 h of incubation in the presence of DTT and sulfonating agents.
6. Acidic dialysis provides tremendous purification from bacterial proteins, most of which precipitate at a pH lower than 6. However, not every recombinant protein remains soluble at acidic pH either. To test the solubility of your protein under these conditions, after at least 18–20 h of basic dialysis, transfer a small aliquot of the protein into a separate dialysis bag and put it in a precooled acidic dialysis buffer for 4–6 h, just long enough for bacterial proteins to form visible precipitation. Once the precipitate is formed, separate it by centrifugation in a table-top microcentrifuge for 5–10 min at 23,500 g. Analyze the presence of your protein in the supernatant and in the pellet by SDS-PAGE or Western blotting. If your protein is found in the supernatant, you can transfer the dialysis bag with the bulk of protein from basic dialysis conditions to acidic dialysis.

7. In our experience, as little as 2–4 h of incubation might be enough for insertion of 85–95% of lipid conjugate into liposome. Prolonged incubation (more than 16 h) does not improve the insertion; in fact, it might result in a decreased amount of liposome-associated protein.

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