METHODS IN MOLECULAR MEDICINE™

Drug Targeting

Strategies, Principles, and Applications

Edited by

G. E. Francis

Cristina Delgado

Humana Press

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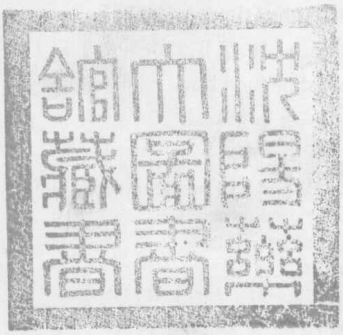
PolyMASC Pharmaceuticals
London, UK



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Preface

Advances in understanding the molecular mechanisms of disease together with the advent of recombinant DNA and other technologies have opened opportunities for a vast array of novel therapeutic biopharmaceuticals and diagnostic agents. However, even natural biomolecules present a myriad of problems that limit their potential as pharmaceutical agents. Rapid degradation and elimination, immunological reactions, and toxicity are often associated with new biopharmaceuticals, much as with conventional agents.

Targeted delivery systems have the potential to increase the efficacy of existing diagnostic and therapeutic agents and also create an opportunity for the use of new pharmaceuticals, substances that themselves can be harmful to normal tissues. Both passive and active targeting have been exploited. Most active targeting strategies have focused on antibody conjugates since preparation of highly specific monoclonal antibodies is well established. However, a new wave of conjugates exploiting other ligands is underway. Access to the target tissue remains an obstacle and is an area where passive targeting can be useful.

In *Drug Targeting: Strategies, Principles, and Applications* we have tried to compile a state-of-the-art volume on current targeting approaches. The first section focuses on certain key strategies applied to date, and how to build the antibody–ligand constructs. This is followed by a section on theoretical considerations for targeting, focusing on approaches relevant to solid tumors. The last section deals with some experimental and clinical applications of targeted drug delivery systems.

Immunotoxins—constructs comprising an antibody to target the appropriate cell tissue or organ and a toxin—were among the first targeted molecules. Chemical construction of immunotoxins is extensively addressed by Ghetie and Vitetta. Ribonuclease–antibody chimeras—a new type of immunotoxin, based on human and humanized ribonuclease proteins that are known to have host–defense activities and expected to be less toxic and less immunogenic than immunotoxins based on plant and bacterial toxins—are discussed by Newton and Rybak in two chapters, one detailing a chemical approach to their synthesis and the second dealing with the preparation of fusion proteins. The production of fusion toxins is also addressed by Pastan.

Raso presents an elegant targeting approach exploiting bispecific antibodies. One antibody binds the effector molecule reversibly, while the second antibody targets the complex to selected sites on the cell membrane. No covalent or chemical

modification of the bioactive molecule is required and thus its structural integrity and full biologic potential are preserved. The properties of enzyme–antibody conjugates are discussed by Muzykantov using an approach based on streptavidin–biotin linkers. Chapters by Cullis et al., Torchilin, and Agrawal disclose strategies for targeting liposomes to tissues other than those of the reticuloendothelial system. The most widely used ligands are again antibodies, but such molecules as oligosacharides, peptides, other proteins, and vitamins have also been employed.

Lee and Low give details on the preparation of folate-bearing conjugates targeted to tumors with folate receptors. Folate has been used in the targeted delivery of proteins, liposomes, gamma imaging agents, oligodeoxyribonucleotides, and gene transfer vectors. Low immunogenicity, rapid extravasation, tumor permeation, and systemic clearance, together with resistance to denaturation, are some of the advantages of folate over the antibody targeting.

Finally, classic targeting methodologies are now being adapted for targeted gene transfer. Pincus discusses the preparation of antibody–virus conjugates to target viruses to the receptor cells. Details of current methods applied to lipid-based plasmid delivery systems are dealt with by Wasan et al.

On the applications of these targeted vehicles, Torchilin deals extensively with targeting myocardial infarction using liposomal systems. Agrawal and Pincus describe the importance of using monoclonal antibodies raised against infected cells to target malaria and HIV-infected cells, respectively. The fusion proteins prepared by Pastan are targeted to leukemia and lymphoma and the pulmonary endothelium is targeted by the antioxidant enzyme–antibody conjugates prepared by Muzykantov.

Targeting of tumors has received the most attention and therefore three chapters giving theoretical aspects have been included. Thomas gives details of both passive and active targeting to tumors. Ching deals with targeting tumor blood flow as a more universal approach for tumor treatment either singly (by generation of hypoxia) or in combination with other therapies (by entrapment of other drugs within the tumor site, including cytotoxic and "bioreductive" drugs that are activated selectively by metabolic reduction under hypoxic conditions).

Drug Targeting: Strategies, Principles, and Applications does not, of course, cover every class of drug and biopharmaceutical targeting to many tissues and diseases. We have tried to include illustrative and interesting examples of the more classical targeting approaches and of the new wave of very promising ligands and constructs, all of which are opening new horizons for targeted drug delivery.

Finally, the editors are grateful to the contributors for the patience and perseverance required to complete this volume.

G. E. Francis
Cristina Delgado

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Contents

PART III. APPLICATIONS

1

Chemical Construction of Immunotoxins

Victor Ghetie and Ellen S. Vitetta

1. Introduction

Immunotoxins (ITs) are chimeric proteins consisting of an antibody linked to a toxin. The antibody confers specificity (ability to recognize and react with the target), whereas the toxin confers cytotoxicity (ability to kill the target) ***(1–3)***. ITs have been used in both mice and humans to eliminate tumor cells, autoimmune cells, and virus-infected cells ***(4–6)***.

The linkage of the antibody to the toxin can be accomplished by one of two general methods, chemical or genetic. Chemical construction of ITs utilizes reagents that crosslink antibody and toxin (**Fig. 1A**) ***(7,8)***. Genetic construction uses hybrid genes to produce antibody-toxin fusion proteins in *Escherichia coli* (**Fig. 1B**) ***(9,10)***. Two major types of chemical bonds can be used to form ITs: disulfide bonds ***(11)*** and thioether bonds ***(12)*** (**Fig. 2**). Disulfide bonds are susceptible to reduction in the cytoplasm of the target cells, thereby releasing the toxin so that it can exert its inhibitory activity only in the cells binding the antibody moiety ***(13)***. This type of covalent bond has been used to construct ITs containing single-chain plant toxins (ricin A chain [RTA], pokeweed antiviral protein [PAP], saporin, gelonin, and so forth). Since mammalian enzymes cannot hydrolyze thioether bonds, thioether-linked conjugates of toxins and antibodies are not cytotoxic to target cells ***(1,14)***. However there are two exceptions. The first is an IT with the intact ricin toxin (RT). RT is composed of two polypeptide chains (the cell-binding B chain [RTB] and the RTA) linked by a disulfide bond. If the antibody is bound to the toxin through the RTB, the toxic chain can be released in the target cell cytosol by reduction of the interchain disulfide bond ***(15)*** (**Fig. 2**). The second exception is an IT prepared with *Pseudomonas* exotoxin (PE). PE can be coupled to antibody by

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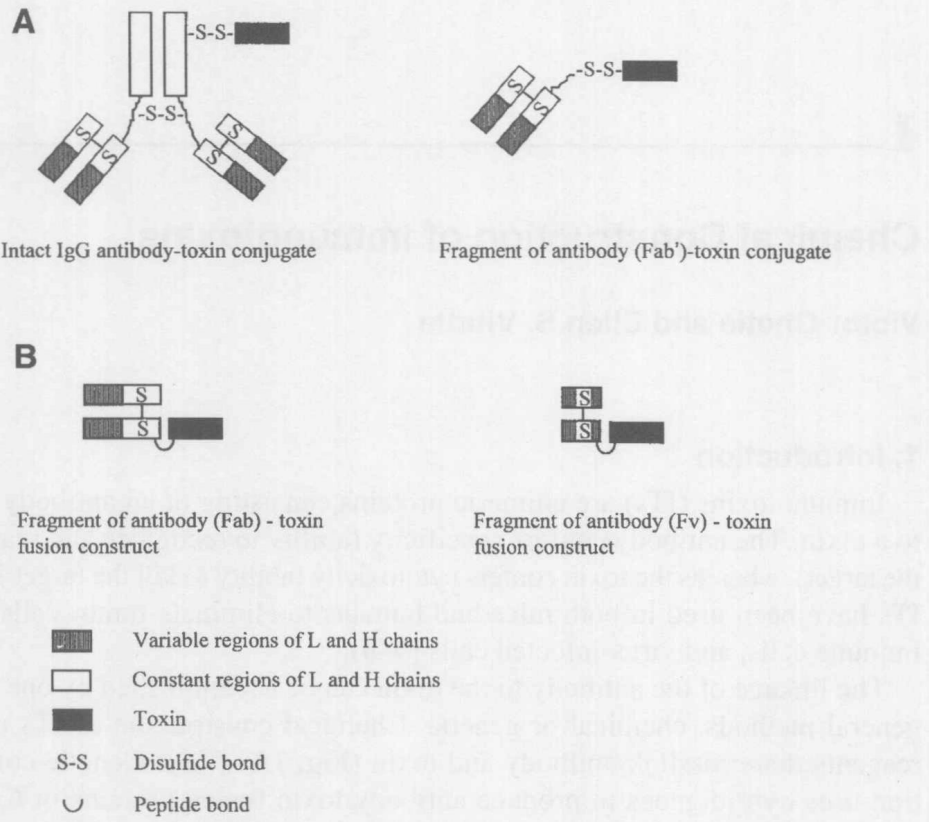

Fig. 1. The structure of antibody–toxin constructs obtained by **(A)** chemical and **(B)** genetic engineering procedures.

a thioether bond, since this toxin contains a protease-sensitive peptide bond that is cleaved intracellularly to generate a toxic moiety bound to the rest of the molecule by a disulfide bond (**Fig. 2**).

This chapter presents methods for preparing ITs with disulfide-linked toxins as exemplified by RTA, PAP, and a truncated recombinant *Pseudomonas* exotoxin (PE35) and with thioether-linked toxins exemplified by blocked ricin (bRT) and truncated recombinant *Pseudomonas* exotoxin (PE38).

2. Materials

The following reagents have been used for the preparation of ITs:

1. From Pharmacia (Piscataway, NJ): Protein A-Sepharose Fast Flow, Protein G-Sepharose Fast Flow, Sephacryl S-200HR, DEAE-Sepharose CL-4B, Sephadex G-25M, Blue-Sepharose CL-4B, Sephadex G-25 MicroSpin, CM-Sepharose CL-4B, SP-Sepharose Fast Flow.

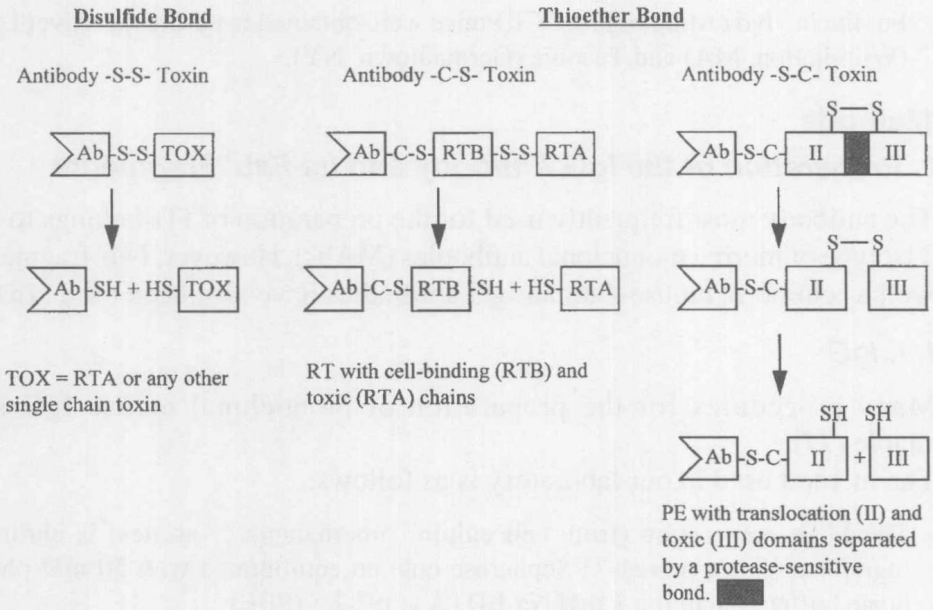

Fig. 2. Covalent bonds crosslinking antibody to toxin.

2. From Pierce (Rockford, IL): 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene (SMPT), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), *N*-succinimidyl 5-acetylthioacetate (SATA), succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), 2-iminothiolane (2-IT), dithiothreitol (DTT), dimethylformamide (DMF), 5,5'-dithio-bis-(2-nitrobenzoic)acid (DTNB), (Ellman's reagent), 2-mercaptoethanol.
3. From Sigma (St. Louis, MO): sodium hydroxyde, sodium chloride, potassium chloride, potassium and sodium phosphate (monobasic and dibasic), ethylenediaminetetra-acetic acid (EDTA; disodium salt), acetic acid (glacial), penicillin G (sodium salt), pepsin (crystallized and insoluble enzyme attached to 4% crosslinked agarose), boric acid, glycine, ricin toxin (Toxin RCA60), ricin A chain, saporin, pockweed mitogen (PAP), pseudomonas exotin A (PE), lactose, galactose, cyanuric chloride, sodium metaperiodate, sodium cyanoborohydride, Trizma hydrochloride (Tris), fetuin, triethanolamine hydrochloride, orcinol, streptomicin sulfate, L-glutamine, RPMI-1640 medium, fetal calf serum.
4. From Amersham (Arlington Heights, IL): ^{35}S-methionine, ^{3}H-thymidine, ^{3}H-Leucine.
5. The following equipment has been used for the preparation of ITs: spectrophotometer (DU640 Beckman, Beckman Instruments, Houston, TX), electrophoresis system (Phastsystem, Pharmacia, Piscataway, NJ), chromatographic system (BioLogic system, Bio-Rad, Hercules, CA), HPLC system (LKB-Pharmacia), HPLC columns (TSK, TosoHaas, Montgomeryville, PA), centrifuge (RC3C, Sorvall, Newton, CT), ultracentrifuge (Optima, Beckman).

6. For the in vivo testing of ITs, SCID mice were obtained from Charles River Labs (Wilmington, MA) and Taconic (Germantown, NY).

3. Methods

3.1. Preparation of the IgG Antibody and Its Fab' Fragments

The antibody most frequently used for the preparation of ITs belongs to the IgG isotype of murine monoclonal antibodies (MAbs). However, Fab' fragments as well as chimeric mouse–human IgG antibodies have also been used *(16)*.

3.1.1. IgG

Many procedures for the preparation of monoclonal mouse IgG are available *(17)*.

The method used in our laboratory is as follows:

1. The MAb preparation (from cell culture supernatants or ascites) is chromatographed over a protein G Sepharose column equilibrated with 50 m*M* phosphate buffer containing 3 m*M* Na_2EDTA at pH 7.5 (PBE).
2. The bound MAb is eluted with 25 m*M* acetic acid and after neutralization is subjected to gel filtration on a column of Sephacryl S-200 HR (length 60–90 cm) equilibrated with PBE containing 0.15 *M* NaCl (PBS) at pH 7.5.
3. The fraction containing purified IgG is concentrated to 5 mg/mL by ultrafiltration (e.g., using the Millipore ultrafiltration centrifugal device) and then used for chemical derivatization.
4. If the MAb is used for the preparation of a clinical IT, an additional chromatographic purification is performed on a DEAE-Sepharose column equilibrated with PBS to remove the murine DNA and bacterial endotoxin contaminating the MAb.

3.1.2. Fab' Fragments

Fab' fragments can be obtained by pepsin digestion of purified IgG molecules. As a result of the hydrolysis, $F(ab')_2$ fragments are obtained. Following reduction with DTT, the $F(ab')_2$ yields two Fab' fragments with one or more free sulfhydryl (SH) groups in the hinge region which are available for crosslinking to the toxin moiety (**Fig. 1A**). Therefore, Fab' fragments do not require chemical derivation with thiol-containing crosslinkers. The pH and duration of pepsinolyis depend on the IgG isotype *(18,19)*. Therefore, preliminary experiments should be carried out to select the optimal conditions for obtaining Fab' fragments with the highest purity and the yields. The method used in our laboratory is as follows *(20)*:

1. IgG is brought to 2.5 mg/mL in 0.1 *M* citrate buffer, pH 3.7, pepsin (Sigma) is added (1 mg pepsin/50 mg), and digestion is performed at 37°C for 2–8 h (depending on the IgG isotype).

2. The pH of the digest is then brought to pH 8.0 with 0.1 *M* NaOH and the mixture is applied to a Sephacryl S-200 HR column equilibrated with PBE.
3. The $F(ab')_2$ is collected and concentrated to 5 mg/mL.
4. DTT is then added to a final concentration of 5 m*M* and the mixture is incubated at room temperature for 1 h in the dark.
5. The reduced Fab' fragments are chromatographed on a Sephadex G-25M column (length 30–60 cm) equilibrated with PBE and flushed with N_2 by loading a volume not greater than 2% of the volume of the gel. Thus, for a column of 1.8×30 cm containing 75 mL gel, <1.5 mL of mixture should be added.
6. The Fab' fraction is eluted in the void volume, concentrated to 5 mg/mL, and treated with a 1/100 volume of DTNB (Ellman reagent) dissolved in DMF (80 mg/mL).
7. After a 1 h incubation at 25°C, the mixture is rechromatographed on a Sephadex G-25M column as described in **Subheading 3.1.2., step 5**.
8. The Ellmanized Fab' eluted in the void volume is collected, concentrated to 5 mg/mL, and stored at 4°C until it can be used for reaction with the toxin.

3.2. Chemical Derivatization of the IgG Antibody

MAbs cannot be linked to toxins unless they are derivatized with crosslinking agents since the IgG molecule, in contrast to Fab', does not contain a free cysteine residue. Disulfide or sulfhydryl groups are therefore introduced into the antibody molecule to form a disulfide bond between the antibody and the toxin. For crosslinking the toxin to the antibody through a thioether bond, a maleimide group should be introduced into the IgG, thus allowing a reaction with the sulfhydryl groups of the toxin.

3.2.1. Introduction of Disulfide Groups

Disulfide groups are introduced using one of two heterobifunctional crosslinkers, which can be obtained commercially in water soluble (sulfo) or insoluble form (Pierce) (**Fig. 3**). We prefer SMPT to SPDP as the pyridyldisulfide crosslinker since it generates a molecule with increased stability in vivo because of the protective effect exerted upon the disulfide bond by the methyl group and the benzene ring on the carbon atoms adjacent to the -ss- bond (**Fig. 3**) *(21,22)*. The procedure used in our laboratory is as follows *(23)*:

1. IgG is dissolved in PBE or PBS, pH 7.5, at a concentration of 5 mg/mL.
2. 10 µL of SMPT (or SPDP) dissolved in DMF (5 mg/mL) or sulfo-SMPT (or Sulfo-SPDP) dissolved in buffer (10 mg/mL) is added to each milliliter of the MAb and the mixture is incubated at 25°C for 1 h.
3. The mixture is chromatographed on Sephadex G-25M as described in **Subheading 1.2.** and the material eluted in the void volume is collected and concentrated to 3–5 mg/mL. This material should be stored at 4°C before mixing it with the toxin.

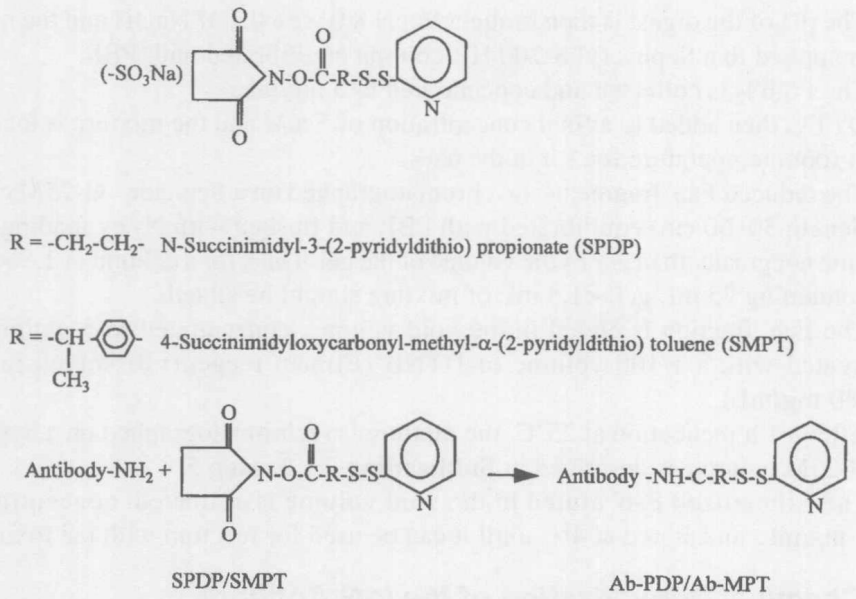

Fig. 3. The structure of the pyridyldisulfide crosslinkers and their reaction with the antibody molecule.

4. The average number of disulfide groups introduced into the antibody molecule can be measured on an aliquot as follows:
   a. To 1 mL of modified IgG with a known absorbance at 280 nm (1–2 absorbance units is optimal), 20 µL of 0.3 *M* DTT is added and the absorbance at 343 nm is measured after an incubation of 5 min.
   b. The MPT/IgG molar ratio (MR) is calculated using the formula: $MR = 26 \times A_{343} / [A_{280} - 0.63 \times A_{343}]$.

The MR of a correctly prepared antibody–MPT derivative should range between 2.0 and 2.5. For example, if A_{280} nm = 1.35 and A_{343} nm = 0.11, MR = 2.86/1.28 = 2.2.

3.2.2. Introduction of Sulfhydryl Groups

Sulfhydryl groups are introduced using one of two reagents that can be obtained commercially: 2-iminothiolane (2-IT) and SATA (**Fig. 4**). SATA contains a protected sulfhydryl group to confer stability on the molecule. When a free sulfhydryl group is needed, it can be generated by treatment with hydroxylamine. (**Fig. 4**).

3.2.2.1. 2-Iminothiolane ***(22,24)***

1. The IgG is dissolved at 10 mg/mL in 50 m*M* borate buffer containing 0.3 *M* NaCl, pH 9.0.
2. 25 µL of 2-IT (4.4 mg/mL in the same buffer) is added and the mixture is stirred at room temperature for 1 h.