2

Construction of Ribonuclease–Antibody Conjugates for Selective Cytotoxicity

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1. Introduction

Immunotoxins based on human and humanized ribonuclease may have potential for cancer therapy while exhibiting less toxic side effects and stimulating less of an immune response in humans than immunotoxins based on plant and bacterial toxins ***(1)***. Both recombinant RNase fusion proteins (***2–4***; *see also* Chapter 6, this volume) and chemical RNase conjugates have been made and characterized. The cytotoxic potential of targeted ribonuclease was first demonstrated with bovine RNase conjugated to transferrin or an antibody directed against the human transferrin receptor ***(5)***. Antibody RNase conjugates have also been shown to have potent anti-tumor activity against human glioma cells in athymic mice ***(6)*** and to enhance the activity of vincristine in mdr1 multidrug-resistant colon cancer cells in vitro and in vivo ***(7)***. Recently, RNase chemically conjugated to an antibody against CD22 was found to specifically kill Daudi lymphoma cells in cell culture at picomolar concentrations (IC_{50}, 10–50 p*M*) and to exhibit potent antitumor activity in SCID mice with disseminated Daudi lymphoma (unpublished data). Methods for linking RNase to specific cell binding ligands are described.

2. Materials

2.1. Derivatization of RNase

1. RNase solution containing 3.2 mg at a concentration ≥3.2 mg/mL.
2. PD-10 columns (Sephadex G-25M) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).
3. Conjugation buffer: 84 mL 0.2 *M* Na_2HPO_4 (35.6 g/L Na_2HPO_4-$2H_2O$), 16 mL 0.2 *M* NaH_2PO_4 (27.6 g/L NaH_2PO_4-H_2O), 1.17 g NaCl + 100 mL H_2O (solution should be pH 7.5).

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4. Centricon 3 and 30 microconcentrators (Amicon Inc., Beverly, MA).
5. *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce Chemical Co., Rockford, IL). Store over Drierite at 4°C.

2.2. Derivatization of Antibody for Disulfide Linkage

1. Antibody (2 mg) at a concentration ≥4 mg/mL.
2. 2-Iminothiolane (2-IT) (Pierce Chemical Co.). Store over Drierite at 4°C.
3. 0.78 *M* sodium borate buffer, pH 8.5: 29.8 g $Na_2B_4O_7 \cdot 10\ H_2O$. Adjust pH to 8.5 with 1 *M* NaOH and make up to a final volume of 0.1 L in distilled H_2O.
4. 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma, St. Louis, MO).

2.3. Derivatization of Antibody for Thioether Linkage

1. Dimethylformamide (DMF) (Sequenal grade, Pierce Chemical Co.). Store in vacuum desiccator over Drierite at 23°C.
2. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce Chemical Co.).

2.4. Conjugation of RNase to Antibody via a Disulfide Linkage

1. Dithiothreitol (DTT) (Sigma).

2.5. Conjugation of RNase to Antibody via a Thioether Linkage

1. 0.1 *M* sodium acetate, pH 4.5, containing 0.1 *M* NaCl; 2.72 g $CH_3COONa \cdot 3H_2O$; and 1.17 g NaCl, adjust to pH 4.5 with concentrated acetic acid. Adjust volume to 200 mL with distilled H_2O.

2.6. Purification of RNase–Antibody Mixture

1. High performance liquid chromatographic (HPLC) system equipped with a suitable high pressure pump, ultraviolet monitoring at 215 nm and a fraction collector.
2. Toyo Soda TSK 3000SW column, 7.5 × 600 mm (Toso Haas Corp., Montgomeryville, PA).
3. HPLC buffer: 0.1 *M* phosphate buffer, pH 7.5: 405 mL 0.2 *M* Na_2HPO_4 + 95 mL NaH_2PO_4 + 500 mL H_2O (*see* **Subheading 2.1., item 3** for formula of 0.2 *M* Na_2HPO_4 and 0.2 *M* NaH_2PO_4).
4. 4–20% sodium dodecyl (SDS)-polyacrylamide gels.

3. Methods

3.1. Derivatization of RNase

1. Apply the RNase solution (3.2–4.0 mg total in a volume ≤1.0 mL) to a PD-10 column equilibrated with conjugation buffer. Collect 0.5 mL fractions, read the absorbance at 280 nm, and pool the peak tubes (total volume 1.5–2.0 mL) (*see* **Note 1**).
2. Determine the concentration of the pooled RNase solution by measuring the optical density of the solution at 280 nm and using the appropriate extinction coefficient.

3. Concentrate the pooled RNase solution to 0.5 mL using a Centricon P-3 microconcentrator. Determine the final volume and concentration of the solution as described in **step 2** (*see* **Note 2**).
4. Prepare a fresh solution of SPDP at 20 µmol/mL in absolute ethanol (*see* **Note 3**).
5. To 3.2 mg RNase solution (a total of 0.23 µmol) (volume ≤0.5 mL) add 29 µL SPDP solution (a total of 0.58 µmol or a 2.5-fold molar excess of SPDP). Incubate the mixture for 30 min at room temperature (*see* **Note 4**).
6. Apply the mixture to a PD-10 column that has been equilibrated with the conjugation buffer to remove excess SPDP. Collect 0.5 mL fractions, read the absorbance at 280 nm, and pool the peak tubes (total volume 1.5–2.0 mL).
7. Remove 50 µL of the pooled modified RNase and determine the degree of substitution (mol of 2-pyridyl disulfide/mol RNase) (*see* **Note 5**).
8. Concentrate the remaining pooled derivatized RNase to 0.5 mL as described in **step 3** and store at 4°C until needed for the reaction (*see* **Note 6**).

3.2. Derivatization of Antibody for Disulfide Linkage

1. Apply the antibody solution (2.5–3.0 mg total) to a PD-10 column equilibrated with conjugation buffer to remove any low-mol-wt materials that may interfere with the reaction. Collect 0.5-mL fractions, read the absorbance at 280 nm, and pool the peak tubes (total volume 1.5–2.0 mL) (*see* **Note 1**).
2. The concentration of the antibody solution should be at least 4 mg/mL. Concentration by Centricon P30 microconcentrator may be required to achieve this (*see* **Note 7**).
3. Just before use, prepare a stock 2-IT solution at 30 m*M* in 0.85 *M* borate buffer, pH 8.5, and DTNB at 10 m*M* in 0.1 *M* Tris, pH 8.0 (*see* **Note 8**).
4. Incubate 2 mg antibody (12.5 nmol) with 250 nmol 2-IT (20-fold molar excess) and 2.5 m*M* DTNB (final concentration) in 100 m*M* sodium borate, pH 8.5 at room temperature for 1 h in a final volume ≤0.5 mL (*see* **Notes 9** and **10**).
5. Apply the reaction mixture to a PD-10 column equilibrated with conjugation buffer to remove the excess 2-IT and DTNB. Collect 0.5-mL aliquots, determine the absorbance at 280 nm, and pool the peak fractions (total volume, 1.5–2.0 mL).

3.3. Derivatization of Antibody for Thioether Linkage

1. Apply the antibody solution (2.5–3.0 mg total) to a PD-10 column equilibrated with conjugation buffer to remove any low-mol-wt materials that may interfere with the reaction. Collect 0.5-mL fractions, read the absorbance at 280 nm, and pool the peak tubes (total volume 1.5–2.0 mL) (*see* **Note 1**).
2. The concentration of the antibody solution should be at least 4 mg/mL. Concentration by Centricon P30 microconcentrator may be required to achieve this (*see* **Note 7**).
3. Prepare a 30-m*M* solution of MBS in dry DMF just before use (*see* **Note 11**).
4. To 2 mg antibody (12.5 nmol) add 62.5 nmol MBS (fivefold molar excess).
5. Incubate at room temperature for 10 min.

6. Apply the reaction mixture to a PD-10 column equilibrated with conjugation buffer to remove excess MBS. Collect 0.5-mL fractions, read the absorbance at 280 nm, and pool the peak tubes (total volume 1.5–2.0 mL) (*see* **Note 12**).

3.4. Conjugation of RNase to Antibody via a Disulfide Linkage

1. Incubate the RNase-2-pyridyl disulfide derivative with 2 mM DTT (final concentration) for 1 h at room temperature to reduce the 2-pyridyl disulfide bond (*see* **Note 13**).
2. To remove excess DTT, apply the reduced RNase solution to a PD-10 column equilibrated with conjugation buffer. Collect 0.5-mL aliquots, determine absorbance at 280 nm, and pool the peak fractions (1.5–2.0 mL) (*see* **Note 14**).
3. Add the RNase solution, which now contains a free sulfhydryl group, to the modified antibody solution. Incubate at room temperature overnight or until the reaction has gone to completion (*see* **Note 15**). The RNase should be present at least as a 10-fold molar excess over antibody (*see* **Note 16**).

3.5. Conjugation of RNase to Antibody via a Thioether Linkage

1. Dialyze the SPDP-RNase against 0.1 *M* sodium acetate, pH 4.5, containing 0.1 *M* NaCl (*see* **Note 17**).
2. Add 0.5 *M* DTT to the SPDP-RNase in acetate buffer **(step 1)** to a final concentration of 25 m*M*. Incubate at room temperature for 30 min (*see* **Note 18**).
3. Apply the mixture to a PD-10 column equilibrated with conjugation buffer to separate the thiolated protein from the low-mol-wt material (*see* **Note 19**).
4. Add the thiolated RNase to the MBS-treated antibody and incubate at 4°C overnight (*see* **Note 20**). The RNase should be at least in a 10-fold molar excess over antibody (*see* **Note 16**).

3.6. Purification of RNase–Antibody Mixture

1. The reaction mixture is chromatographed on a Toyo Soda TSK 3000 SW column (7.5 × 600 mm) equilibrated in 100 m*M* phosphate buffer, pH 7.5, at 0.5 mL/min (*see* **Note 21**).
2. Collect 1 min fractions and determine the absorbance at 280 nm.
3. Several peaks will be observed in addition to the RNase–antibody conjugate (*see* **Note 22**). The predominant peak (retention time of 27–31 min) represents RNase–antibody conjugate (also some free antibody if the reaction did not go to completion; retention time, 31 min) (*see* **Note 23**) and that at 41–42 min represents SPDP-RNase. Pool the RNase–antibody conjugate (*see* **Note 24**).
4. The yield of conjugate is variable ranging from 30–60%. This will depend on how much of the very-high-mol-wt material is formed, the antibody, the ability of the antibody/RNase/conjugate to be concentrated, as well as the various reaction times and temperatures (*see* **Note 25**).
5. Determine the level of substitution by running a sample of the pooled RNase–antibody conjugate on an SDS-polyacrylamide reducing gel and comparing the density of the bands of the RNase and the heavy and light chains of the antibody with a standard curve of known concentrations of RNase and antibody.

4. Notes

4.1. Derivatization of RNase

1. It is advisable to chromatograph the RNase and antibody on a PD-10 column before use to remove any low-mol-wt materials that may interfere with the reaction between the RNase and SPDP or the antibody and 2-IT. Since SPDP and 2-IT react with free amino groups, the choice of buffer is also important. If the proteins are stable to dialysis, they may also be dialyzed against the conjugation buffer. Dialysis may be preferred over gel filtration since it will eliminate the need to concentrate the protein, as described in **Subheading 3.1., step 3** (*see* **Note 2**).
2. For some RNases, especially recombinant RNases, there can be a large loss of protein after concentration with a Centricon microconcentrator. Other methods of concentration, such as Diaflo ultrafiltration (Amicon Inc.) using a YM3 membrane, have not been successful in these cases. This concentration step can be avoided by either dialyzing as described in **Note 1** or by starting with more material, such as 1 mL of a 10–20 mg/mL RNase solution. The final protein concentration should be 6.4 mg/mL.
3. SPDP is a heterobifunctional cleavable crosslinker containing a *N*-hydroxysuccinimde residue and a pyridyl disulfide residue to react with primary amines and sulfhydryls, respectively ***(8)***. SPDP is stable as a solution in ethanol at room temperature as long as it is kept free of moisture; thus a 20-m*M* solution may be prepared and used for several days. The powder form of SPDP should be stored surrounded by silica gel (or another drying agent) because it is very unstable in water.
4. The ratio of SPDP to RNase (2.5 mol SPDP/mol RNase) consistently results in 0.9–1.1 mol 2-pyridyl disulfide groups/mol RNase for such RNases as bovine pancreatic RNase A ***(6)***, EDN (unpublished observations), human pancreatic RNase (unpublished observations), and Onconase ***(9,10)***. A higher level of substitution may result in complete inactivation of the protein or in multiples of antibody conjugated to the RNase.
5. To calculate the level of substitution of the RNase ***(8)***, remove 50 µL from the pooled derivatized RNase solution and adjust the volume to 0.5 mL with conjugation buffer. Determine the concentration of the modified RNase by measuring the optical density at 280 nm. Since the 2-pyridyl disulfide group also absorbs at 280 nm (molar extinction coefficient, 5.1×10^3 at 280 nm), its contribution to the optical density should be taken into account as follows; concentration of pyridine-2-thione $\times$ 5100 = A_{280} nm resulting from pyridine-2-thione. Add 25 µL of freshly prepared 50 m*M* DTT to the diluted RNase tube and determine the optical density at 343 nm. The mols of pyridine-2-thione released upon reduction can be calculated using the molar extinction coefficient for pyridine-2-thione at 343 nm (8.08×10^3). Do not recombine this sample with the original substituted RNase pool.
6. The SPDP-modified RNase is stable at 4°C for at least 1 wk.

4.2. Derivatization of Antibody for Disulfide Linkage

7. Applying more of a more concentrated solution of antibody will eliminate the need for this concentration step, i.e., 1 mL of 10 mg/mL solution.

8. 2-IT reacts with primary amines to introduce a sulfhydryl residue. It is stable in solution at acidic to neutral pH ***(11)***.
9. Before beginning a preparative conjugation, the optimal ratio of 2-IT to antibody should be determined. A pilot study in which the ratio of 2-IT to antibody varies between 10 and 40 mol 2-IT to 1 mol antibody should be performed and the reaction analyzed by HPLC as described in **Subheading 3.6.** The reaction conditions should be adjusted such that there is little remaining unreacted antibody and the level of high-mol-wt species of conjugate is minimal.
10. DTNB is employed in concert with 2-IT for three reasons: The number of thiol groups introduced onto the antibody can be followed by monitoring the absorbance at 412 nm (*see* **Note 15**), the 5-thio-2-nitrobenzoic acid is a very good leaving group in the formation of a disulfide linkage between the antibody and RNase, and the reaction between the antibody and RNase can be quantitated by following the absorbance at 412 nm (*see* **Note 15**) ***(12)***.

4.3. Derivatization of Antibody for Thioether Linkage

11. At a pH above neutrality, MBS hydrolyzes to maleamic acid and thus should be prepared just before use ***(13)***.
12. Studies by Liu et al. ***(13)*** show that the maleimide group on the protein is not stable at neutral pH. Also, the maleimide group reacts with both amino groups and sulfhydryl groups on the same or different molecules leading to dimerization/multimerization. Therefore, both the RNase and antibody should be prepared simultaneously so that both solutions are ready to be mixed immediately.

4.4. Conjugation of RNase to Antibody via a Disulfide Linkage

13. The disulfide bonds of the RNase are not affected by this concentration of DTT (2.0 m*M*) ***(14)***. If the RNase is stable to dialysis and concentration, the 2-pyridyl disulfide bond can be reduced under acidic conditions. At pH 4.5, the reduction of the protein-2-pyridyl disulfide is very specific. At this pH, the 2-thio-pyridine is a good leaving group ***(8)***. To perform the reduction under acidic conditions, follow **steps 1–3** in **Subheading 3.5.**
14. Do not let this reaction sit or the free sulfhydryl groups will interact with each other and form RNase dimers ***(8)***.
15. Follow the reaction between the RNase and antibody by observing the appearance of thionitrobenzoate (TNB) ***(12)***. TNB is released from the antibody as disulfide bonds between the RNase and antibody are formed. This can be observed spectrophotometrically at 412 nm using the molar extinction coefficient of TNB of 13,600. By comparing the number of mols of TNB released with the number of mols of antibody, the number of mols of RNase conjugated per mol of antibody can be determined.
16. The reaction must be driven to completion by a large excess of RNase, because it is very difficult to separate free unconjugated antibody from the RNase–antibody conjugate. The molecular weights of free antibody (160,000 kDa) and RNase-modified antibody differ only by 10–30 kDa (170,000–200,000 kDa) resulting in

retention times on the sizing column that differ by <1 min. Therefore, to minimize the interference that may result from any unreacted antibody, the reaction is driven to completion by a large excess of SPDP-modified RNase.

4.5. Conjugation of RNase to Antibody via a Thioether Linkage

17. The buffer may be exchanged by PD-10 chromatography on a column pre-equilibrated with 0.1 *M* sodium acetate, pH 4.5, containing 0.1 *M* NaCl. Before it can be used, the volume must be concentrated to 0.5 mL using a Centricon P3 microconcentrator. Another method of exchanging buffer is to dilute the SPDP modified-RNase with the sodium acetate buffer and concentrate via the Centricon P3 microconcentrator. Repeating this step several times will result in an exchange of buffers (*see* **Note 2**).
18. Reduction in the presence of acid pH will result in the reduction of the 2-pyridyl disulfide bond without affecting the disulfide bonds of the native protein.
19. The protein should be stored in the pyridyl disulfide-modified form until just before use. The thiol group is very reactive and unwanted conjugations will result if the thiol form is allowed to remain for any length of time in the absence of the antibody ***(8)***.
20. Incubation should be at 4°C because maleimide residues hydrolyze more slowly at lower temperatures.

4.6. Purification of RNase–Antibody Mixture

21. The reaction may be concentrated with a Centricon P30 microconcentrator before application to the sizing column to reduce the number of chromatographic columns that must be performed. Before concentration, however, an analytical run of the reaction before and after concentration should be performed to ensure that the concentration step does not result in an increase of higher-mol-wt aggregates. We find that some RNase–antibody conjugates can not be concentrated without a loss (in some cases up to 50%) of conjugate.
22. There may be some peaks appearing at the void volume of the column (19 min) and at 21–23 min. The material eluting at these retention times most likely includes multimers of at least two molecules of antibody and an unknown number of RNase molecules. This material should not be included in the pool of RNase–antibody conjugate.
23. Because there is such a small difference in molecular weight between unconjugated antibody (160,000) and conjugate (170,000–200,000), the conjugates are not cleanly separated from unconjugated antibody. When pooling the conjugate, pool narrowly on the downside of the peak in order to minimize any further contamination of the conjugate with free antibody. Similar results have been noted by Lambert and Blattler for antibody–gelonin conjugates ***(15)*** and Myers et al. for antibody–pokeweed antiviral protein ***(16)***. If the level of free antibody present in the conjugate solution interferes with the activity of the conjugate, several methods for further purification of the conjugate are described ***(15,16)***. RNases are very basic proteins ***(17)*** and therefore bind to CM Sephadex

C-50. In contrast to free RNase, at neutrality the RNase conjugates will not adhere to this resin. Decreasing the ionic strength and altering the pH of the conjugate to 5.0 allows some RNase conjugates to bind to CM-Sephadex C-50 while the unconjugated antibody passes through the column. The RNase conjugate can then be eluted by increasing the pH to 7.8 and increasing the NaCl concentration to 0.5 *M* (unpublished observation).

24. At this stage, the pooled RNase–antibody reaction may be concentrated on a Centricon P30 microconcentrator, however, the conjugate should be concentrated with caution because some RNase–antibody reactions will result in as much as a 50% loss of material as a result of aggregation.
25. To sterilize the RNase conjugate, use Millipore Millex-HV (Millipore Products Division, Bedford, MA) filters.

Abbreviations

SPDP, *N*-Succinimidyl 3-(2-pyridyldithio) propionate; 2-IT, 2-iminothiolane; DTNB, 5,5'dithiobis(2-nitrobenzoic acid); DMF, dimethlformamide; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; DTT, dithiothreitol.

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