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Monoclonal antibody-conjugated dendritic nanostructures for siRNA delivery

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Monoclonal antibody conjugated dendritic nanostructures for siRNA delivery

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Abstract

Small interfering RNA (siRNA) is a promising tool for gene therapy-based disease treatments. However, delivery of siRNA to the target cells requires a specific and reliable carrier system. Herein we describe a targeted carrier system that can deliver siRNA to cancer cells overexpressing the human epidermal growth factor 2 (HER2) receptor. Trastuzumab-conjugated poly(amido)amine dendrimers can be synthesized using the protocols described here.

Key words: siRNA delivery, Dendrimers, Trastuzumab, Bioconjugation, HER2 receptors

1. Introduction

In recent years, RNA interference (RNAi) has emerged as a powerful technique for treating diseases by interfering with the activity of disease-causing or promoting genes. RNAi is attracting increased attention due to several intrinsic properties that include effective gene silencing, high specificity, minor side effects as well as easy and low cost of synthesis (1-3). ShRNAs/siRNAs are a class of short double-stranded RNA molecules which can effectively silence a specified target gene *in vitro/in vivo* However, to date, shRNA/siRNA-based gene therapy suffers from several drawbacks (4,5). Firstly, naked or unmodified siRNAs are highly unstable in the blood stream and are rapidly removed enzymatically by endo- and exo-nucleases. Secondly, given its anionic nature, siRNA cannot cross the cytoplasmic membrane by simple diffusion (6). Thirdly, siRNAs are rapidly cleared from the body due to high aqueous solubility (7). Fourthly, the potential for siRNA off-target effects. Therefore, the use of siRNAs/shRNAs in the human body is a challenging task that necessitates a carrier system to deliver the therapeutic siRNA to its target site (8).

Although various gene-carrier systems have been investigated in the past decade, dendrimers are amongst the most attractive carrier system due to several inherent gene-carrying properties (9-12). Dendrimers (Dend) are molecules consisting of radially symmetric tree-like arms or branches that compose into a well-defined, homogeneous, and monodisperse nano-structure (13). Due to their structure, there are several void spaces within the dendrimers where siRNAs/shRNAs can be encapsulated providing high chemical and physical stability to the cargo within biological systems. Furthermore, cationic dendrimers can be easily complexed with anionic nucleic acid molecules by electrostatic interactions into “ready to go” nanoparticles; which also have a plethora of surface functional groups that facilitates specific targeting of dendrimers in order to avoid off-target effects of the therapeutic siRNA.

This chapter illustrates a dendrimer-based strategy for site-specific delivery of a siRNA to human epidermal growth factor 2 (HER2) receptor-positive breast cancer cells. About 20% of breast cancers express a high level of HER2 receptors (14, 15). Trastuzumab (TZ) is a humanized monoclonal antibody that binds specifically to HER2 receptors and is currently used for the treatment of HER2-positive breast cancer (15, 16). Therefore, TZ-conjugated dendrimers represent an efficient delivery system for the targeted delivery of drugs and genes to HER2 positive breast cancer cells.

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2. Materials

1. G4 PAMAM dendrimers (MW: 14215 Da) with a diaminobutane core.
2. Trastuzumab (TZ), Nava Sanjivani Drugs (Hyderabad, India).
3. MAL-PEG-NHS (MW: 865.92 Da).
4. Nylon membrane filters (pore size of 0.22 μm).
5. Fluorescein isothiocyanate (FITC).
6. Dialysis tubing (molecular weight cut off 2000).
7. Dulbecco's modified Eagle' medium (DMEM).
8. 0.25% Trypsin–EDTA (1x) phenol red.
9. Antibiotic/antimycotic solution.
10. PBS (Ca^{2+}, Mg^{2+} free).
11. 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).
12. Dimethyl sulfoxide (DMSO).
13. Annexin V-FITC apoptosis detection kit.
14. Acridine orange (AO)
15. Ethidium bromide (EB).
16. Fetal bovine serum.

17. Human breast cancer cell lines MDA-MB-453 (HER2-positive) and MDA-MB-231 (HER2-negative).

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3. Methods

3.1 Synthesis of FITC-labelled dendrimers

1. To synthesize FITC-labelled dendrimers (FITC-Dend), in a round bottom flask resuspend G4 PAMAM dendrimers in PBS (pH 7.4) to a final dendrimer concentration of 5 mg/mL (Note 1).
2. Dissolve enough FITC in acetone to achieve a 1:6 molar ratio of FITC to dendrimers.
3. Add FITC solution to dendrimer solution and stir at room temperature for 12 h.
4. Remove unconjugated FITC by dialysis against PBS for 24 h at room temperature.
5. Measure the absorbance at 495 nm using a UV-VIS spectrophotometer and calculate the number of FITC molecules conjugated to per molecule of dendrimers. The amount of FITC conjugated to the dendrimers is measured by standard calibration of FITC.

3.2 Conjugation of hetero-cross-linker (NHS-PEG-MAL) to FITC-Dend

1. For the generation of Dend-PEG-MAL molecules, NHS-PEG-MAL is mixed with FITC-Dend conjugates in a molar ratio of 3:1 in PBS (pH 8.0) and allowed to react by stirring for 30 minutes at room temperature.
2. Purify the FITC-Dend-PEG-MAL conjugate by dialysis against PBS for 12 h at room temperature. Dialysis will remove the unconjugated or free NHS-PEG-MAL.

3. Lyophilize the purified FITC-Dend-PEG-MAL to obtain in powder form for ^{1}H-NMR spectroscopy (Note 2).
4. Dissolve about 5 mg of conjugate in D_2O and run a ^{1}H-NMR to confirm the conjugation of FITC-Dend-PEG-MAL.

3.3 Bio-conjugation of TZ with dendrimers

1. For thiolation of TZ, dissolve the TZ in PBS and add 2-iminothiolane hydrochloride in 1:10 molar ratio. Stir the solution for 2 h at room temperature.
2. Place the solution in a dialysis bag and dialyse against PBS to remove free 2-iminothiolane hydrochloride.
3. For the bio-conjugation of thiolated TZ and dendrimer, thiolated TZ is coupled to FITC-Dend-PEG-MAL (10:1 ratio).
4. The FITC-Dend-PEG-MAL dendrimer solution is mixed with the thiolated TZ solution and allowed to react overnight by stirring at room temperature.
5. FITC-Dend-PEG-TZ conjugates are purified by PBS (pH 7.4) elution through a size exclusion chromatography Sephadex column (G-25 M).
6. The chemical reactions for section 3.1-3.3 are presented in Figure 1.

3.4 Measurement of the Zeta potential of FITC-Dend-PEG-MAL-TZ conjugates

1. Determine zeta potential of different formulations using Zetasizer Nano-ZS. Dilute the samples in deionized water and analyse at 25 ^{o}C (Note 3).

3.5 Preparation of Dendrimer-siRNA complex

1. Resuspend dendrimer conjugates (FITC-Dend, FITC-Dend-PEG- TZ) in water and mix with siRNA at different N/P ratios (Note 4). The N/P ratio is defined by the number of amine groups present in the dendrimers and the number of anionic phosphate groups from siRNA.

2. Vortex dendrimer-siRNA mixture for 1 min and incubate at room temperature for 30 min in order to ensure the formation of the complexes (Figure 1).
3. Store the prepared dendrimer-siRNA (siRNA/FITC-Dend, siRNA/FITC-Dend-PEG- TZ) complexes in freezer (–4 °C) until use.

3.6 Cell culture and Anti-proliferation assay

1. Grow MDA-MB-453 (HER2-positive) and MDA-MB-231(HER2-negative) human breast cell lines in DMEM medium supplemented with 10% fetal bovine serum 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine. Cells should be maintained at 37 °C and 5% CO_2 environment in a CO_2 incubator.
2. Seed the cells in 96-well plates at a density of 5×10^3 cells per well in 100 μL of medium and allow them to adhere overnight before incubation with non-conjugated siRNA, and conjugated siRNA/FITC-Dend-PEG-MAL or siRNA/FITC-Dend-PEG-MAL-TZ at equivalent siRNA concentrations (0-100 nM) for 48 h.
3. Replace the media with serum-free DMEM containing MTT (0.5 mg/mL) and incubate for 4 h.
4. Gently remove the media and add 150 μL DMSO.
5. Measure the absorbance at 570 nm using a microplate reader.
6. Untreated cells are used as a negative control (100% viability) and IC_{50} is calculated by fitting the curve of cell viability against the drug concentration.

3.7 Cellular uptake studies

1. Seed cells in 12-well plates and allow them to adhere for 24 h.
2. Incubate cells with siRNA/FITC-Dend-PEG-MAL or siRNA/FITC-Dend-PEG-MAL-TZ conjugates, at an equivalent FITC concentration (100 μg/mL).

3. For competitive binding studies, preincubate the cells with an excess (20 mM) of TZ for 2 h and then treat with siRNA/FITC-Dend-PEG-MAL or siRNA/FITC-Dend-PEG-MAL-TZ.
4. Remove the culture media, wash the cells twice with cold PBS and observe the cells using a fluorescence microscope.
5. For quantitative studies, add 0.1% Triton X-100 in 0.2 M NaOH, then add into each well to lyse cells.
6. Measure the fluorescence intensity using a microplate reader at an excitation wavelength of 495 nm and emission wavelength of 520 nm.

3.8 Stability Study

1. To check the stability of final formulation (siRNA/FITC-Dend-PEG-MAL-TZ), store the samples at 4 °C.
2. Monitor the formulations for any changes in consistency and sign of precipitation.
3. Measure the zeta potential using Zetasizer Nano ZS at predetermined time intervals.

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4. Notes

1. The chemical reactions and purification must be performed in the dark. FITC is a light-sensitive compound.
2. Before lyophilization, sample should be pre-freeze using liquid nitrogen.
3. During the measurements of zeta potential using Zetasizer Nano ZS, the samples should be diluted appropriately to achieve count rate of between 50-300.
4. Dendrimer-siRNA complex can also be prepared in HEPES buffer pH 7.4.

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Figures

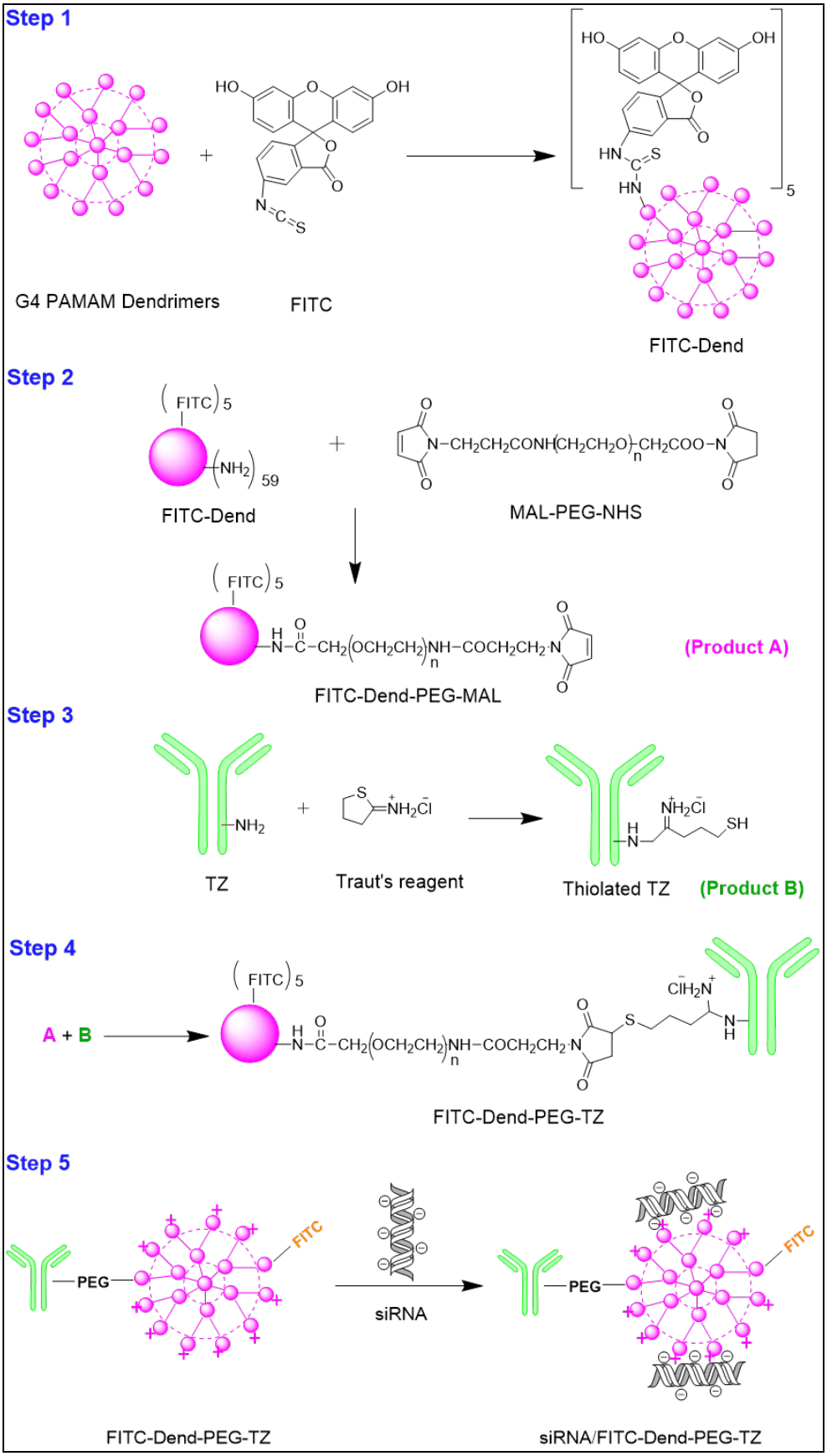
Step 1
G4 PAMAM Dendrimers
FITC
FITC-Dend
Step 2
FITC-Dend
MAL-PEG-NHS
FITC-Dend-PEG-MAL
(Product A)
Step 3
TZ
Traut's reagent
Thiolated TZ
(Product B)
Step 4
A + B
FITC-Dend-PEG-TZ
Step 5
PEG
FITC
siRNA
FITC-Dend-PEG-TZ
siRNA/FITC-Dend-PEG-TZ

Figure 1: Schematic diagram illustrating the synthesis of Trastuzumab (TZ)-grafted, fluorescein isothiocyanate (FITC)-labelled G4 poly(amido) amine (PAMAM) dendrimers siRNA/FITC-Dend-PEG-TZ. **Step 1**, FITC was conjugated to G4 PAMAM dendrimers FITC-Dend. **Step 2**, FITC-labelled dendrimers were crosslinked with MAL-PEG-NHS to give FITC-Dend-PEG-MAL (Product A). **Step 3**, TZ was thiolated using Traut's reagent (2-iminothiolane) to give thiolated TZ. **Step 4**, FITC-Dend (Product A) was reacted to thiolated TZ (Product B) to synthesize TZ-grafted FITC-labelled G4 PAMAM dendrimers (FITC-Dend-PEG-TZ). **Step 5**, siRNA is reacted to generate siRNA/FITC-Dend-PEG-TZ.