RAFT Polymerzation for Delivery of Bioactives

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ABSTRACT

This paper describes recent efforts on the use of Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerization for synthesis of well-defined polymer conjugates for delivery of bioactives. The research is illustrated by work on with the antitumor drug SN38, to improve water solubility and cell viability, and the delivery of siRNA, for gene silencing. The RAFT polymers are conjugated to the active through a covalent linkage, in the case of anticancer drug SN38, and by ionic association, for the case of siRNA. Preliminary results are extremely encouraging. The synthesized RAFT ABA triblock polymers provided 100% siRNA uptake and gave gene silencing similar to that of the transfection reagent Lipofectamine™. In both examples RAFT-acive conjugates polymers were well-tolerated showing no discernable cell cytotoxicity.

Keywords: RAFT polymerization, SN38 antitumor drug, siRNA, bioconjuates

1 INTRODUCTION

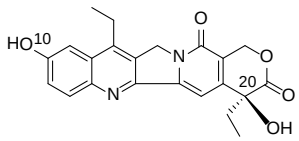
RAFT polymerization, a reversible deactivation radical polymerization (RDRP),[1] is arguably the most versatile method for providing living characteristics to radical polymerization. The method, developed at CSIRO laboratories,[2] is now well-established as providing exceptional control over molecular weight, molecular weight distribution, composition and architecture. The process can be applied to most monomers polymerizable by radical polymerization and offers a convenient route to well-defined homo-, gradient, diblock, triblock and star polymers as well as more complex architectures that include microgels and polymer brushes.[3-8] Moreover, RAFT polymerization, like conventional radical polymerization, is tolerant of functionality in monomer, solvent (it can be carried out in aqueous media), it is compatible with bulk, solution, suspension and emulsion processes.[2-8]

Therapeutics delivery is devoted to the design, synthesis and application of materials capable of delivering bioactives safely and efficiently into the body of a patient. Despite extensive research efforts made by academics and pharceutical industries, there are still many challenges and limitations that need to be overcome before therapeutics based on small drug molecules, peptides, proteins and genes see widespread clinical use.[9-14]

The RAFT process is well placed to meet these challenges due to its robustness, high flexibility, mild experimental conditions and ability to insert functional groups within the polymer chain. This produces well defined polymers needed to construct well-defined polymer-therapeutic conjugates. RAFT polymerization opens up the opportunity to produce polymers with the desired feature for delivery of bioactives. The present study uses RAFT polymerization with specific RAFT agents to produce polymer-drug conjugates and a series of three ABA triblock copolymers with neutral and cationic blocks, and explores the ability of these polymers to bind, and deliver siRNA into cells to allow silencing of target genes.

2 POLYMER-SN38 DRUG CONJUGATES

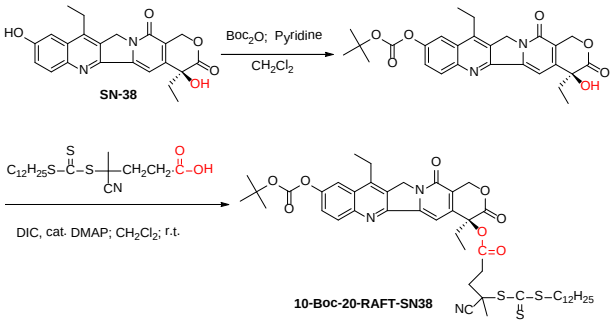
Camptothecin and its derivatives such as 7-ethyl-10-hydroxycamptothecin (SN38) are an important class of antitumor agent.[15] They inhibit the topoisomerase I of tumors[16] and suppress the proliferation of cancer cells to elicit antitumor effect.[17-19] Unfortunately, poor solubility in water and in physiologically acceptable organic solvents presents a serious barrier of campothecin drug family and SN38 in practical use. We have overcome this by making polymer-drug conjugates using RAFT polymerization with a water soluble, FDA approval monomer, *N*-(2-hydroxypropyl) methacrylamide (HPMA).



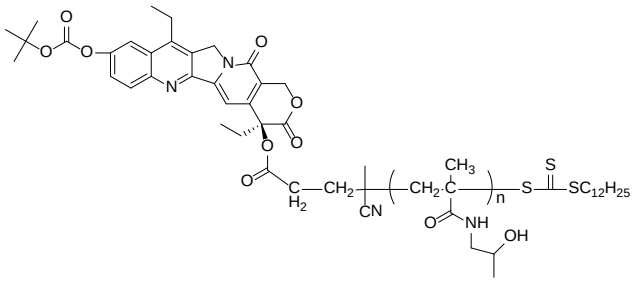
SN-38

In this study, the SN38-RAFT agent, 10-Boc-20-RAFT-SN38, was synthesised using a two step syntheses as shown in Scheme 1. The 10-hydroxy group of SN38 was protected by a Boc-group in quantitative yield, then the 20-hydroxy group of the resulting product was allowed to react with an equimolar amount of the RAFT agent, 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid[20,21] in the presence of the coupling agent,

diisopropylcarbodiimide (DIC) with N,N-dimethylaminopyridine (DMAP) as catalyst in dichloromethane at room temperature to yield the final product in 55% yield after silica-gel column chromatography.



Scheme 1: Synthesis of 10-Boc-20-RAFT-SN38



Poly(HPMA)-SN38 conjugate

With the 10-Boc-20-RAFT-SN38 on hand, radical polymerization of N-(2-hydroxypropyl) methacrylamide (HPMA) with different concentration of SN38-RAFT agent in DMF solvent with AIBN as initiator at 60°C for 24 h were carried out. The results are summarized in Table 1.

Table 1: Poly(HPMA)-SN38 conjugates

Entry	[RAFT] $\times 10^3$ M	M_n [a] $\times 10^{-3}$	M_w/M_n [a]	$Mn(calc) \times 10^{-3}$
D	32.7	6.7	1.27	6.6
C	16.36	11.9	1.19	13.2
B	8.18	21.8	1.17	26.4
A	4.09	25.2	1.29	47.0

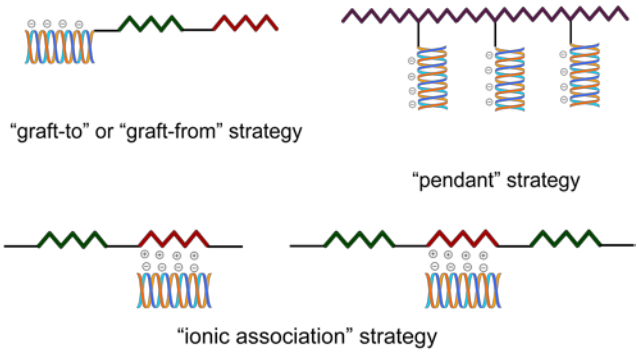
[a] GPC (DMF solvent) in polystyrene equivalents.

Results shown that conjugation of SN38 to poly(HPMA) dramatically improves the solubility and penetration through tissue. Cell viability assays were perfomed with the L929, Cos, X63-Ag8, B16-F10 and HeLa cell lines.

3 POLYMER-siRNA CONJUGATES

Ribonucleic acid (RNA) is a bio-macromolecule used by cells for defence and to regulate gene expression. The RNAi process involves the incorporation of specific siRNA into the specific target messenger RNA (mRNA) to inhibit the synthesis of the encoded protein. Small interfering RNAs (siRNAs), 19-23 mer double-strand oligonucleotides, play a fundamental role in RNA interference, and are a very powerful tool for reducing the expression of genes which are specifically targeted.[22-24] Although the significant potency of siRNAs to treat gene-related diseases, their poor in vivo delivery remains a major challenge medical therapies.[14,25] Thus, the therapeutic value of siRNAs under in vivo conditions is still controversial due to their fast degradation in physiological media (e.g., low stability against enzymatic digestion in the blood stream), poor cellular uptake, inefficient translocation into the cytoplasm, rapid renal clearance after systematic administration, and lack of targeting activity. For instance, the target delivery of siRNA is one of the main obstacles in cancer therapy by inhibiting the translation of oncogenes through the RNA interference.[22, 26-27]

In this study, we have used RAFT polymerization to produce a series of neutral and cationic polymers via 'graft-to' or 'graft-from', 'pendent' and 'ionic association' strategies to test the binding and cellular delivery of siRNA.



Formation of Polymer/siRNA complexes

Molar ratios of polymer to 50 nM siRNA or siDNA were calculated. Complexes were formed by the addition of OPTIMEM media (Invitrogen, USA) to eppendorf tubes. The required amount of polymer resuspended in water was added to the tubes and the mixture vortexed. 50nM of anti-GFP siRNA (si22) or DNA oligonucleotides corresponding to anti-GFP siRNA sequence (di22) was then added to the tubes and the sample vortexed. Complexation was allowed to continue for 1 h at RT.

Agarose gel

Samples at different molar ratios of polymer to 50 nM siRNA were electrophoresed on a 2% agarose gel in TBE at 100V for 40 min. siRNA was visualised by gel red (Jomar Bioscience) on a UV transilluminator with camera, the image was recorded by the GeneSnap program (Syngene, USA).

Silencing Assay

CHO-GFP cells were seeded at $1x10^4$ cells in 96-well tissue culture plates in triplicate and grown overnight at 37 ºC with 5% CO_2. For positive and negative controls siRNAs were transfected into cells using Lipofectamine™ 2000 (Invitrogen, USA) as per manufacturer's instructions.

Briefly, 50 picomole of the relevant siRNA were mixed with 1 µl of Lipofectamine™ 2000 both diluted in 50 µl OPTI-MEM (Invitrogen, USA) and incubated at room temperature for 20 mins. The siNA: Lipofectamine™ mix was added to cells and incubated for 4 h. Cell media was replaced and incubated for further 72 h.

For RAFT/siRNA complexes cell media was removed and replaced with 100 µl OPTI-MEM and incubated for a further 72h. Cells were washed twice with PBS, trypsinised and washed once with FACS wash (PBS with 1% FBS). Cells were subjected to flow cytometry and EGFP silencing was analyzed as a percentage of the non-silencing siRNA or RAFT/diRNA complexes mean EGFP (measured on FITC wavelength) fluorescence.

Results

The three RAFT polymers (samples 422-1, 422-2 and 422-3) with different neutral and cationic blocks were analyzed for their ability to bind siRNA at different molar ratios. Two of the polymers (samples 422-2 and 422-3) could bind strongly the siRNA at low ratios whilst the third (sample 422-1) was less efficient (Figure 1).

Figure 1. Gel electrophoresis of association of triblock copolymer with siRNA as a function of polymer:siRNA ratio (w/w).

These samples were then analyzed for the ability to silence the target gene green fluorescent proteinin CHO-GFP cells. Only sample 422-3 demonstrated significant silencing (>75%) comparable to the use of transfection reagents Lipofectamine™ (Figure 2), indicating that the configuration of the blocks has a significant impact on both the ability to bind siRNA and induce silencing.

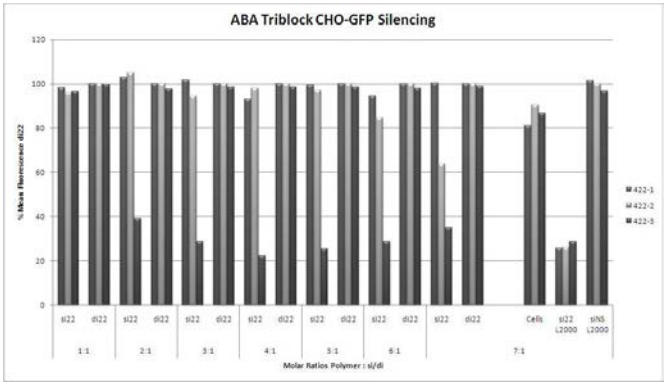


Figure 2. Gene silencing in CHO-GFP cells for different siRNA:RAFT copolymer combintations presented as a percentage of non-silencing siRNA complexes mean EGFP (measured by FITC wavelength) fluorescence.

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