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The effect of RAFT-derived cationic block copolymer structure on gene silencing efficiency

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ABSTRACT

In this work a series of ABA tri-block copolymers was prepared from oligo(ethylene glycol) methyl ether methacrylate ($OEGMA_{475}$) and *N,N*-dimethylaminoethyl methacrylate (DMAEMA) to investigate the effect of polymer composition on cell viability, siRNA uptake, serum stability and gene silencing. Reversible Addition–Fragmentation Chain Transfer (RAFT) polymerization was used as the method of polymer synthesis as this technique allows the preparation of well-defined block copolymers with low polydispersity. Eight block copolymers were prepared by systematically varying the central cationic block (DMAEMA) length from 38 to 192 monomer units and the outer hydrophilic block ($OEGMA_{475}$) from 7 to 69 units. The polymers were characterized using size exclusion chromatography and ^{1}H NMR. Chinese Hamster Ovary-GFP and Human Embryonic Kidney 293 cells were used to assay cell viability while the efficiency of block copolymers to complex with siRNA was evaluated by agarose gel electrophoresis. The ability of the polymer–siRNA complexes to enter into cells and to silence the targeted reporter gene enhanced green fluorescent protein (EGFP) was measured by using a CHO-GFP silencing assay. The length of the central cationic block appears to be the key structural parameter that has a significant effect on cell viability and gene silencing efficiency with block lengths of 110–120 monomer units being the optimum. The ABA block copolymer architecture is also critical with the outer hydrophilic blocks contributing to serum stability and overall efficiency of the polymer as a delivery system.

1. Introduction

RNA interference (RNAi) is a naturally occurring mechanism found in both plants and animals that uses short RNA molecules (21–23 nucleotides) to degrade or sequester messenger RNA (mRNA) resulting in specific gene suppression [1,2]. This mechanism has been taken advantage of by artificially introducing short interfering RNAs (siRNAs) into cells to suppress genes of interest. The use of siRNAs as therapeutics for genetic disorders, cancer and as antivirals is a fast growing field of research with several siRNAs already in clinical trials. siRNAs targeting HIV-1 [3], Hepatitis B [4], Influenza A [5], and foot and mouth disease virus [6] have been demonstrated to be effective at silencing these viruses both *in vitro* and *in vivo*. Thus, the therapeutic potential of siRNA is well recognized but the lack of effective delivery vehicles remains a major hurdle to the advancement of siRNA therapeutics in the clinic. The obstacles still needed to be overcome include nuclease degradation in serum, rapid clearance by the kidneys, activation of the immune system, entry into target cells and poor endosomal release of siRNA [7].

Many different delivery strategies have been attempted with some showing promising results with phase 1 trials already in progress [8–10]. Among the evaluated delivery systems are cationic lipids [11], chitosan [12], peptide conjugates [13] and polymer conjugates containing polymers such as polyethylene glycol (PEG) and polyvinylethers (PVE) [14]. One of the major issues confronting the clinical use of lipidic systems for siRNA delivery is their toxicity depending on the lipid used [15], and activation of inflammatory cytokines and interferon responses [16]. Cationic polymers also have issues with toxicity due to the disruption of the plasma membrane [17]. The other main problem with systemic delivery is sequestration of the vehicle by the reticulendothelial system (RES), resulting in the majority of the particles being present in liver and spleen macrophages [18]. Many of the reported delivery systems

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are aimed at targeting cancerous lesions based on upregulation of certain receptors on the target cancer and the enhanced permeability and retention (EPR) effect common to this disease. However, most of these systems still show a high proportion of the treatment in the liver and spleen [19]. Hence, a reliable, easily reproducible system that can deliver siRNA systemically to the desired cell types is still required. This is particularly important for long term RNAi treatment for genetic disorders or cancer, where knockdown of the endogenous gene in the wrong cell type i.e., healthy cells and not cancerous cells could have unforeseen consequences [20].

Reversible addition–fragmentation chain transfer (RAFT) polymerization is a controlled "living" radical polymerization technique that allows the synthesis of well-defined polymer architectures with low dispersity [21–28]. RAFT polymerization has been used to synthesize many different polymers including poly(acrylamides) and poly(methacrylamides) [29,30], as well as the synthesis of functional and stimuli-responsive block copolymers [31]. Another key feature of RAFT polymerization is the ability to further modify polymer end functional groups with active agents and targeting moieties to enhance the functional performance of the polymer [24–26,32]. For these reasons, the RAFT technique is attractive to design and synthesize polymers for advanced drug and gene delivery systems.

A number of literature studies report on the use of the RAFT technique to synthesize polymers for the delivery of deoxyribonucleic acid (DNA) and siRNA [33]. For example, Scales et al. have prepared [33,34] diblock copolymers of *N*-(2-hydroxypropyl) methacrylamide (HPMA), *N*-[3-(dimethylamino)propyl] methacrylamide (DMAPMA), and *N*-(3-aminopropyl) methacrylamide (APMA) and demonstrated their effectiveness to bind siRNAs and their potential as gene delivery vehicles. This study further demonstrated that in HMPA-DMAPMA diblock copolymers, the length of the cationic block has a strong influence on the binding affinity to siRNA; longer cationic block lengths resulted in particle aggregation due to interpolymer bridging. The diblock copolymers protected the siRNA from enzymatic degradation [34], whereas the RAFT polymers containing APMA have also shown gene suppression [35]. In another study, Heredia et al. [36] synthesized RAFT polymers from poly(ethylene glycol acrylate) (PEGA) and DMAPMA, and conjugated thiol functional siRNA to polymer with pyridyl disulfide end groups, demonstrating the versatility of this polymerization technique. Another study reported the synthesis of siRNA polyplexes via a combination of RAFT polymerization and thiolene chemistry utilizing HPMA as hydrophilic monomer; it was found that an increase in the length of the hydrophilic block decreases the efficiency of siRNA complexation [37]. The use of other controlled radical polymerization techniques has also been explored for the preparation of polymer materials for gene delivery systems [38]. In this later work, the statistical copolymerization of oligo(ethylene glycol) methyl ether methacrylates with *N,N*-dimethylaminoethyl methacrylate (DMAEMA) via atom transfer radical polymerization (ATRP) was utilized for the preparation of a series of copolymers as nonviral gene transfer agents. The influence of the macromolecular structures on the gene vector particle properties and transfection efficiency was investigated; the transfection efficiency of the most efficient copolymer was found to be 10-fold lower than the well-investigated branched polyethylenimine (PEI) 25 kDa [38].

For polymeric gene delivery vehicles, cationic units on the polymer chains interact with siRNA through ionic interactions which generally results in the formation of nanoparticle complexes [39]. In this regard, the chemical structure, molar mass, polydispersity, macromolecular architecture and composition of these polymers can play an important role in determining the nature of the complexes formed with siRNA. From a biological viewpoint, these properties in turn will influence the cell viability, cellular uptake and efficiency of gene silencing. While many cationic polymers have been investigated for gene delivery [39], the influence of the above factors is not well understood due mainly to the limited availability of well-defined polymeric materials to perform systematic biological investigations.

Here we report the ability of a series of well-defined ABA triblock copolymers – synthesized by RAFT polymerization. These copolymers are based on oligo(ethylene glycol) methyl ether methacrylate $OEGMA_{475}$ (A) and DMAEMA (B) and designed to bind siRNA, protect it from serum degradation, facilitate entry into cells and to silence the targeted reporter gene enhanced green fluorescent protein (EGFP). EGFP from the jellyfish *Aequorea victoria* has become widely used as a reporter gene due to its ability to independently fluoresce similar to fluorescein when expressed in cells and tissues. The ABA tri-block copolymer series was synthesized with varying lengths of both hydrophilic (A) and cationic (B) blocks to determine the most efficient ABA polymer structure to deliver active siRNA whilst retaining serum stability and producing minimal toxicity.

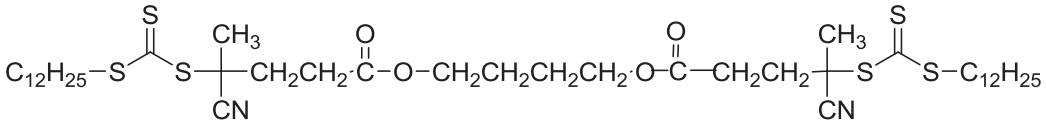
2. Materials and methods

Eight ABA tri-block copolymers were synthesized via RAFT polymerization in an automated parallel synthesizer (Method 1) and in Schlenk flasks (Method 2) utilizing similar experimental synthetic techniques as reported elsewhere [30,40,41].

2.1. Materials

N,N-dimethylaminoethyl methacrylate (DMAEMA) and oligo(ethylene glycol) methyl ether methacrylate ($OEGMA_{475}$, Mn ~ 475 g mol^{-1}) monomers were purchased from Aldrich and purified by stirring in the presence of inhibitor-remover for hydroquinone or hydroquinone monomethyl ether (Aldrich) for 30 min prior to use. Bis-RAFT agent 4-cyano-4-(dodecylthiocarbonothioylthio)pentanoyloxy)butyl 4-cyano-4-(dodecylthiocarbonothioylthio)pentanoate (I) was prepared according to the procedure described below. 1,1′-Azobis(cyclohexanecarbonitrile) (VAZO-88) initiator (DuPont) was used as received. *N,N*-Dimethylformamide (DMF) (AR grade, Merck) was degassed by sparging nitrogen for at least 15 min prior to use. Dicholormethane (DCM), *n*-heptane, diisopropyl ether, methyl iodide, methanol, and other chemical substances were commercial reagents and used without further purification.

Synthesis of bis-RAFT agent 4-cyano-4(dodecylthiocarbonothioylthio)pentanoyloxy)butyl 4-cyano-4-(dodecylthiocarbonothioylthio)pentanoate (I) $C_{42}H_{72}N_2O_4S_6$*; MW 861.42:*



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