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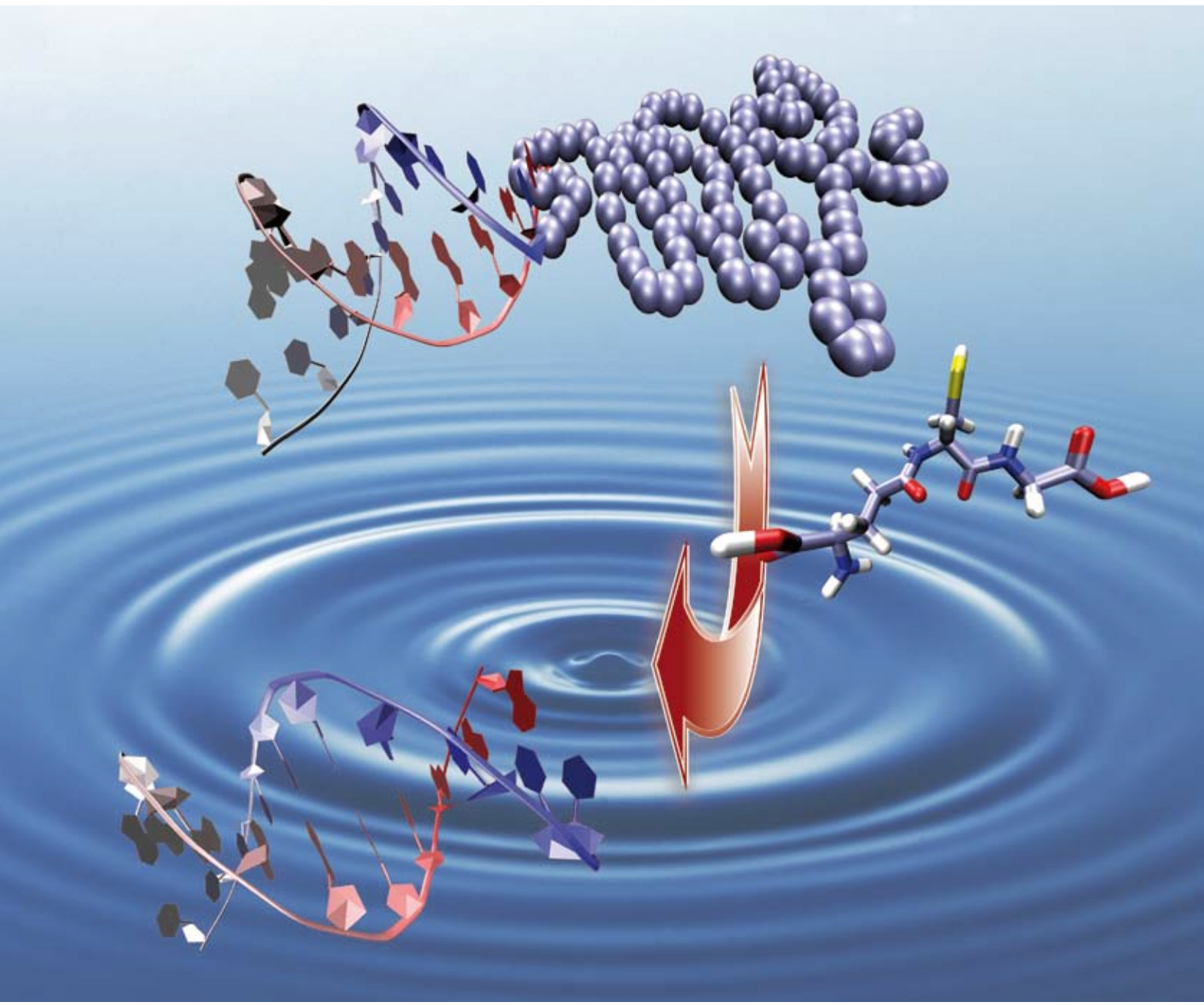

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Reversible siRNA–polymer conjugates by RAFT polymerization

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Reversible siRNA–polymer conjugates by RAFT polymerization†

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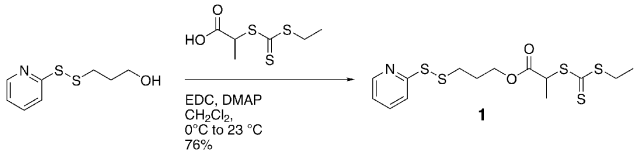
A straightforward synthetic method to prepare pyridyl disulfide end functionalized poly(PEG acrylate) by RAFT polymerization for efficient and reversible conjugation to siRNA is described.

RNA interference (RNAi) using double stranded short interfering RNA (siRNA) is a promising therapeutic strategy for a wide spectrum of diseases.[1,2] However, a bottleneck for efficient therapy *via* specific gene silencing lies in the inability to effectively deliver the siRNA. As a result, significant effort has been focused on developing methods to stabilize, increase circulation life times, and deliver siRNA to the cytoplasm of target tissues.[3] For example, conjugation of siRNA to proteins,[4] cholesterol,[5–7] sugars,[8] cell-penetrating/targeting peptides,[9,10] and formation of polyplexes with cationic polymers[3,11] and dendrimers[12] have been effective. In some of these cases, poly(ethylene glycol) (PEG) was employed to help protect the siRNA.[8,11,13] It was found that covalent attachment of the PEG to the siRNA or its delivery system enhanced stability and efficient delivery to targeted tissue and that by introducing a reversible disulfide linkage between the siRNA and the delivery system the siRNA could be released under normal reductive intracellular conditions. The latter is important to liberate the siRNA to achieve full biological activity.[11] In order to circumvent modification steps normally required to prepare a suitable PEG or polymeric delivery system, we focused on developing a straightforward approach for synthesis of polymers capable of making reversible covalent conjugates with siRNA.

Living radical polymerizations (LRPs) provide polymers with narrow molecular weight distributions,[14–19] and in recent years have been explored to make polymers for biomedical applications.[20] Although LRP has been used to prepare cationic polymers to form non-covalent complexes with siRNA,[21] to our knowledge there have been no reports of covalent conjugates using these strategies. Herein we describe the synthesis of a thiol-reactive poly(PEG acrylate) (pPEGA) for conjugation to the 5′-thiol-modified sense strand of siRNA. The strategy is different from reported LRP strategies to make thiol-reactive polymers[22–25] and involves synthesis of a novel chain transfer agent (CTA) for reversible addition–fragmentation chain transfer (RAFT) polymerization. RAFT is a LRP technique that provides a wide range of functional polymers with defined architectures.[16–18] It is mediated by a CTA, which typically consists of a dithioester or trithiocarbonate, and the resultant polymer contains the same functionality at the chain-end. Protein-reactive R- and Z-group modified CTAs have been employed to prepare α- and ω-functional polymers, respectively, that are directly reactive toward proteins.[24,26–29] In this report we designed the CTA to have the activated disulfide R-group. This is advantageous compared to Z-group modification in that the end-group is linked to the polymer through a stable C–C bond, rather than through the dithioester or trithiocarbonate bond which is potentially susceptible to hydrolysis *in vivo*. We describe the straightforward synthesis of a pyridyl disulfide CTA, controlled polymerization of PEGA by RAFT polymerization, and efficient conjugation of the resulting polymer to siRNA.

We envisioned that by employing a CTA containing a pyridyl disulfide R-group, the polymer could be directly conjugated to siRNA containing a thiol group, since these are readily available. The final coupling step of the synthesis of the targeted CTA (**1**) is outlined in Scheme 1. The R-group contains the required activated disulfide moiety. CTA **1** was synthesized in 76% yield *via* carbodiimide-mediated coupling between pyridyl disulfide propanol[30] and 2-(ethyl trithiocarbonate)propionic acid.[31] ^{1}H NMR spectroscopy indicated formation of the desired CTA **1** (ESI, Fig. S1†). Mass spectrometry and UV–Vis analysis confirmed that the CTA had been synthesized because the expected mass was obtained and the absorbance indicative of the trithiocarbonate was observed (λ_{max} = 307 nm).

We chose to polymerize PEGA because, similar to PEG, pPEGA should facilitate passive targeting, longer circulation lifetimes, and protection of the siRNA.[32,33] To ensure a controlled polymerization of PEGA, kinetic analysis of the polymerization was performed. 2,2′-Azobisisobutyronitrile (AIBN) initiated polymerizations (Scheme 2) were initially conducted using molar ratios of 1 : 30 : 0.1 of **1** : PEGA :



Scheme 1 Synthesis of pyridyl disulfide functionalized CTA (**1**).

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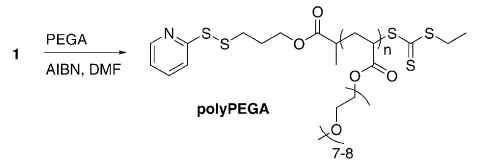

Scheme 2 RAFT polymerization of PEGA with the pyridyl disulfide CTA.

AIBN in *N*,*N*-dimethylformamide (DMF) at 70 °C. At this temperature, gel permeation chromatography (GPC) analysis indicated that the polydispersity index (PDI) of the resultant polymer approached 1.2. However, the natural logarithmic plot was not perfectly linear with respect to time, indicating that the polymerization was not controlled (ESI, Fig. S2†). To achieve control, the temperature was lowered to 60 °C. At this temperature, it was imperative that the AIBN was freshly purified, otherwise the polymerization did not proceed. It may have been alternatively possible to utilize more equivalents of AIBN. After an inhibition period of approximately 20 minutes, the natural logarithmic plot was linear with respect to time and conversions of approximately 80% were achieved (Fig. 1a). The PDI remained below 1.2 during the entire polymerization and the molecular weight of the growing polymer was linear with respect to conversion (Fig. 1b). The molecular weight *versus* percent conversion did not pass through the origin. There are a number of explanations that could be tendered for this minor anomaly including our use of non-authentic GPC standards or possible initialization phenomena in the RAFT pre-equilibrium. Taken together, these results are indicative of a controlled RAFT polymerization. The final polymer had a number average molecular weight (M_n, GPC) of 13 400 and a PDI of 1.17 (GPC traces, ESI Fig. S3†).

Retention of the pyridyl disulfide end-group is essential for conjugation to thiol bearing biomolecules. We synthesized a low molecular weight polymer in order to be able to better observe the end-group by ^{1}H NMR spectroscopy. Shown in Fig. 2 is the typical ^{1}H NMR spectrum of a pyridyl disulfide end-functional pPEGA prepared in this manner (M_n, ^{1}H NMR = 7170; M_n, theory = 5040; PDI = 1.12). Comparison of the integrations of the methylene protons adjacent to the disulfide (labeled e) with the proton *ortho* to the nitrogen (labeled a) revealed a 2 : 1 ratio, which indicated retention of the activated disulfide end-group. Further, the peak centered at 4.77 ppm (labeled l) which is the proton adjacent to the trithiocarbonate chain-end, integrated to 1. This indicated that the polymer chains with the pyridyl disulfide end-group contained the trithiocarbonate moiety at the ω chain-end, further demonstrating that the polymerization was controlled.

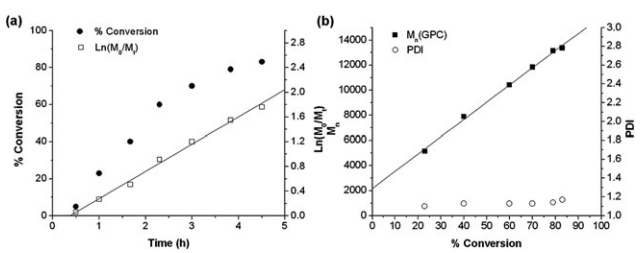

Fig. 1 RAFT polymerization of PEGA: kinetic study at 60 °C. (a) Kinetic trace and conversions determined by ^{1}H NMR spectroscopy. (b) Molecular weight and PDI determined by GPC.

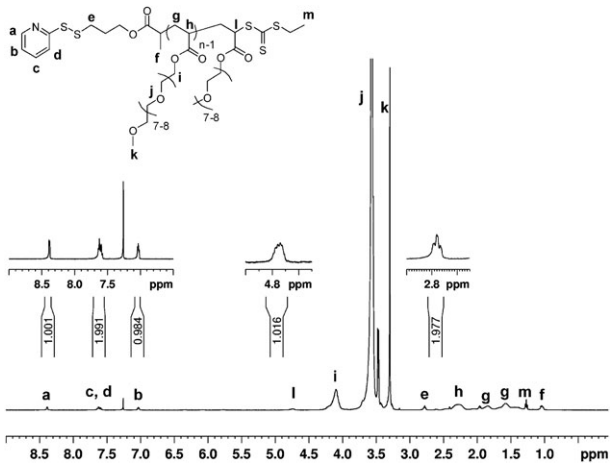

Fig. 2 ^{1}H NMR spectrum ($CDCl_3$) of pyridyl disulfide end-functional pPEGA.

Bioconjugate formation was pursued with 5′-thiol modified siRNA. As a model system, we chose siRNA with a sequence specific for mRNA of enhanced green fluorescent protein (EGFP).[34] Hybridization of the complementary strands was performed and polyacrylamide gel electrophoresis (PAGE) was used for verification (ESI, Fig. S4†). Next, the thiol protecting group on the sense strand was removed by incubation with dithiothreitol (DTT), and the deprotected siRNA was purified by precipitating in ethanol followed by mixing directly with a solution of the pPEGA (M_n, ^{1}H NMR = 6130; PDI = 1.10) in 100 mM sodium bicarbonate buffer (pH 8.5).[35] The resulting conjugates were analyzed by PAGE (Fig. 3); conjugation was evident by a shift to higher molecular weight (lanes 5 and 6) compared with the double stranded siRNA (lane 4). The conjugation was quantified by standard imaging of the gels, and the average yield of the conjugate was 88.3 ± 6.5% from five replicates. Importantly, under reducing conditions, the conjugate was no longer visible (lanes 1 and 2) and

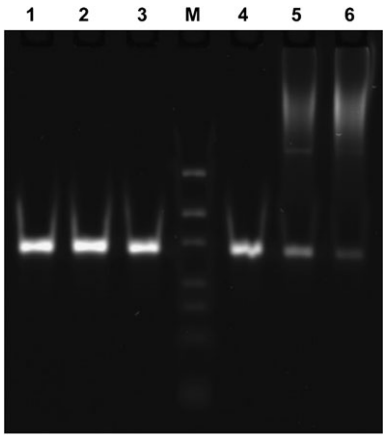

Fig. 3 Polyacrylamide gel electrophoresis of the siRNA–pPEGA conjugates. Lanes 1–3 are under reducing conditions, lanes 4–6 are under nonreducing conditions. Lanes 1 and 5: poly(PEGA)–siRNA replicate #1; lanes 2 and 6: poly(PEGA)–siRNA replicate #2; lanes 3 and 4: double stranded siRNA; M: 10/60 oligo ladder (Integrated DNA Technologies, Inc., IA).

the band looked identical to unmodified siRNA (lane 3). These results demonstrate that conjugation of the pyridyl disulfide end-functionalized polymer synthesized by RAFT to siRNA is both efficient and reversible.

We report a general and straightforward approach to synthesize polymers for conjugation to siRNA. We have shown that a CTA modified at the R-group with pyridyl disulfide was readily synthesized and effective for controlled polymerization by RAFT. Although the synthesis of pPEGA was demonstrated, a variety of polymers containing an α-thiol reactive group could be prepared utilizing this same strategy. The α-functional pyridyl disulfide pPEGA was conjugated to siRNA in high yield and the conjugation was reversible. We anticipate that this synthetic strategy will be useful as an alternative to PEGylation of siRNA for therapeutic applications, and we are currently examining the *in vitro* stability of the siRNA–polymer conjugates prepared by RAFT polymerization.

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