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How to cite:

Da Pieve, Chiara; Williams, Paul; Haddleton, David M.; Palmer, Richard M. J. and Missailidis, Sotiris (2010). Modification of Thiol Functionalized Aptamers by Conjugation of Synthetic Polymers. *Bioconjugate Chemistry*, 21(1) pp. 169–174.

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Version: Accepted Manuscript

Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.1021/bc900397s>

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Modification of thiol functionalised aptamers by conjugation of synthetic polymers

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ABSTRACT

Aptamers are known for their short *in vivo* circulating half-life and rapid renal clearance. Their conjugation to polyethylene glycol (PEG) is a way to improve their residence in the body. Two aptamers (AptD and AptF), having a disulfide protected thiol modification on the 3' end, have been conjugated to maleimide activated PEGs of various molecular weights and structures (linear PEG20, branched PEG20 and 40, PolyPEG[®] 17, 40 and 60 kDa). The high yield coupling (70-80% in most of the cases) could be achieved using immobilised *tris*[2-carboxyethyl]phosphine hydrochloride (TCEP) as reducing agent at pH 4. The affinity of PEGylated AptD for its target was reduced by conjugation to linear PEG20 and branched PEG40, but not to branched PEG20 and PolyPEG[®]s. This work demonstrates an alternative approach to PEGylation of aptamers, and that the effect of PEG on the affinity for the target varies according to the structure and conformation of the synthetic polymer.

INTRODUCTION

Aptamers are nucleic acid-based molecules (usually 8-15 kDa) that can selectively bind to almost any target such as extracellular proteins, peptides, enzymes, and small molecules. Aptamers can be selected using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method from libraries of oligonucleotides having random sequences. Moreover, they have shown low toxicity and immunogenicity. These characteristics make aptamers interesting and potentially powerful molecules for targeted therapy (1-4). Since aptamers are small and highly charged molecules, they are subject to rapid renal elimination, resulting in a short *in vivo* half-life. Conjugation to synthetic polymers such as poly(ethylene glycol) (PEG) is frequently used in order to improve the performance of biomolecules as therapeutics (5-11). The increase of the hydrodynamic volume/molecular weight of aptamers above the renal filtration cut-off, achievable by PEGylation, can overcome this potential limitation (12, 13). PEG shows low toxicity, non-immunogenicity, high solubility in water, and is commercially available in various configurations (linear, branched or comb shaped), molecular weights, and with a large variety of terminal functional groups suitable for conjugation (14-17). A few examples of PEGylated aptamers can be found in the literature and they have been prepared by coupling the activated polymer to amino-modified aptamers (18-21). In addition, comb polymers of PEG acrylate containing a pyridyl disulfide terminus have been reversibly conjugated to siRNA (22).

In this present work, a 3'-thiol-modified (disulfide protected) 25 base DNA aptamer, previously selected against the protein core of MUC1 glycoprotein (AptD) (23) has been successfully conjugated via a maleimide functional group to a range of PEG-

based polymers of varying topology and molecular weight. This includes conventional linear PEG (LinPEG20 kDa) and branched PEGs (BrPEG20 and 40 kDa), and three novel PolyPEG[®] structures (PolyPEG[®]17, 40 and 60 kDa). PolyPEG[®]s were selected as novel pegylating agents due to their “comb-like” architecture based on a poly(methacrylate) backbone with PEG “teeth” attached to it by ester bonds. These α -functional polymers are prepared by transition-metal-mediated living radical polymerisation (TMM-LRP) and have previously been shown to conjugate proteins and peptides via a range of functionalities (24-26). Recent studies have demonstrated that PolyPEG[®] is particularly effective for conjugation to peptides such as salmon calcitonin, retaining bioactivity and conferring an increased half-life *in vivo* (27). The comb shaped polymer has been shown to have interesting additional properties to linear PEGs such as reduced viscosity (16). Moreover, because the PEG “teeth” in PolyPEG[®] are attached by ester bonds to the backbone, its structure could degrade to small units that are readily excreted over time from the body, avoiding the potential toxicological problems due to renal epithelial vacuolisation on administration to animals shown by linear PEG (28, 29).

The affinity of the various PEGylated aptamer D for their target has been studied using a convenient fluorescent intercalator displacement (FID) assay to detect any influence of the polymers on the binding.

The optimised conjugation conditions were used to PEGylate a second 25 base thiol-modified DNA aptamer (AptF) previously selected for the tumor glycosylated MUC1 glycoprotein (30) in order to demonstrate that the high yield conjugation reaction is not dependent on the aptamer sequence.

EXPERIMENTAL PROCEDURES

Materials and methods. All polymerisations were carried out using standard Schlenk techniques under an inert atmosphere of nitrogen. Poly(ethylene glycol) methyl ether methacrylate (MeOPEGMA $M_n = 1100 \text{ g.mol}^{-1}$) was purchased from Aldrich (Gillingham, UK). *N*-(Ethyl)-2-pyridylmethanimine and 2-bromo-2-methyl propionic acid 2-(3,5-dioxo-10-oxa-4-azatricyclo[5.2.1.0^{2,6}]dec-8-en-4-yl) (**1**) were synthesised as previously reported (26, 31). Copper(I) bromide (Aldrich, 98%) was purified as described elsewhere (32). Hydrosart[®], stabilised cellulose, MWCO 10 kDa, filtration area 0.1 m² was purchased from Sartorius AG (Goettingen, Germany).

The sequences of AptD and AptF are respectively: 5'-GCA GTT GAT CCT TTG GAT ACC CTG G-3', and 5'-GGC GTA CGG TAG GCG GGG TCA ACT G-3'. Both aptamers have a 3'-thiol-modification protected as disulfide (-S-S-C₃H₆-OH) (IDT Integrated DNA Technologies, Leuven, Belgium). The control aptamer (AptX) is a 25 base oligo of random composition (12%A, 20%C, 32%G, and 36%T).

Linear PEG20 kDa, and branched PEG20 and 40 kDa were acquired from Nektar Therapeutics (Huntsville, AL, USA). PolyPEG[®]17, 40, and 60 kDa, were provided by Warwick Effect Polymers Limited (Coventry, UK). Immobilised *tris*[2-carboxyethyl]phosphine hydrochloride (TCEP) disulfide reducing gel was purchased from Pierce (Illinois, USA). Agarose and PAGE gels were stained with ethidium bromide and imaged on Kodak Image Station 440.

[Ru(phen)₂DPPZ](PF₆)₂ was synthesised according to literature (33). The MUC1 core peptide (APDTRPAPG) was prepared by the Oligonucleotide and Peptide Synthesis Unit, University of Nottingham. The emission measurements were recorded in the range 450-700 nm on FluoroMax-P Fluorometer (Jobin Yvon Horiba).

Synthesis of maleimide functional PolyPEG[®]s (3). In a typical polymerisation, a mixture of **1** (0.535 g, 1.49 mmol), poly(ethylene glycol) methyl ether methacrylate (50 g, 46.3 mmol) and copper(I) bromide (0.214 g, 1.49mmol) in toluene (100 mL) was deoxygenated by a series of five freeze-pump-thaw cycles. Degassed *N*-(ethyl)-2-pyridylmethanimine (0.9 mL, 6.57 mmol) was added and the resultant solution was stirred at 50°C. Samples were removed periodically for molecular weight and conversion analysis. At the end of the polymerisation, the solution was diluted with toluene (200 mL) and bubbled with air for 7 h. The solution was then filtered through a column containing basic alumina and the solvent removed under reduced pressure to yield an off-white solid which was dissolved in water and purified by dialysis using Hydrosart[®] membrane (MWCO 10 kDa). The solution was freeze-dried and the product **2** obtained as a white powder.

A solution of polymer **2** (3 g, 0.079 mmol) in toluene (30 mL) was warmed to reflux for 6 h. The solvent was then removed under reduced pressure and the off-white solid dissolved in water and purified by dialysis as above. The solution was freeze-dried and the product **3** obtained as a white powder.

Synthesis of the PEGylated conjugates. In a typical PEGylation reaction, the 3'-disulphide protected aptamer (3 nmol) in 20 µl of 18 mM ammonium formate pH 4, was added to 30 nmol of each PEG (1:5 reduced aptamer to PEG molar ratio), followed by 3 µl of immobilised *tris*[2-carboxyethyl]phosphine hydrochloride (TCEP) disulfide reducing gel. The mixture was mixed for one hour at 37°C, centrifuged, and the supernatant lyophilised.

High Performance Liquid Chromatography (HPLC) analysis and isolation of PEGylated aptamer D. The HPLC analyses and isolation of the various PEGylated conjugates of AptD are described in the supplementary information section and supplementary figures S3 and S4. A 0.02 mM aqueous solution of AptD was used as standard.

Fluorescent Intercalator Displacement (FID) assay. A 50 nM solution of either AptD, PEGylated AptD, or the control AptX in 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mM NaCl (pH 7.2) was titrated with a 0.1 mM solution of $[\text{Ru}(\text{phen})_2\text{DPPZ}](\text{PF}_6)_2$ in 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mM NaCl (pH 7.2) until saturation. The displacement of the aptamer-bound ruthenium complex was studied at the excitation wavelength of 434 nm by adding aliquots of a 20 mM solution of target peptide (MUC1 core peptide) in 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mM NaCl (pH 7.2).

RESULTS

Synthesis of maleimide functionalised PolyPEGs[®]. The maleimide functionalised PolyPEG[®]s used in this study were prepared via copper catalyzed living radical polymerisation. The protected maleimide initiator (**1**) was used for the polymerisation of (methoxyPEG₁₁₀₀) methacrylate in the presence of $\text{Cu}^{\text{I}}\text{Br}$ and an iminopyridine ligand (**31**), followed by the reintroduction of the maleimide functionality by a retro-Diels-Alder reaction (**26**) (Scheme 1). Figures S1 and S2 (in supplemental data) show the ¹H-NMR analyses of products **2** and **3**.

Scheme 1. Synthesis of α -maleimide functional PolyPEGs[®] and subsequent conjugation to an aptamer. Reagents and conditions: (a) Cu^IBr/*N*-(ethyl)-2-pyridylmethanimine/(methoxyPEG1100)methacrylate, toluene, 50°C; (b) toluene reflux, 6 h; (c) Immobilised TCEP, Ammonium formate buffer pH 4, 37°C.

Separation of un-conjugated and PEGylated aptamer D by anion-exchange HPLC. Conjugation reactions were monitored by anion-exchange chromatography, and quantified from the peak areas using a 0.02 mM aqueous solution of AptD as standard. The un-conjugated aptamer had a R_t of *ca.* 18.0 minutes while the PEGylated products eluted earlier at *ca.* 12, 13 or 14 minutes, depending of the size and structure of the PEG (Figures S3 and S4 in supplemental data). The conjugation yields were determined by absorbance at 260 nm, after confirming that the PEG moieties did not contribute to the absorption of the conjugates by comparing UV-Vis calibration lines of the aptamer and the PEGs at this wavelength (data not shown). AptD-PolyPEG[®]40 and AptD-PolyPEG[®]60 did not elute consistently from the column under standard conditions, which was most probably due to their structure.

Therefore their conjugation yields were quantified by imaging of the agarose gel. Different HPLC conditions (eluent and gradient) for these conjugates need to be further investigated.

Optimising the reaction conditions for a high yield PEGylation. All PEGylations were performed using immobilised TCEP gel as reducing agent at pH 4. Very low conjugation was achieved using other reagents such as dithiotreitol (DTT) and non-immobilised TCEP. Preliminary aptamer-PEG conjugation reactions were performed at two different temperatures (25 and 37°C). Anion-exchange HPLC analyses showed that an increase of the conjugation yield was achieved at 37°C (Figure 1). Subsequently, the effect of molar ratio of aptamer to PEG on conjugation was studied at 37°C (Figure 1).

Figure 1. Conjugation yields of AptD with LinPEG20 kDa at different temperatures (left), and reduced aptamer to PEG molar ratios (right).

Optimal conjugation yields were achieved when the reaction was performed at 37°C for 1 hour using a aptamer:PEG molar ratio of 1:5 (reduced aptamer:PEG). A kinetic

study of the conjugation of the thiol-modified aptamer to other polymers (branched PEG20 and 40 kDa, PolyPEG[®]17, 40 and 60 kDa) using these optimised conditions also produced the highest yields of conjugate (>78%) after one hour (Figure 2). The extension of the reaction time to 2 hours did not improve the PEGylation yield further.

Figure 2. Kinetic study of the conjugation of AptD to various PEGs at 37 °C, and 1:5 molar ratio (reduced aptamer:PEG).

Identification of conjugates by gel electrophoresis. The PEGylated aptamers (AptD-LinPEG20, AptD-PolyPEG[®]17, AptD-BrPEG20, AptD-PolyPEG[®]40, AptD-BrPEG40, and AptD-PolyPEG[®]60) were isolated by preparative anionic-exchange HPLC and analysed by agarose gel electrophoresis for the PolyPEG[®] derivatives, and native polyacrylamide gel electrophoresis (PAGE) for the linear and branched PEGs. The use of different gel matrices was due to the poor migration of the branched PEG 40 kDa conjugate in agarose gel. Un-conjugated aptamer and PEGs were used as controls. The shift of the aptamer to higher molecular weights confirmed its successful conjugation (Figure 3).

Figure 3. Agarose (2%) (left) and native PAGE (7%) gel (right) of the AptD-PEG conjugates. Lines L: 25 base pair ladder; 1 and 8: AptD; 2: AptD-PolyPEG[®]17; 3: free PolyPEG[®]17; 4: AptD-PolyPEG[®]40; 5: free PolyPEG[®]40; 6: AptD-PolyPEG[®]60; 7: free PolyPEG[®]60; 9: AptD-BrPEG40; 10: free BrPEG40; 11: AptD-LinPEG20; 12: free LinPEG20; 13: AptD-BrPEG20; 14: free BrPEG20. The running buffer was 1xTBE (Tris-Borate-EDTA).

Effect of conjugation on AptD affinity for its target peptide. $[\text{Ru}(\text{phen})_2\text{DPPZ}]^{2+}$ shows a strong photoluminescence only when in contact with DNA (double or single stranded) (34, 35). The FID assay studies the decrease in fluorescence due to the displacement of the ruthenium complex bound to the aptamer by adding the target peptide. The percent decrease in fluorescence and the extent of binding of the target to the aptamer are directly related. A control 25 base DNA aptamer (AptX), having unspecific binding affinity for the same peptide, and the un-modified aptamer D were used as controls. All compounds were dissolved in the same solution previously used for the selection of the aptamer (23). The data shows that the displacement of the ruthenium complex from AptD, either un-modified or PEGylated, by the target

peptide is cooperative (Figure 4). The midpoints and cooperativity values are summarised in Table 1. The data shows that the various conjugates have different influences on binding, depending on the molecular weight and structure of the PEG component.

Figure 4. FID binding curves of the target peptide against AptD-PEG conjugates, AptD and control aptamer. Displacement of the aptamer-bound ruthenium complex was studied at an excitation wavelength of 434 nm.

Conjugate	Midpoint [mol. equiv. of target]	Cooperativity [n]
Un-modified AptD	1140.50	3.92
AptD-LinPEG20	1420.07	4.80
AptD-BrPEG20	1100.80	4.62
AptD-BrPEG40	1375.76	4.22
AptD-PolyPEG [®] 17	1185.02	3.57
AptD-PolyPEG [®] 40	1051.30	3.01
AptD-PolyPEG [®] 60	1150.00	3.20

Table 1. Midpoints and cooperativity values of AptD and PEGylated AptD obtained from the FID studies. The calculations were done using the Hill equation: $y = y_{\infty} + (y_0 - y_{\infty})/[1 + (x/K)^n]$ (42), where y is the spectroscopic response, x is the concentration of

the added reagent, y_0 is the response when $x = 0$, y_∞ is the maximal response when $x = \infty$, K is the concentration of x at the midpoint, and n is the degree of cooperativity.

The midpoint values of AptD-LinPEG20 and AptD-BrPEG40 are higher compared to the un-conjugated aptamer, whereas those for AptD-BrPEG20 and all of the AptD-PolyPEG[®]s are similar to the un-conjugated aptamer. In addition, the cooperativity of the PEG20 and BrPEG40 conjugated aptamer were increased, whereas those for BrPEG20 and all PolyPEG[®]s were similar to that for the un-modified aptamer.

DISCUSSION

The conjugation of high molecular weight polymers, such as PEG, to small biomolecules has been shown to prolong their circulating half-life *in vivo* by preventing rapid renal clearance. So far, the only examples of PEGylated aptamers in the literature have been prepared by coupling the activated polymer to an amino modification on the aptamer. Therefore, the aim of our work is to propose an alternative way to PEGylate aptamers *via* a maleimide-thiol reaction. Additionally, new comb shaped PEGs (PolyPEG[®]s) were synthesised and then conjugated to the aptamer as for the conventional and commercially available PEGs.

PolyPEG[®] is a comb shaped molecule with a polymethacrylate backbone and pendant PEG teeth formed by polymerisation of commercially available PEG methacrylate monomers. This is prepared by living radical polymerisation (36-38) which allows the incorporation of specific functionality on the chain end which can be subsequently used for conjugation reactions (26, 39, 40). As expected for living polymerisations, the reaction exhibited a linear kinetic with respect to monomer concentration and the polymers had narrow molecular weight distributions. Monomer conversions were

calculated via ^1H NMR spectroscopy by monitoring the decrease of the integrals of the monomer vinyl signals (6.2 and 5.6 ppm) using the sharp peak at *ca.* 3.4 ppm (MeO end group) as the internal standard. The molecular weight of the polymer was calculated by comparing the relative integrals of the vinyl signal at 6.5 ppm and the MeO groups of the PEG branches (3.35 ppm) (Figure 5)

Figure 5. Typical first-order kinetic plot for the polymerisation of a protected maleimide functional PolyPEG[®] (PolyPEG40) (**2**)

The optimised conjugation conditions (reduced aptamer:PEG ratio of 1:5, for 1 hour at 37°C) were used to PEGylate a second thiol-modified DNA aptamer (AptF). The resulting conjugates were analysed by gel electrophoresis (Figure 6). The conjugation yields, quantified by anion exchange HPLC using a 0.02 mM aqueous solution of AptF as standard and confirmed by band intensity on the gels, were >75%.

Figure 6. Agarose (left) and native PAGE (right) gels of the AptF-PEG conjugates. Lines L: 25 base pair ladder; 1 and 8: AptF; 2: AptF-PolyPEG[®]17; 3: free PolyPEG[®]17; 4: AptF-PolyPEG[®]40; 5: free PolyPEG[®]40; 6: AptF-PolyPEG[®]60; 7: free PolyPEG[®]60; 9: AptF-LinPEG20; 10: free LinPEG20free; 11: AptF-BrPEG20; 12: free BrPEG20; 13: AptF-BrPEG40; 14: free BrPEG40.

Post-selection modifications on an aptamer can alter its original structure and overall charge and thus its binding affinity for the target. A convenient fluorescent intercalator displacement (FID) assay using $[\text{Ru}(\text{phen})_2\text{DPPZ}]^{2+}$ was performed to test the retention of affinity for its target of the isolated AptD-PEG conjugates (33-35, 41). Two parameters have been considered for the evaluation of the aptamer-peptide binding affinity: the concentration of peptide needed to displace 50% of the metal (midpoint), and the alteration in binding of a peptide molecule to the aptamer due to the prior binding of another peptide molecule to the same aptamer. The binding can be enhanced (positive cooperativity) or decreased (negative cooperativity). All FID plots in Figure 4 are sigmoid, showing a positive cooperative displacement of the Ru^{2+}

moiety from the aptamer (either un-modified or PEGylated) by the addition of the target. Branched PEG40 kDa and linear PEG20 kDa clearly interfere with the binding of the aptamer to its target decreasing the affinity. Compared to the un-modified aptamer, higher quantities of peptide are needed to displace 50% of the bound Ru^{2+} (higher midpoints) despite the greater effect on the displacement shown by the increase of the target concentration (higher cooperativities). The binding is, however, not completely compromised for the BrPEG40 conjugate as the maximum effect is not reduced. In contrast, AptD-BrPEG20 shows a lower midpoint value, most possibly due to the higher cooperativity. The three aptamer-PolyPEG[®] conjugates display similar binding affinities for the target, which were alike to that of the un-modified aptamer. In each case, although the PolyPEG[®] products show a slight decrease in cooperativity, the midpoints are reached at very similar molar equivalents of target peptide to that required for the un-modified AptD used as a positive control. As expected, the 25 base aptamer having unspecific binding affinity (control AptX) showed only a 33.5% decrease in fluorescence after the addition of more than 3 times the molar equivalent of peptide necessary to reach the midpoint for the un-modified AptD.

In summary, we report an efficient, high yielding method for the PEGylation of two thiol-modified aptamers using a range of PEG-based polymers of differing structure and molecular weight. This procedure can be considered as an alternative to the most common approach of using PEG active esters and amino modified aptamers. We have shown that PEGylation has a distinct influence (exemplified by a variation of midpoints and cooperativity values) upon the binding affinity of AptD for its target. Reduced binding affinity was only apparent for the AptD-LinPEG20 and AptD-BrPEG40 conjugates. This suggests that it is the physical size and conformation of the

20 kDa PEG (random coil) and 40 kDa (2x20 kDa) branched PEG that causes the reduction in affinity, since it is not observed with either branched 20 kDa (2x10 kDa) PEG and even larger molecular mass PolyPEG polymers, which are 'brush shaped'. Further *in vivo* studies of the pharmacokinetic and biodistribution properties of the aptamer-PEG conjugates will be undertaken.

ACKNOWLEDGEMENTS

The work was supported by the Breast Cancer Campaign [2005May11]. The authors thank Warwick Effect Polymers Ltd, Graham Jeffs, Huma Kahn, Sergey Bylikin, and Dave Lambert for their help and assistance.

Supporting Information Available: ^1H NMR spectra of compounds **2** and **3**, and HPLC traces of AptD- LinPEG20, ApD-BrPEG20 ApD-BrPEG40, and ApD-PolyPEG17. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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