Comparison of metal free polymer-dye conjugation strategies in protic solvents

Jens Gaitzsch,*[,1,2] Maarten Delahaye,[3] Alessandro Poma,[1] Filip Du Prez[3] and Giuseppe Battaglia[1]

[1] Department of Chemistry, University College London, London WC1H 0AJ, United Kingdom

[2] Department of Chemistry, University of Basel, 4056 Basel, Switzerland

[3] Department of Organic and Macromolecular Chemistry, Polymer Chemistry Research Group, Ghent University, Krijgslaan 281 S4, B-9000 Ghent, Belgium

Corresponding author: jens.gaitzsch@unibas.ch

Abstract

Polymer-dye conjugations are one of the common examples for polymer modifications. They represent a crucial step for imaging in optical microscopy or for tracing and marking macromolecules. In this work, we present a comparison between quick and efficient conjugation reactions that do not rely on the use of a metal catalyst, which could contaminate the resulting polymer and hinder its use for subsequent biological applications. Moreover, since the conjugation strategy needs to be compatible with the solvent system used to solubilise the polymer, we focussed our attention on assessing these conjugation approaches for their feasibility with a sample polymer that is only soluble in protic solvents. The methods studied include the ring-strain promoted azide-alkyne click reaction, thiol/maleimide conjugation as well as the 1,2,4-triazoline-3,5-dione (TAD) with a diene moiety, one of the latest click chemistries available.

Introduction

The chemical structure of a polymer backbone, either as single polymer or in bulk, is often not suitable to be used in practical applications. May it be for molecular recognition, imaging or tracing, polymers often need to be conjugated with another substance. The latter may belong to a large variety of molecules, with proteins and dyes being two of the most prominent examples.[1-5]

The conjugation process can be performed before or after the actual synthesis of the polymer. Conjugation before the polymer synthesis is usually achieved by preparing a modified initiator molecule. This bares the advantage that such a species can be purified easily by column chromatography, eventually leading to a pure compound. A necessary precondition of this conjugation approach, however, would be that the conjugate does not interfere with the polymerisation process.

There is, on the other hand, also the alternative conjugation strategy, i.e. when the polymer is conjugated after the polymerisation process. It is usually conducted by exploiting an anchoring group with an orthogonal reactivity to the polymerisation reaction. With this approach it is of paramount importance to deploy highly efficient reactions, because it is in most cases extremely difficult to separate a non-conjugated polymer from a conjugated one.

There are a variety of conjugation methods available,[6] including the copper catalysed azide-alkyne click reaction (CuAAC),[4, 7-9] the thiol-ene reaction[10-12] and the amine/NHS ester reaction (especially for proteins) amongst a few others.[10, 13-16] One of the latest reported example of an efficient polymer analogous reaction is the Diels-Alder reaction of a 1,2,4-triazoline-3,5-dione (TAD) with a diene, which is currently the fastest click reaction available.[17, 18] All these conjugation strategies have been proven successful in a variety of

examples.[6] Nonetheless, each of these methods also has drawbacks and may or may not be compliant with the needs of a specific research approach.

The CuAAC reaction, for example, involves the use of copper as a catalyst, which represents an issue for biological applications.[4, 19] In this instance, the ring-strain promoted azide-alkyne reaction (SPAAC) becomes of interest as it is additive-free and reported to occur almost instantaneously.[19, 20] The ring strain is present in the alkyne, which is integrated in a [6,1,0] bicyclic nonyne (Figure 1). Due to its high ring tension, however, this moiety can also react with other functional groups, thus resulting in lack of a stability and selectivity, ultimately limiting the scope of SPAAC.[21-23]

Thiol-ene based reactions often require an additive to prevent the (re)formation of the disulphide bond, but the phosphines typically used for this purpose are not reported to be toxic. Thiols, however, are neither always easily incorporated into polymers nor into small molecules. Moreover, thiol-maleimide conjugates are known to lack stability *in vivo*.[24, 25]

These examples suggest that each conjugation strategy exhibits specific limiting factors, and that an optimal conjugation reaction is likely to differ from one polymer system to another.

Amongst the various types of polymeric materials, there is a particular interest in pH-sensitive amphiphilic block copolymers because of their importance for drug delivery applications.[26-28] An important system in this respect are polymersomes, which are vesicular self-assembly structures from amphiphilic block copolymers.[27, 29, 30] We often reported on poly(methacrylic phosphoryl choline)-poly(diisopropylaminoethylmethacrylate) ($PMPC_{25}$-$PDPA_{70}$, 23 kg/mol – referred to as PMPC-PDPA from now on). Our research focus on this particular polymeric material derives from its potential for biological applications. PMPC-PDPA is indeed

biocompatible, pH-sensitive, recognised by cell receptors and is able to self-assemble into polymersomes suitable for drug-delivery.

Nevertheless, if these nanostructures are meant to be employed for biological applications, they need the capability to be imaged within a biological environment. It is, for example, necessary to monitor their cellular uptake and follow their pathway across tissue barriers.[31, 32] As the unmodified polymer itself and thus also the polymersomes produced with it are not detectable via optical imaging, conjugating the polymers with dyes or other reporter molecules is of vital importance for biological applications.[29, 33] Conjugation reactions of PMPC-PDPA, however, are not straightforward, because this polymer is only soluble in protic solvents such as chloroform/methanol, hexafluoroisopropanol (HFIP) or acidic water.[34, 35]

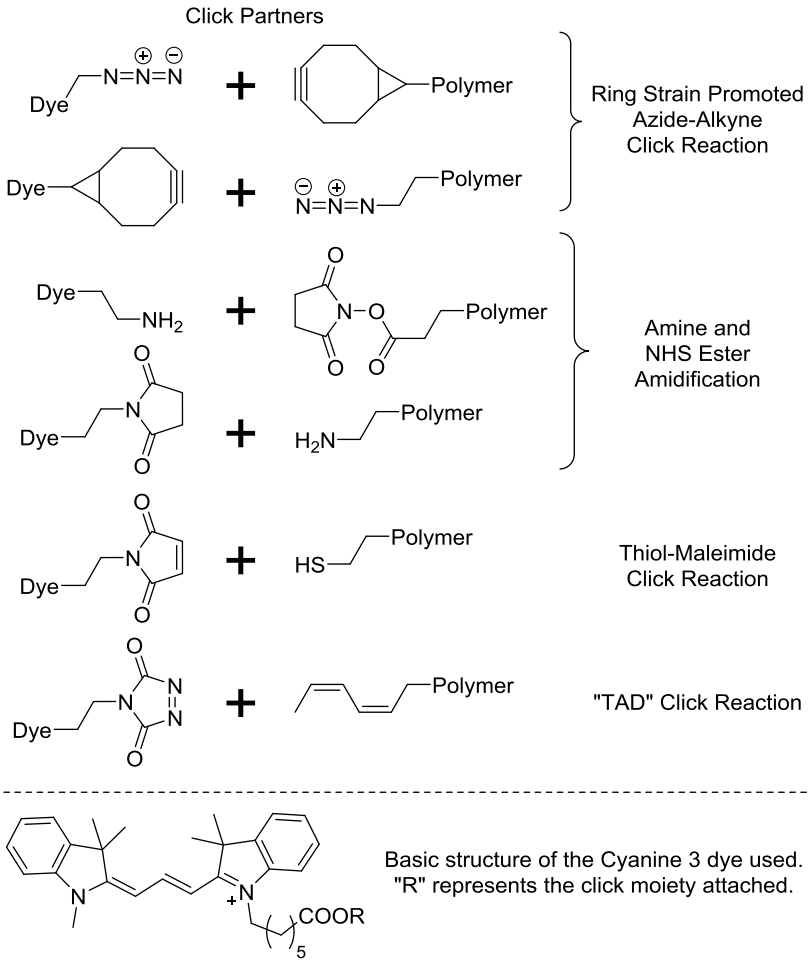


Figure 1: Overview on the investigated conjugation strategies between dyes and polymer chains and the structure of the Cyanine-3 dye used for all conjugations but the TAD ligation. These include the azide-alkyne click reaction, amine-NHS ester linkage, thiol-maleimide reaction and TAD chemistry.

In this context, we have conducted a systematic study on an array of conjugation methods to investigate which ones are applicable for working in an additive-free manner on PMPC-PDPA. This means specifically that the conjugation has to be conducted in a protic solvent and should not interfere with the atom transfer radical polymerisation (ATRP) used to produce the polymer. We tested the SPAAC reaction, the amine-NHS ester conjugation, the thiol-maleimide click reaction as well as TAD chemistry (Figure 1). Our results will highlight the advantages and disadvantages of each system with regard to the PMPC-PDPA polymer system. It is also important to note that the conclusions derived from this study can be well

extended to the other polymeric materials that exhibit comparable physico-chemical properties.

Materials and Methods

Materials

2-(Methacryloyloxy)ethyl phosphorylcholine monomer (MPC, 99.9% purity) was donated by Biocompatibles U.K. Ltd. Diisopropylaminoethyl methacrylate (DPA), sorbic alcohol, potassium carbonate, ethyl acetate (EtOAc), trifloroacetic acid (TFA), alpha-bromo-isobutyryl bromide, N-hydroxy succinimide, bis[2-(2'-bromoisobutyryloxy)ethyl]disulfide] (98 %), silica gel 0.2 - 0.5mm 30-70 mesh chromatograph, copper(I) bromide (Cu(I)Br, 99.999%), 2,2'-bipyridine (bpy, 99%), anhydrous tetrahydrofuran, chloroform, poly(ethylene glycol) azide (Mn = 1500), ethyl-4-bromobutyrate (95 %), ethyl carbazate (97 %), *n*-hexane (≥ 97 %), hydrochloric acid in dioxane (4M) were purchased from Sigma Aldrich UK. Ethanol and methanol (Normapur) were purchased from Merck KG (Darmstadt, Germany). HPLC grade dichloromethane, sodium chloride and magnesium sulphate were purchased from Fisher Scientific (Loughborough, UK). Cy-3 alkyne, Cy3-azide, Cy-3 maleimide and Cy-3 amine (Cy = Cyanine) were purchased from Lumiprobe (Hannover, Germany, structure see Fig. 1 and SI). Both bicyclic [6,1,0] cyclic nonynes (alcohol and amine derivative, see main paper) were purchased from Synaffix (AC Oss, Netherlands). BocN(H)-PEG-OH was purchased from Iris Biotech (Marktredwitz, Germany). Diphenyl phosphoryl azide (≥ 96 %) and Sudan II (≥ 90 %) were purchased from TCI Europe. *N*,*N*-dimethylformamide (99.8 %) and triethylamine (99 %) were purchased at Acros Organics (Loughborough, UK). Concentrated hydrochloric acid (36 %) was bought from Chem-lab and potassium carbonate (≥ 99 %) was purchased from Carl

Roth (Karlsruhe, Germany). All of the above were used as received. Semi-permeable cellulose dialysis tubing (Spectra/Por 6 MWCO 1,000) was purchased from Spectrum Labs (Breda, Netherlands). Toluene (Sigma-Aldrich, 99.9%) was distilled over $CaCl_2$. Diaminobicyclo[2,2,2]octanylbromid (DABCO-Br) was synthesised as described previously.[17]

Methods

NMR was performed on an Avance III 600 spectrometer and an Avance 300 spectrometer from Bruker (Billerica, USA). HPLC was performed on a Dionex Ultimate 3000 chromatography system, equipped with a UV and fluorescence detector and methanol with 0.05 vol% TFA and water with 0.05 vol% TFA (95% water to 20% water, gradient elution) using a Dionex C18-15cm column, including guard column (Fisher Scientific, UK). GPC was performed on a GPCMax equipped with an RI detector from Malvern Technologies (Greater Malvern, UK) with acidic water (0.25 vol% TFA in water) as solvent on a Novamax column, incl. guard column from PSS Polymers (Mainz, Germany). Electrospray mass spectra (ESI-MS) were recorded with a single quad MS detector (VL Detector) with electrospray ionisation (complete system from Agilent Technologies, US).

General method for initiator synthesis: We adopted a method that was published earlier.[36] Here, 2 mmol of the corresponding alcohol is dissolved in 20 mol THF in a round-bottom flask (PEG-OH being dried in vacuum for 30 min). An amount of 1,21 g (5 mmol) 2-bromoisobutyric acid bromide was then dissolved in 1.5 ml dry THF and subsequently added to the solution. The flask is now cooled with ice and 0,38 g (3,75 mmol) dry triethylamine is added. The gloomy mixture is stirred for 2 h at 40°C. The reaction mixture is then poured into an excess of water and extracted three times with EtOAc. The combined organic layers are dried with magnesium

sulphate, filtered to remove the drying agent and concentrated *in vacuo*. The crude mixture is finally purified using flash chromatography with a hexane/ethyl acetate mixture (3:1 vol/vol with gradient towards 1:1 vol/vol) to give the pure products.
For PEG-Br: Similar procedure, but the mixture is left stirring for 40 h at RT, the reaction is quenched with trifluoroacetic acid and the product is precipitated in hexane before being dialysed three times against methanol - finally the solvent is removed *in vacuo*.

Initiator from bicyclic nonyne: NMR (ppm, ^{1}H, 600 MHz): 0.94 (2H, CH, m), 1.53 ppm (3H, CH/CH_2, m), 1.88 (6H, CH_3, s), 2.21 ppm (6H, CH_2, m), 4.20 ppm (2H, CH_2, d, *j* = 8.3 Hz)

Initiator with N_3 moiety: NMR (ppm, ^{1}H, 600 MHz): 1.93 (6H, CH_3, s), 3:42 (2H, CH_2, d, *j = 16.6 Hz*), 3:82 (2H, CH_2, d, *j = 16.2 Hz*)

Initiator from N-hydroxy succinimide: NMR (ppm, ^{1}H, 600 MHz): 2.08 (6H, CH_3, s), 2.87 ppm (4H, CH_2, s)

NH_2-PEG(113)-Br Initiator (ppm, ^{1}H, 600 MHz): 1.91 (6H, CH_3, s), 3.61 (452 H, CH_2, s)

Initiator from sorbic alcohol: NMR (ppm, ^{1}H, 600 MHz): 1.75 (3H, CH_3, d, *j* = 6.8 Hz), 1.91 (6H, CH_3, s), 4.65 (2H, CH_2, d, *j* = 6.6 Hz), 5.62 (1H, CH, m), 5.74 (1H, CH, m), 6.04 (1H, CH, dd, j_1 = 14.3 Hz, j_2 = 11.3 Hz), 6.28 (1H, CH, dd, j_1 = 15.2 Hz, j_2 = 10.4 Hz),

General method for ATRP procedure: POEGMA-PDPA, PMPC-PDPA and PEG-PDPA were synthesised using standard literature procedures for ATRP described previously [31, 37] using the initiators synthesised as described above or commercially acquired (disulphide initiator) for this project. We refer to the supporting information (SI) for synthetic and characterisation details of all polymers.

General method for ligations: In a typical experiment, equimolar amounts of the polymer (predissolved in chloroform/methanol 3:1 vol/vol (2 mg/ml)), except for conjugating H_2N-PEG-PDPA where THF was used) and the corresponding modified dye (0.9 equivalents) were stirred for 5min (TAD) to 24hrs (other conjugations) before the mixtures were analysed by HPLC. For the thiol-maleimide coupling, the solvents were purged with N_2 gas and 200 mol-% of triphenylphosphine were also added to the solution at the beginning of the reaction. The polymers were then purified by filtration through silica gel, and dialysis (MWCO 1kDa) against ethanol (2x) and water (2x) before being freeze-dried.

Synthesis of TAD-modified Sudan-Red II (see scheme 1 - reactions 1-3 in SI, key reactions 4 and 5 shown here).

*Synthesis of Sudan-urazole (**6**), using a reported method.* [38, 39]

A suspension of Sudan-semicarbazide (**5**, 0.859 g, 1.85 mmol, 1 eq) and K_2CO_3 (1.02 g, 7.42 mmol, 4 eq) in 20mL ethanol was placed under an inert atmosphere and stirred at 65 °C for 24 hours. The excess of base was filtered off and the filtrate was acidified with HCl (4M solution in dioxane) to a pH of 4. The precipitate was filtered off and the filtrate was concentrated *in vacuo* to give Sudan-urazole (**6**, 0.489 g, 1.17 mmol, 63 %).

Brutoformula: $C_{23}H_{23}N_5O_3$. MW.: 417.47 g/mol. ESI-MS (m/z): 418.2 $[MH]^+$.

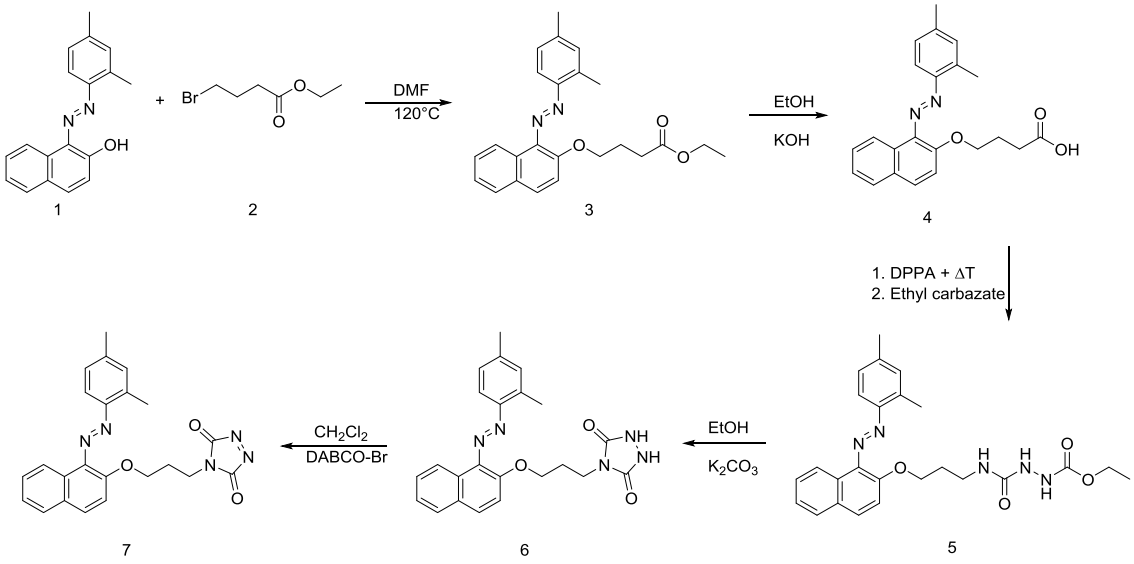
^{1}H-NMR (300 MHz, DMSO d_6): (ppm) = 2.00 (quint, 2 H, CH2-*CH2*-CH2), 2.38 (s, 3 H, Ar-CH3), 2.64 (s, 3 H, Ar-CH3), 3.54 (t, 2 H, CH2-N), 4.21 (t, 2 H, Ar-O-CH2), 7.17 (d, 1 H, Ar-H), 7.27 (s, 1 H, Ar-H), 7.46 (m, 1 H, Ar-H), 7.57 (m, 3 H, Ar-H), 7.95 (d, 1 H, Ar-), 8.01 (d, 1 H, Ar-H), 8.33 (d, 1 H, Ar-H), 10.07 (s, 2 H, N-H).

*Synthesis of Sudan-triazolinedione (**7**)*

A suspension of 50.0mg Sudan-urazole (**6**, 0.120 mmol, 1 eq) and 37.7mg 1,4-diazabicyclo[2.2.2]octane bromide complex (DABCO-Br, 0.0240 mmol, 0.2 eq) was stirred at room temperature for 4 hours. The mixture was filtered off to remove the heterogeneous oxidant and the filtrate was concentrated *in vacuo* to give Sudan-triazolinedione.

(**7**, 15.9 mg, 0.0383 mmol, 31 %).

Brutoformula: C23H21N5O3. MW.: 415.45 g/mol (calculated). 1H-NMR (300 MHz, DMSO d_6): (ppm) = 2.07 (m, 2 H, CH2-*CH2*-CH2), 2.37 (s, 3 H, Ar-CH3), 2.65 (s, 3 H, Ar-CH3), 3.68 (t, 2 H, CH2-N), 4.26 (t, 2 H, Ar-O-*CH2*), 7.17 (d, 1 H, Ar-H), 7.27 (s, 1 H, Ar-H), 7.45 (m, 1 H, Ar-H), 7.57 (m, 3 H, Ar-H), 7.95 (d, 1 H, Ar-H), 8.02 (d, 1 H, Ar-H), 8.33 (d, 1 H, Ar-H)



Scheme 1: Overview of the reaction scheme for the synthesis of Sudan-triazolinedione.

Results and Discussion

1. Ring-strain promoted azide-alkyne click reaction

The sample dye for all the conjugations approaches tested was Cyanine-3 (Cy-3, see Figure 1 and SI) with its required modifications to suit the needs of the specific reactions.

For the ring-strain promoted azide-alkyne click reaction (SPAAC), the bicyclic cyclononyne needed to be attached either to the polymer or to the dye. An alcohol derivative of the cyclononyne moiety is commercially available and can be easily converted into an ATRP initiator. At this point, two alternative strategies have been pursued, namely conjugating the dye before or after the polymerisation.

Performing the conjugation before the polymerisation proved to be a very efficient reaction, which is in line with reports in literature.[19, 20] The azide-containing dye reacted instantaneously and in a quantitative manner with both the unmodified alcohol-containing bicyclic nonyne as well as with the one modified with the ATRP-initiating moiety (Figure 2a). The HPLC traces for both reactions show no remaining free dye, indicating that the esterification of the alcohol with the ATRP initiator left the triple bond of the cyclononyne unimpaired.

The Cy-3 modified initiator was then used to grow the modified PMPC-PDPA directly from the dye. The GPC trace of the final polymer, however, revealed severe problems with this specific approach as it exhibited a multimodal distribution with at least 3 distinguishable peaks (Figure 2a). Considering that the ^{1}H-NMR spectrum of the non-conjugated initiator showed a pure compound, any disturbance of the polymerisation seems to have originated from the dye. Indeed, as Cy-3 contains a variety of double bonds, it could easily interfere with a radical polymerisation process. Initially, we hypothesised that the concentration of monomer was high enough to prevent such hindrance. In light of the discovered multimodal distribution, however, the level of interference is too high to achieve a clean dye-labelled polymer with this specific SPAAC approach.

We then tried to polymerise the PMPC-PDPA onto the modified cyclononyne initiator before conjugation with Cy-3. The GPC trace of the product corresponded in this case to a clean polymer with a narrow and monomodal distribution (dispersity of 1.25), which could be used for further experiments (Figure 2b). Judging from this GPC trace, the triple bond did not interfere with the polymerisation since no shoulder to either side was visible in the chromatogram. Nonetheless, to our surprise, even after 24 hours no successful conjugation could be observed when this polymer was reacted with the azide-modified Cy-3 dye (Figure 2b). The HPLC trace still showed the unreacted dye and no sign of a modified polymer. These results indicate that the reaction does not occur on the polymer although the initiator reacted instantaneously with the dye as described above. Since the GPC trace of the polymer exhibits a monomodal distribution and the molar mass is as expected, the triple bond did not prevent a successful ATRP reaction to form PMPC-PDPA. However, it must have reacted during the polymerisation since no modified polymer could be obtained. This could not be verified by NMR spectroscopy, due to overlapping signals with the polymer and low intensity of the signal from the end group in comparison to the signals of the polymer. From those experiments, we concluded that SPAAC is not a suitable platform for a successful conjugation in the case investigated, neither before nor after polymerisation.

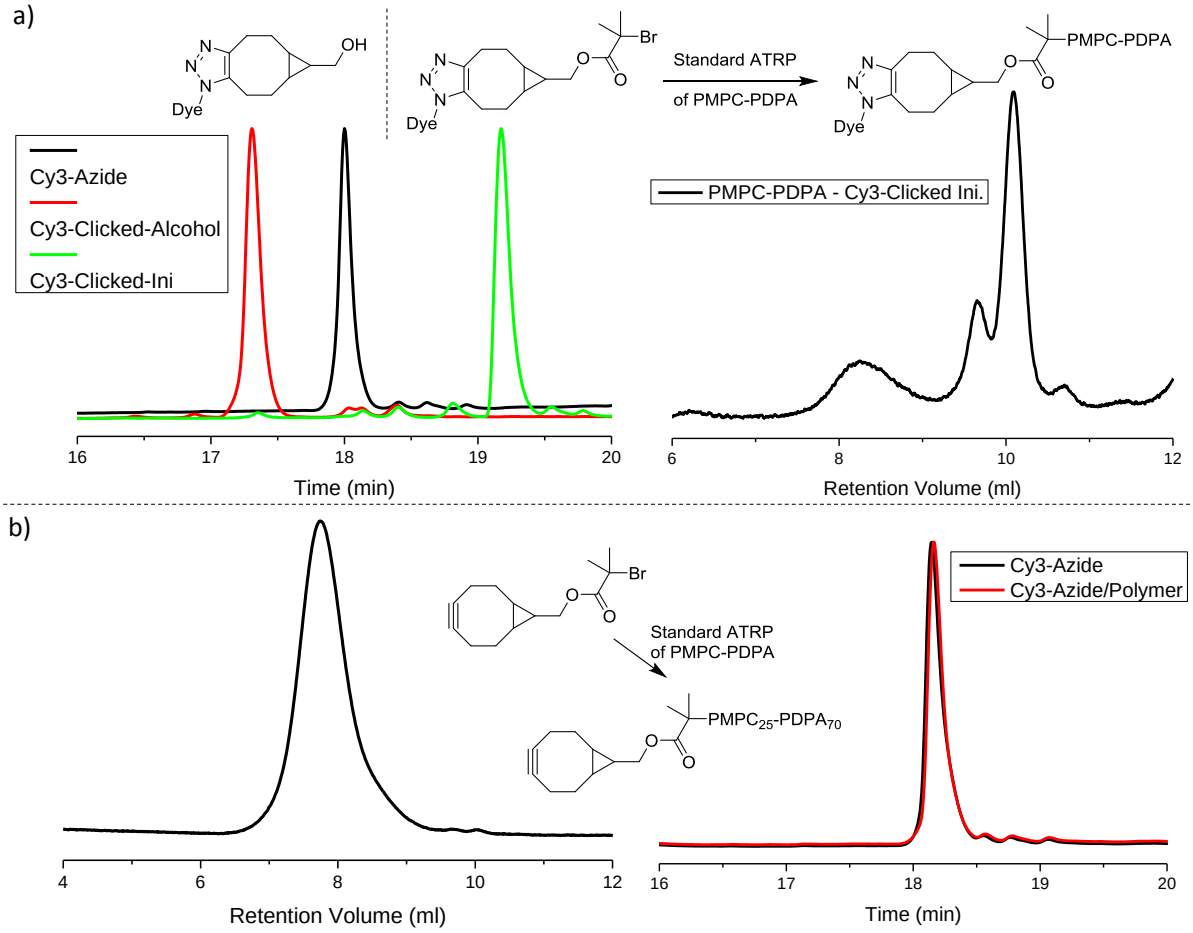


Figure 2: a) HPLC trace of the pure Cy-3 azide (black) and the corresponding reaction products (red and green), together with GPC trace of the corresponding polymer. b) If the dye is not attached beforehand, a clean GPC trace is reached (left), but no conjugation could be achieved (HPLC with overlapping traces, right).

The click reaction can also be performed with reversed functionalities, i.e. with the azide moiety on the polymer and the alkyne group on the dye (as it was shown for other polymers previously).[9] The preparation of an ATRP initiator from an azide alcohol was achieved by a standard esterification and the following polymerisation proceeded without any notable problems (Figure 3). Since the bicyclic nonyne is not readily available with the sample-dye (Cy-3) used for this study, it was conjugated in a separate reaction. Specifically, we applied a method often used in peptide chemistry, i.e. reacting an amine (here on the alkyne portion of the molecule) with an NHS ester. Our choice was driven by the fact that NHS-ester derivatives are commercially available for a vast amount of functional molecules (including

dyes and peptides), allowing us to draw more general conclusions from the obtained results. The HPLC trace showed a high conversion towards the modified amide dye, which was subsequently exploited in the conjugation attempt (Figure 3).

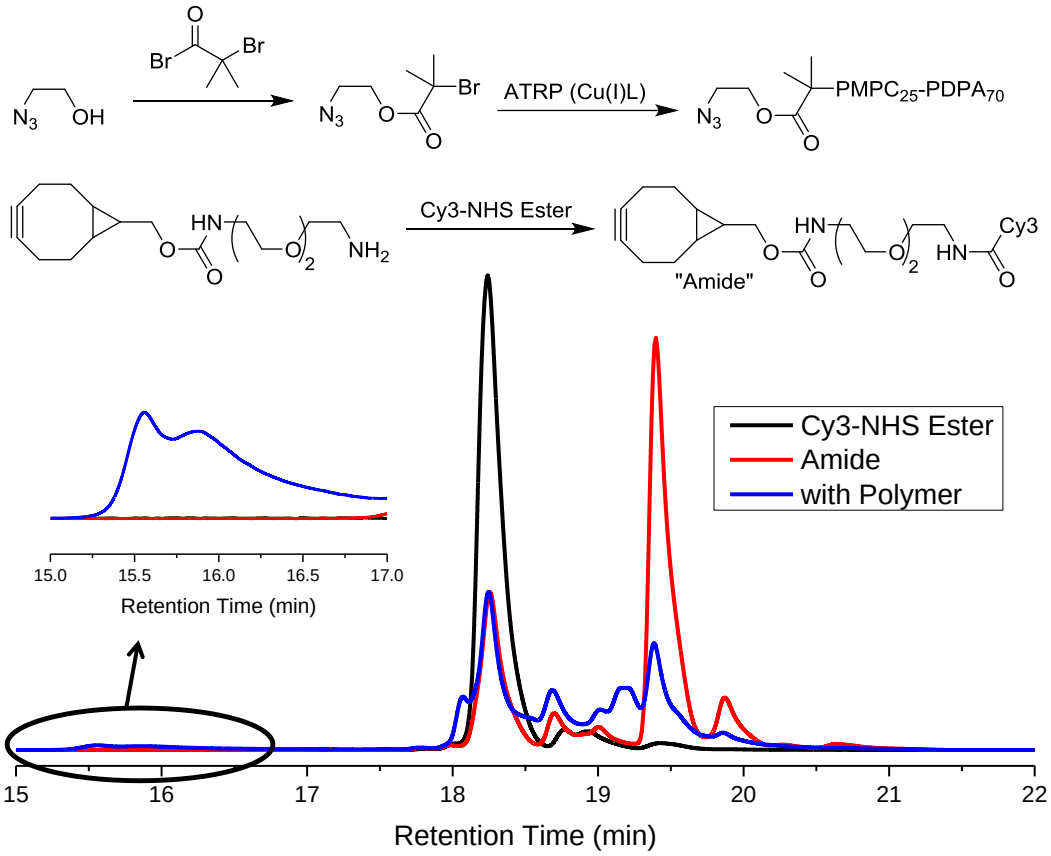


Figure 3: Reactions and HPLC trace for the reaction cascade involving the azide in the polymer and the bicyclic nonyne on the dye (pure dye – black, conjugated version in red, attempted polymer conjugate in blue). Only a small amount of modified polymer was formed (very small peak at 15-16 min).

Theoretically, the azide-containing polymer should have reacted quantitatively with all the bicyclic nonyne-modified dye to form the conjugated polymer. Nevertheless, although most of the modified dye is consumed during the reaction, only a negligible amount of it is actually linked to the polymer. The peak at 19.5 min retention time (modified dye) considerably decreases in intensity, but the peak appearing at about 15.5 min (polymer peak), is barely visible (Figure 3). We concluded that although the bicyclic nonyne could be attached to the dye, it was not possible to use it for a conjugation reaction with the PMPC-PDPA polymer. At

this stage, we can only postulate that this is due to an instability of the azide moiety during a radical polymerisation, an assumption which is partially supported[40] and partially discouraged[9, 41] by literature

2. Amine-NHS ester conjugation

From the previous results, it became apparent that the amine-to-NHS ester conjugation is an effective reaction, although probably not running to completion. It still seemed reasonable to test a straight NHS ester-to-amine conjugation, which is also known to work for polymers.[16] An alcohol derivative on an N-hydroxysuccinimide (with an ethyl spacer) is commercially available and an ATRP initiator alongside the corresponding polymer can be synthesised easily in good quality (See also SI). One of the aims of this study, however, was to investigate the feasibility of a conjugation strategy compatible with protic solvents. The conjugation in question is an amidification, which relies on the nucleophilicity of amines. Although primary amines are more nucleophilic than primary alcohols, an alcohol used as a solvent results in a much higher concentration of hydroxyl groups than the amines. As a consequence, the alcohol has the potential to also react with the NHS ester itself, thus making the conjugation process impossible. This was the case for PMPC-PDPA, which can only be polymerised in protic solvents (ethanol in our case), consequently leading to a complete lack of reactivity with an amine-containing dye (See SI). To further assess this hypothesis, we also tried to use POEGMA-PDPA, which can be polymerised in THF. However, since the workup of this polymer also involves protic solvents, we did not observe any reaction in this case as well (See SI). We concluded that polymers that require a protic solvent during their production and/or purification protocol therefore cannot be conjugated successfully with this approach.

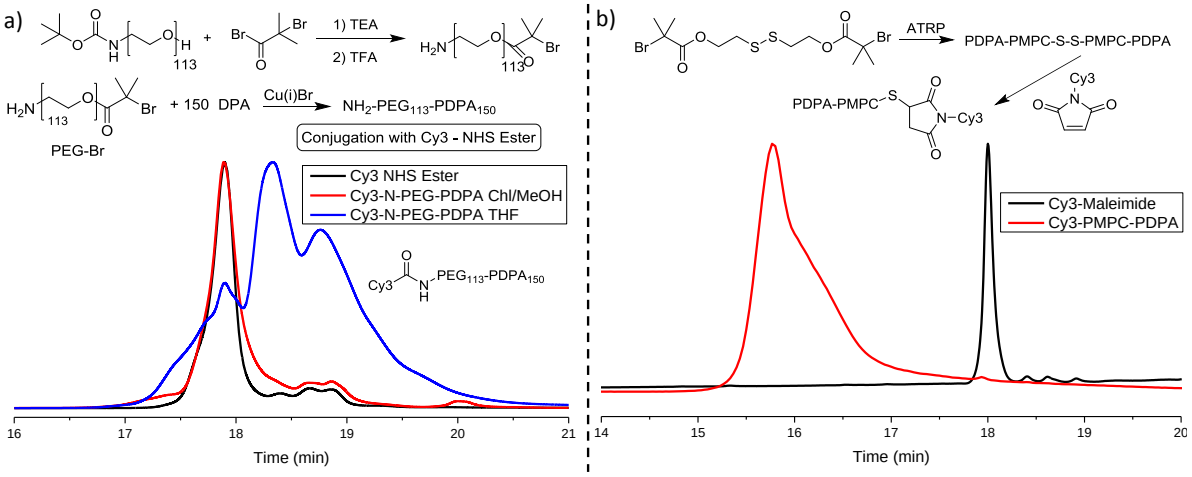


Figure 4: Polymer modification using the NHS/amine link (a). The PEG macroinitiator is BOC deprotected and then used for polymerisation. The final PEG-PDPA is then conjugated with Cy-3-NHS ester where a conversion can be observed using THF as a solvent. When using a thiol-maleimide link (b), a full conversion with no residual free dye could be observed with PMPC-PDPA.

Since the NHS ester is apparently cleaved during the polymer preparation, the reverse approach was attempted next. For this strategy, a similar problem as for the one discussed above could be foreseen. Since PMPC-PDPA is only soluble in protic solvents, any NHS ester present on a dye would be cleaved by the solvent instead of reacting with the primary amine on a polymer. We thus decided to synthesise PEG-PDPA (also soluble in THF) and then test the conjugation in a protic and non-protic environment. A BOC-protected heterobifunctional PEG [(Boc)NH-PEG-OH] was converted into an ATRP initiator and the BOC group cleaved during the workup procedure. The resulting free amine moiety did not interfere with the consecutive ATRP (Figure 4a and SI). After the polymerisation, we then tested the conjugation in two different solvent systems: once in chloroform/methanol for a potential application with PMPC-PDPA and once in anhydrous THF. As expected, the protic solvent mixture of chloroform/methanol did not result in any modified polymer as the methanol deactivates the NHS ester. In dry THF though, an almost complete conversion could be observed and only little remaining free dye was present in the solution (Figure 4a). Interestingly, this free dye

seems to be physically bound to the polymer as it cannot be removed during the workup process.

3. Thiol-Maleimide reaction

With respect to click chemistry, thiols are applied in various reactions.[10, 37, 42-45] The conjugation is based on the reactivity of thiols towards double bonds, especially to the ones in maleimides.[46] Since maleimides conjugate well to thiols but are far less reactive towards alcohols or amines, side reactions as with the previous approach can be avoided. For this conjugation approach, however, the maleimide moiety cannot be part of the polymer initiator, because it can act as a monomer during the radical polymerisation.[47] Thus, a thiol-containing initiator must be used. The thiol unit, however, needs to be masked during the polymerisation because it can interfere with the radical reactions by forming a radical itself. We circumvented this problem by employing a bifunctional ATRP initiator with a disulphide link, which is commercially available. PMPC-PDPA could then be synthesised simultaneously onto both sides of the initiator (Figure 4b). An advantage of this linking method is that maleimide-derivatives are highly available for a wide range of functional molecules. We have indeed used this linking method earlier to attach the DOTA ligand onto PMPC-PDPA.[37] Following the same strategy, also Cy-3 could be conjugated efficiently and a complete conversion was observed (see HPLC analysis, Figure 4b).

Although these results indicate that this is a very feasible general linking method, it does unfortunately exhibit a major drawback, namely the instability of the linkage. It has been reported that the thiol-maleimide link is unstable in a biological environment, but initial studies on brominated maleimides show indications that this problem could be overcome.[24, 25, 48] This holds important implications when such conjugates are used for tracking polymers

in cells or *in vivo*. Due to the instability, it is not certain whether one observes the actual polymer or the free dye after it has been cleaved off from the conjugate.

4. TAD Modification and conjugation

Up to this point, we discussed established linking methods and how well they can be applied towards the PMPC-PDPA diblock copolymer. In 2014, however, Du Prez et al., also co-authoring the present study, presented a novel click chemistry platform, which makes use of a well-known reaction between triazolinedione (TAD) and dienes such as sorbic alcohol. Both compounds react quickly in an additive-free Diels-Alder reaction, and the reaction is already known to have a wide scope.[17, 18, 49] For this reason, we decided to apply it for conjugating a dye to a polymer chain end. The TAD, or its urazole precursor, could either be attached to the initiator or to the polymer. When attached to the polymer, however, it could potentially react with a variety of double bonds available with dye molecules (see Fig. 1 for Cy-3 and Scheme 1 for Sudan red II) and thus interfere with their functionality as a chromophore. We then decided to conjugate the urazole to the dye and oxidise it *in situ*. In this way, any side reactions of the TAD-dye conjugate with itself would be kept to a minimum. Since purifying the polymer from the by-products of side reactions can be achieved by standard methods, the strategy described would allow to obtain a clean polymer. However, this choice made it necessary to attach a sorbic alcohol unit to the polymer. Sorbic alcohol contains the conjugated double bond required for the subsequent coupling with the TAD unit, and it was not clear whether this double bond would still be present after a radical polymerisation process.

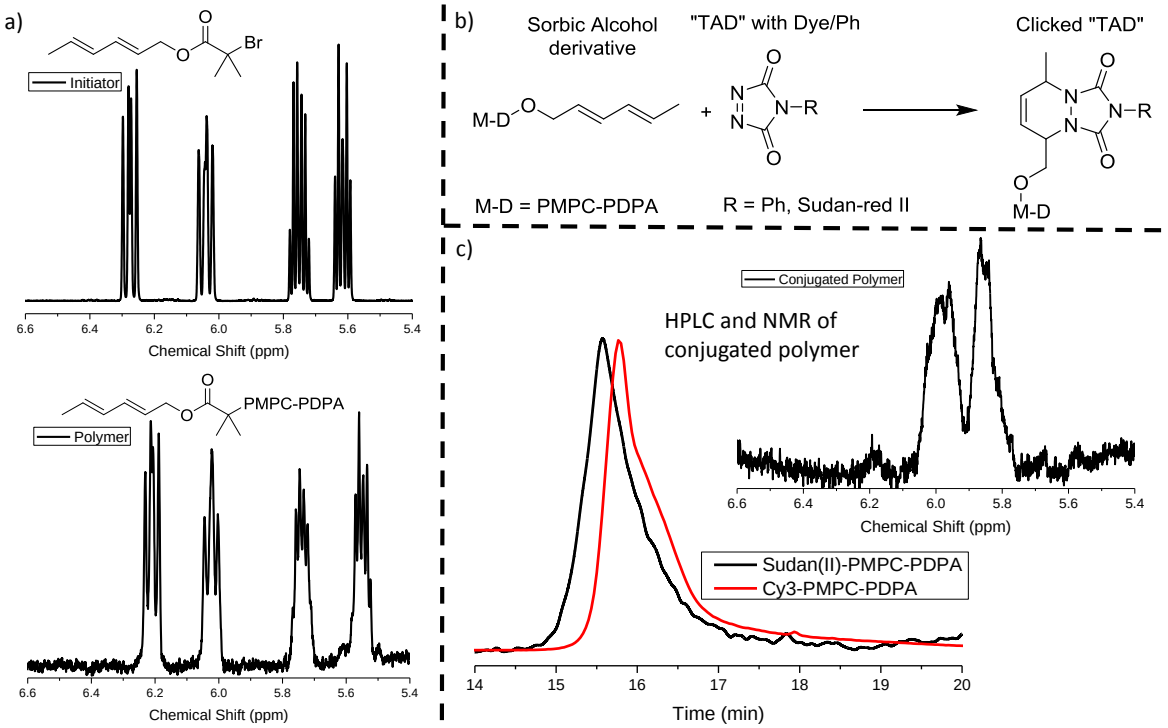


Figure 5: a) NMR of the sorbic-alcohol based initiator and the corresponding polymer focussing on the area of the protons associated with the conjugated double bond. All protons are still present in the polymer. (b) This polymer is coupled with a TAD modified Sudan-red II and phenyl-TAD (structure Sudan-red-II-TAD in scheme 1). c) NMR shows that the protons of the Diels-Alder adduct appear (R = Ph) and HPLC provides proof for Dye conjugation (R = Sudan-red). For HPLC, the Cy-3-PMPC-PDPA trace is shown for comparison as control.

To our surprise, the conjugated double bond was still present after the ATRP. This is confirmed by the ^{1}H-NMR spectrum of the product in the region of 5-7 ppm (where protons in double bonds occur). Before and after the reaction, all four protons associated with the conjugated double bond are present (Figure 5). Their integrational values do not differ from one another and even the multiplicity of the peaks is preserved during the reaction. Once the presence of the sorbic alcohol unit on the polymer was confirmed, we initially attempted to conjugate the polymer with a phenyl-derivatised TAD, of which we reported the click reaction earlier.[17] NMR-analysis of the product confirmed that the protons from the sorbic alcohol unit vanished and that protons originating from the new double bond formed in the Diels-Alder reaction appeared in the spectrum (Figure 5). Considering the promising results, the obvious following

step was to equip a feasible dye with an urazole moiety. It was important that it would retain its stability once the TAD is formed in order to ensure that the conjugation reaction with the polymer could take place. We screened a variety of dyes and finally settled with the plain Sudan-red II, since its alcohol moiety could be converted in a short series of reactions into the urazole, the precursor of the TAD functionality (Scheme 1). In contrast to our previously published procedures, we now show that not only amines and isocyanates can be used as a precursor, but also alcohols. In this case an alcohol is initially converted into a carboxylic acid, and subsequently into an isocyanate moiety exploiting a Curtius rearrangement. Introducing the isocyanate group into the dye is an important step in developing the TAD chemistry for a larger scope of starting materials.[50] In accordance to our previously published procedure,[17] this isocyanate can be converted into the urazole and eventually into the TAD derivative (Scheme 1). As already mentioned, the conversion from the urazole to the TAD occurred *in situ* with the coupling reaction to supress side reactions as much as possible. It is relevant to note that TAD can react with alcohols, although much more slowly than with dienes. Since we had to use a solvent mixture containing methanol, side reactions were possible, especially because we attempted a polymer analogous reaction. Low molar mass dienes react with TAD in less than a second, but the reaction rate slows down to the range of minutes in the case of polymer analogous reactions.[17] We aimed at counteracting these issues by using one of the best dienes available for the TAD conjugation (sorbic alcohol derivative).[17] Despite these possible issues, we were delighted to observe a successful conjugation reaction, as confirmed by HPLC (Figure 5). Some side products did form, but could be removed easily during purification. Despite its drawback of being synthetically more demanding (TAD synthesis), this method combines the speed of the TAD conjugation with the stability of a Diels-Alder adduct, thus making it a very useful dye-conjugation tool for the polymer chemistry community.

Conclusion

We have discussed a variety of metal-free conjugation methods with respect to their feasibility to attach dyes to an amphiphilic block copolymer in protic solvents. First of all, from our results it seems more advantageous to conjugate the dye after the radical polymerisation has taken place, as it can interfere with the polymerisation process. We can also postulate that the properties of the dye as chromophore were not influenced significantly since we did not have to adjust the settings on the detector of the HPLC but detected all compounds with the same ones.

The SPAAC chemistry does not appear to be feasible in the case of radical polymerisations. Our results indicate that both the bicyclic nonyne as well as the azide experience a loss of their reactivity towards their respective counterparts during the ATRP process.

Although it is possible to incorporate an amine into the polymer, for the amine-NHS ester coupling the NHS ester can be cleaved at any stage due to solvolysis in the presence of a protic solvent. Coupling the polymer containing a primary amine is thus only possible in a non-protic solvent.

The thiol-maleimide click reaction yields a clean conjugated polymer even in a protic environment. The drawback for this technique is that an additive (a phosphine) is needed and that the final bond has been reported to be partially unstable *in vivo*.

Finally, the TAD-coupling does provide a stable bond and works in additive-free conditions to give a complete conversion of the reactant. Once more TAD-modified dyes will become widely available, we believe that this conjugation strategy will become the best and most widely exploited conjugation method available.

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Associated Content:

Supporting information on selected GPC traces and synthetic data on TAD synthesis is available free of charge via the internet.

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