Supramolecular behaviour and fluorescence of rhodamine-functionalised ROMP polymers†

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Inherently fluorescent polymers are of interest in materials and medicine. We report a ring-opening meta-
thesis polymerisation (ROMP) platform for creation of amphiphilic block copolymers in which one block is
formed from rhodamine B-containing monomers. The polymers self-assemble into well-defined micelles
which are able to sequester molecular dyes and further interact with them by energy transfer. Despite
incorporating a cationic dye known to bind DNA, the polymer micelles do not interact with DNA, indicat-
ing that they are potentially safe for use in bioanalytical applications.

We here report the synthesis and analysis of a new fluorophore-rich block copolymer prepared by ROMP. Previous
work in our laboratories has focused on polymer–drug
conjugates23,24 and we wished to explore the incorporation of
fluorophores25 as these have a range of important medical
applications, enabling visualisation of cellular uptake, and are
complementary to our luminescence investigations of uptake
of potential therapeutics in resistant cancer lines.26 We were
particularly interested in self-assembled systems which
possess “stealth” properties and to this end we used polyethyl-
enene glycol (PEG) as a clinically established copolymerisation
adduct; PEG interacts minimally with biological fluid com-
ponents, is hydrophilic, biologically inert, and can be used to
control self-assembly outcomes, which in turn affects blood
circulation and elimination.5 Rhodamine B (RhB) was chosen
as the fluorophore. The rhodamine group of dyes are second
only to the xanthene fluorescein group with respect to polymer
dye conjugates used for labelling2 and yet there are few ROMP
copolymer conjugates examples in the literature.20,25,27–32 Our
ROMP polymer shows robust self-assembly into well-defined
nanoparticles, which can interact both supramolecularly and
photophysically with molecular dyes, while showing no inter-
actions with DNA which could lead to toxicity. Such nano-
particles which display high fluorescence and minimal inter-
actions with biomolecules could be useful in monitoring drug-
delivery processes at the cellular level.

Results and discussion

Monomer synthesis

The incorporation of PEG units onto ROMP polymers is well-
established with some early examples reported by the Grubbs
and Nguyen groups amongst others.33–36 For our studies, we
chose to synthesise the norbornene PEG-derivative, 5, as it possesses a symmetrical structure which minimizes head-to-tail effects, and its preparation and polymerisation characteristics have previously been reported by our group.\textsuperscript{37} The synthesis is a three step procedure from \textit{exo}-hemic anhydride 2 (Scheme 1), itself prepared from the commercially available \textit{endo}-hemic anhydride 1 according to a well-established procedure.\textsuperscript{38} The reaction sequence was first tested with the more readily available \textit{endo}-hemic anhydride 1 (Scheme 1). The derivative, 3, is a known solid and here we report single crystal diffraction data and structure for the first time (see ESI). RhB possesses a carboxylic acid group, and we originally envisaged synthesizing the required monomer derivative, \textit{exo}-7, via a Steglich esterification\textsuperscript{39} with \textit{N}-(hydroxypenta-nyl)-cis-5-norbornene-\textit{endo}-2,3-dicarboximide, 6, the latter available following a literature procedure.\textsuperscript{40} The reaction was performed starting with the more readily available \textit{endo}-hemic anhydride to establish suitable reaction conditions. The desired ester derivatives, \textit{endo}- and \textit{exo}-7, were obtained but repeated purification steps were required to remove urea by-products, and traces of RhB persisted, suggesting that hydrolysis occurred during the purification process, giving low overall yields. We therefore switched our attention to an amide derivative of RhB as these are less prone to hydrolysis and the overall yields. We therefore switched our attention to an amide derivative, 8, as it possesses a symmetrical structure which minimizes head-to-tail effects, and its preparation and polymerisation characteristics have previously been reported by our group.\textsuperscript{37} The synthesis is a three step procedure from \textit{exo}-hemic anhydride 2 (Scheme 1), itself prepared from the commercially available \textit{endo}-hemic anhydride 1 according to a well-established procedure.\textsuperscript{38} The reaction sequence was first tested with the more readily available \textit{endo}-hemic anhydride 1 according to a well-established procedure.\textsuperscript{38} The derivative, 3, is a known solid and here we report single crystal diffraction data and structure for the first time (see ESI). RhB possesses a carboxylic acid group, and we originally envisaged synthesizing the required monomer derivative, \textit{exo}-7, via a Steglich esterification\textsuperscript{39} with \textit{N}-(hydroxypenta-nyl)-cis-5-norbornene-\textit{endo}-2,3-dicarboximide, 6, the latter available following a literature procedure.\textsuperscript{40} The reaction sequence was first tested with the more readily available \textit{endo}-hemic anhydride to establish suitable reaction conditions. The desired ester derivatives, \textit{endo}- and \textit{exo}-7, were obtained but repeated purification steps were required to remove urea by-products, and traces of RhB persisted, suggesting that hydrolysis occurred during the purification process, giving low overall yields. We therefore switched our attention to an amide derivative of RhB as these are less prone to hydrolysis and the overall yields. We therefore switched our attention to an amide derivative, 8, as it possesses a symmetrical structure which minimizes head-to-tail effects, and its preparation and polymerisation characteristics have previously been reported by our group.\textsuperscript{37} The synthesis is a three step procedure from \textit{exo}-hemic anhydride 2 (Scheme 1), itself prepared from the commercially available \textit{endo}-hemic anhydride 1 according to a well-established procedure.\textsuperscript{38} The reaction sequence was first tested with the more readily available \textit{endo}-hemic anhydride 1 according to a well-established procedure.\textsuperscript{38} The derivative, 3, is a known solid and here we report single crystal diffraction data and structure for the first time (see ESI). RhB possesses a carboxylic acid group, and we originally envisaged synthesizing the required monomer derivative, \textit{exo}-7, via a Steglich esterification\textsuperscript{39} with \textit{N}-(hydroxypenta-nyl)-cis-5-norbornene-\textit{endo}-2,3-dicarboximide, 6, the latter available following a literature procedure.\textsuperscript{40} The reaction sequence was first tested with the more readily available \textit{endo}-hemic anhydride to establish suitable reaction conditions. The desired ester derivatives, \textit{endo}- and \textit{exo}-7, were obtained but repeated purification steps were required to remove urea by-products, and traces of RhB persisted, suggesting that hydrolysis occurred during the purification process, giving low overall yields. We therefore switched our attention to an amide derivative of RhB as these are less prone to hydrolysis and the overall yields.

### Scheme 1 Monomer synthesis.

The block copolymerization was performed starting with 5 which is known to polymerise within 10 min,\textsuperscript{24,31} and so we focused on investigating the homopolymerisation of \textit{exo}-norbornene-RhB monomer \textit{exo}-10. The Grubbs G3 initiator was chosen as this has been shown to possess excellent activity even with complex pendant groups.\textsuperscript{13,47} A kinetic study of the polymerisation in deuterated-chloroform showed that the polymerisation commenced rapidly and the majority of the monomer was consumed after 30 min, although approximately 80 min were required for complete conversion as evidenced by the disappearance of the monomer peak at 2.5 ppm (Fig. 2).

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final polymer product with an average molecular weight of 21.8 kDa and a \(\bar{M}_n\) of 1.29 (Fig. S22, ESI†). NMR analysis of poly 5-b-exo-10 was consistent with that expected from a combination of the homopolymer spectra.

Self-assembly of block copolymer

Poly 5-b-exo-10 was tested under multiple buffer conditions to assess its interaction with both single-stranded and double-stranded DNA. The polymer (1 mg) was dissolved in acetone (100 \(\mu\)L) and 900 \(\mu\)L of the aqueous medium of choice (unbuffered water; tris-borate-EDTA (TBE, pH 8) buffer; tris-acetate-magnesium (TAMg, pH 8) buffer; and acetate (pH 5) buffer) was added in portions (15 \(\times\) 10 \(\mu\)L, 10 \(\times\) 20 \(\mu\)L, 5 \(\times\) 50 \(\mu\)L, 3 \(\times\) 100 \(\mu\)L) whilst stirring.

DLS analysis of the micellar solutions (Fig. 3a, S23–S25, ESI†) gave particle diameters of 27 nm (water), 38 nm (TBE), and 37 nm (TAMg). The size distributions of the pH buffered particles were very low (dispersity indices of 0.04 and 0.05 for TBE and TAMg respectively), while the distribution in unbuffered water was noticeably broader (dispersity index of 0.24). Examination of the size distribution graphs (Fig. S23–S25, ESI†) makes it clear that taking into account the broader distribution in water, the differences in average size are not significant. The size of the micelles was not concentration dependent. The emission intensity of the RhB unit was also measured (Fig. S28–S30, ESI†) and again the buffered systems were distinctly different from the unbuffered solution, which was noticeably less intense – this suggests that the interior arrangement of chains in water promotes self-quenching to a greater degree. The micelles in water were also analysed by TEM (Fig. 3a, b and S26, S27, ESI†), giving size distributions slightly smaller than those in solution using DLS (22 ± 2 nm). This is as expected, since TEM does not record a hydration sphere and uses a vacuum system which frequently contracts the diameter of the self-assembled systems, whereas DLS observes particles including their solvation sphere. The size distribution was remarkably uniform, showing that the low dispersity polymers also have minimal variation in aggregation number as well.

Fluorescence and energy transfer

Typically in fluorophore-functionalised ROMP polymers, fluorescent monomers are copolymerised with monomers that provide different imaging functionalities such as MRI.
When copolymerised, the fluorescent monomers tend to undergo quenching to varying degrees and this can be caused by several phenomena including aggregation-caused quenching (ACQ) and chemical interaction with other functional groups. In the system reported here, since the hydrophobic block is formed from just one monomer, it is possible for quenching or other photophysical interactions to occur both at intra- and interchain levels. Intrachain effects will be independent of the local concentration of the polymer, whereas interchain effects will depend on self-assembly and change of local environment. Fluorescence measurements at concentrations between 0.0001 to 1 mg mL$^{-1}$ show that emission intensity increases with concentration above that expected for simple concentration effects, indicating increased efficiency of emission likely due to a more hydrophobic environment, as a result of micellization. However, no further increase is observed with concentration, and from 0.01 to 1 mg mL$^{-1}$ emission intensity decreases, consistent with significant interchain quenching (Fig. 4a).

Förster resonance energy transfer (FRET), which is dependent upon the distance between a pair of donor-acceptor fluorophores, was used to further probe the polymer micelles using a supramolecular approach through encapsulation of hydrophobic dyes within the polymer micelles. For efficient FRET, there must be both a short distance between the two dyes (<10 nm), and good spectral overlap between the emission

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**Fig. 3** Characterisation of poly 5-b-exo-10 in water by (a) DLS and (b) TEM imaging (stained with uranyl acetate) and (c) counting of TEM particle sizes. Data for TBE and TAMg can be found in the ESI.$^\dagger$

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**Fig. 4** (a) Effect of dilution upon fluorescence intensity in water (note log scales on both axes). The samples were excited at 550 nm, and emission collected over 560–760 nm, with $\lambda_{\text{max}} = 580$ nm. (b) Emission spectra for FRET experiments. $\lambda_{\text{ex}}$ (proflavine) = 460 nm, $\lambda_{\text{ex}}$ (6-FAM) = 490 nm, $\lambda_{\text{ex}}$ (RhB) = 550 nm.
peak of the directly excited (donating) dye and the excitation peak of the accepting dye. The overall effect of FRET is a reduction in the emission intensity of the donating dye and an increase in that of the accepting dye. 6-Carboxyfluorescein (6-FAM) was selected for these studies as its emission maximum 520 nm (excitation maximum 490 nm) is broad enough to overlap with RhB excitation ($\lambda_{\text{exc}} = 550$ nm). At constant 6-FAM concentration, the presence of poly 5-b-exo-10 micelles in water caused the 6-FAM emission at 520 nm to drop by 11%, while a shoulder appeared at 580 nm on the emission spectra, corresponding exactly to RhB emission (Fig. 4b). Changing 6-FAM for proflavine (emission $\lambda_{\text{exc}} = 510$ nm) the same effect was seen, with the proflavine peak decreasing coincidentally by the same value (11%) while RhB emission appeared at 580 nm. In both cases, the integrated emission intensity decreased by 14%, indicating very little non-radiative energy loss, given that under optimal conditions, the quantum yield for direct excitation of RhB is no higher than 0.8, and typically closer to 0.6. These results indicate that the micelles are both capable of noncovalently binding organic molecules and participating in FRET processes with such guests. This is potentially a useful tool for study of polymer-mediated drug delivery: if an encapsulated drug (e.g. doxorubicin) changes the photophysics of the polymer (either by FRET or some other measurable effect), it becomes possible to distinguish between the bound and free drug using fluorescence microscopy without making assumptions about colocalisation.

Interaction with DNA

Due to its cationic and aromatic nature, RhB can bind to DNA and has been found to interact with the minor groove of the B-DNA double helix, leading to a reduction in fluorescence. For our system to be useful as a tool for examining drug delivery, binding of DNA should be minimised. We examined the interaction of poly 5-b-exo-10 with both single-stranded and double stranded DNA (ssDNA and dsDNA) oligomers (20 mers). We observed no meaningful changes in dimension (DLS Fig. S31–S33, TEM Fig. S35–S40, ESIF) or fluorescence (Fig. S34, ESIF) upon mixing at a 1:1 ratio of DNA bases to RhB monomer, in either water, or TBE, or TAMg buffer. The reason for this lack of interaction became clear when we performed analysis by agarose gel electrophoresis (Fig. 5, S43, ESIF): the micelle migrated in the same direction as DNA, towards the anode, forming a very well-defined band, which again demonstrates their uniformity. The direction of mobility remained the same despite adjusting the pH to 5 (acetate buffer) to be entirely sure that the RhB units are in their cationic form, and even after annealing the samples from 95 °C to 4 °C over an hour to overcome kinetic effects. Electrophoretic mobility is determined by the surface charge, and in this case, there is PEG on the surface, not cationic RhB, regardless of pH effects. The surface charge includes associated ions, and this is usually modelled as the electrostatic double layer – the chemistry on the exterior binds either anions or cations as a first layer, and a second layer forms of the opposite ions. Here, we believe that the PEG binds cations through the oxygen atoms, resulting in an anionic second sphere which leads to the observed electrophoresis result. The negative surface charge would therefore repel the similarly-charged DNA and prevent colocalisation.

Conclusion

We have shown that ROMP can be used to create RhB-rich block copolymers which self-assemble into well-defined micelles whose fluorescence can be modulated through non-covalent inclusion of molecular dyes. Despite the propensity of RhB to interact with DNA, the micellization results in safe confinement of the RhB within the micelle core, while the PEG shell is expected to provide biological “stealthing”. The true innocence of fluorescent dyes used to image processes such as cellular uptake of nanostructures can be dubious; we believe that this system provides a robust route to high emission/low interaction nanostructures which could be used to enhance analytical studies in bionanotechnology.

Conflicts of interest

The authors declare no competing financial interests. All authors have given approval to the final version of the manuscript.

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