Biomacromolecules

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Synthesis of Rhodamine 6G-Based Compounds for the ATRP Synthesis of Fluorescently Labeled Biocompatible Polymers

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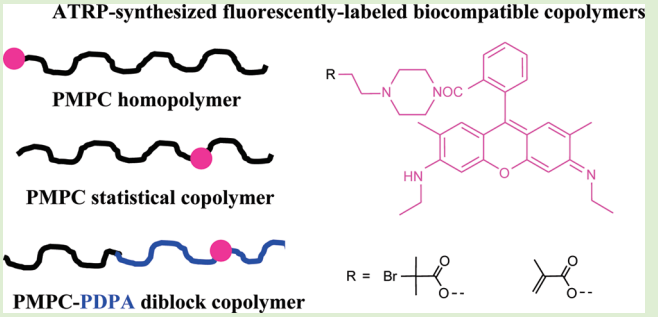
Supporting Information

ABSTRACT: Facile derivatization of rhodamine 6G in the 2′ position by direct reaction with secondary amines is reported. If the secondary amine contains a hydroxy group, the hydroxyl-functional intermediate can be readily esterified to give either fluorescent initiators for atom transfer radical polymerization (ATRP) or a fluorescent methacrylic comonomer. In contrast to rhodamine dyes functionalized using primary amines, which are only fluorescent at low pH, these compounds are highly fluorescent at physiological pH. These new compounds were subsequently used to prepare a range of fluorescently labeled biocompatible polymers based on the biomimetic monomer, 2-(methacryloyloxy)ethyl phosphorylcholine (MPC), for biomedical studies.



INTRODUCTION

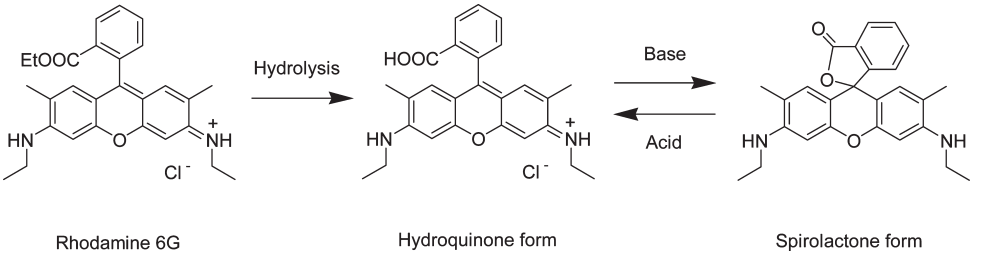
The use of synthetic polymers for the intracellular delivery of drugs requires a detailed knowledge of the final fate of the macromolecular vector. One important tracking approach is to fluorescently label the polymer chains. This allows their diffusion within tissue and live cells to be monitored in situ using established techniques such as fluorimetry and confocal fluorescence microscopy.[1−4] Ideally, the dye label should emit in a spectral region where there is minimal autofluorescence from either cells or body fluids/organs. In addition, high quantum yields are clearly advantageous, since this can either increase sensitivity or minimize the degree of labeling required. In the latter case, this allows significant cost savings for relatively expensive dyes and reduces possible toxic side effects due to the dye label. Finally, dyes with high photostabilities are preferred. Rhodamines are one class of dyes that fulfill all of the above requirements, because they have high quantum yields, emit in the red part of the visible spectrum, are relatively cost-effective, and offer good photostability.[5]

In general, there are several possible methods for the covalent attachment of a dye label onto a polymer chain. A range of functionalized rhodamines are commercially available for coupling via various chemistries.[6] These dyes are commonly used for labeling specific sites in biological macromolecules.[7,8] Recently, alternative dyes have also been used for labeling synthetic polymers.[9] However, such reactive labels are significantly more expensive than their nonreactive counterparts.[10] In addition, there are several literature examples of polymerizable vinylic rhodamine derivatives,[11−14] including at least one commercially available rhodamine-labeled monomer.[15] Usually, such dye-functionalized monomers are copolymerized with conventional vinyl monomers to give statistical copolymers with relatively low dye contents. In contrast, using monofunctional fluorescent initiators allows the chromophore to be placed precisely at the polymer chain end.

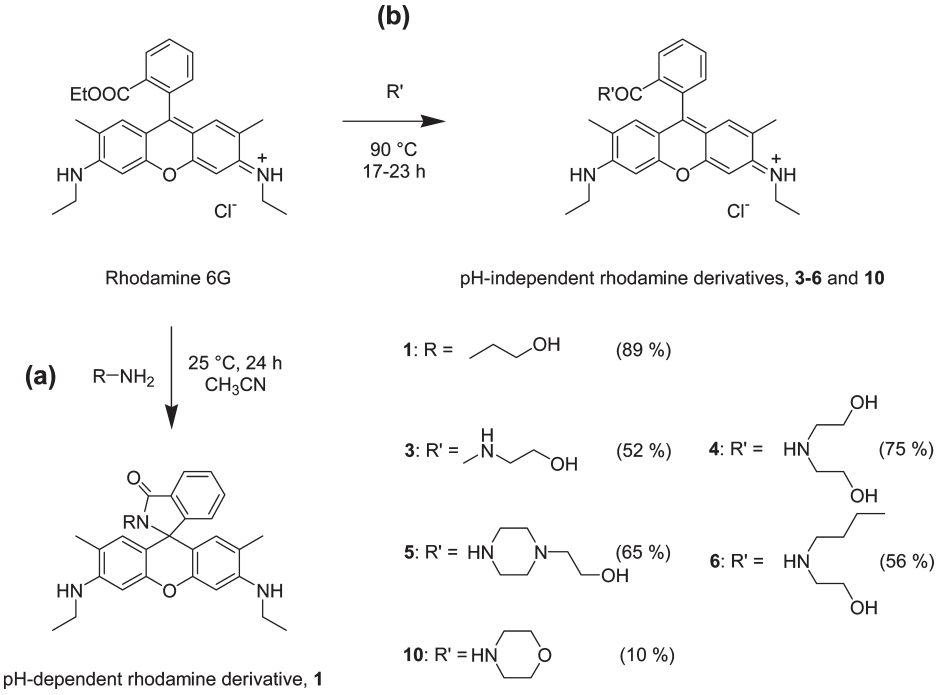
There appear to be no reports of rhodamine-based initiators for atom transfer radical polymerization (ATRP), although a number of other fluorescent dye initiators have been used to prepare labeled copolymers.[16−23] For example, a 2-bromoisobutyrate ester of fluorescein allowed good control to be obtained for the polymerization of N-isopropylacrylamide.[16] However, the relatively poor photostability of fluorescein[5] combined with the hydrolytic instability of aromatic esters[24] suggests that such labeled polymers may not be ideal for biomedical applications that require prolonged monitoring over extended time periods (days to weeks) in aqueous solution. According to Zhang and co-workers, an ATRP initiator based on phenyloxazole[17] is reasonably efficient. However, this chromophore has an emission maximum at 370 nm, and autofluorescence of cellular constituents is likely to be problematic at this wavelength. Similarly, the anthracene-based initiator reported

Received: March 7, 2011
Revised: April 8, 2011
Published: April 11, 2011

Scheme 1. Base-Induced Conversion of 2′-Substituted Rhodamine 6G from its Hydroquinone Form to its Spirolactone Form



Scheme 2. General Reaction between Rhodamine 6G and Amines to Form Various Substituted Amides[a]



[a] Numbers in parentheses are isolated yields after purification by recrystallization.

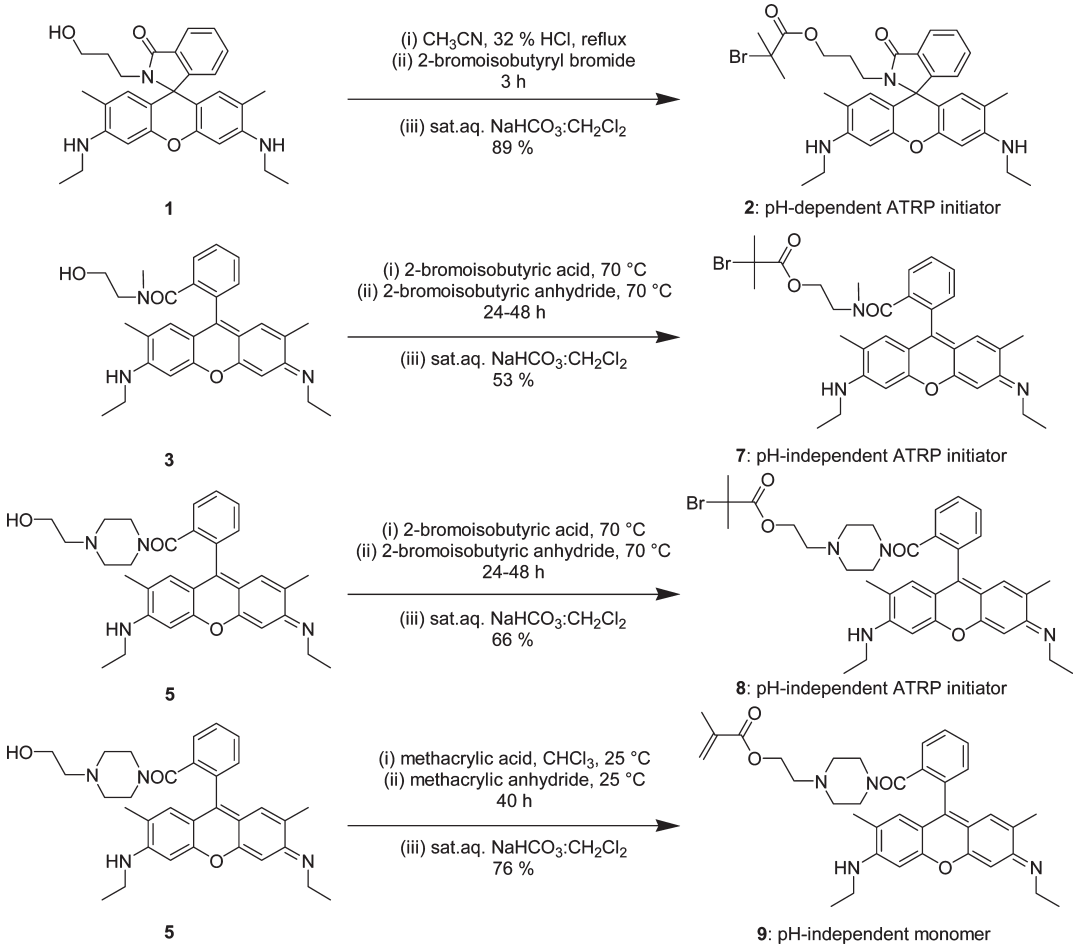
by Klumperman's group[18] presumably has an emission maximum at around 400 nm (i.e., similar to that of native anthracene), which may also lead to autofluorescence problems. Initiators based on substituted naphthalimides exhibit maximum emissions at around 500 nm,[18,21] which is close to that of fluorescein. Thus, the former could also be a useful alternative label to the latter commonly used dye. On the other hand, rhodamine dyes are generally more photostable than fluorescein[5] and are also relatively water soluble compared to naphthalimide-based dyes.[25,26]

Here we focus on modifying the commercially available dye, rhodamine 6G, because this compound has an exceptionally high quantum yield (> 0.9) compared to rhodamine B, which typically has quantum yields of the order of 0.3−0.4.[10] Moreover, given the very high absorption coefficient of rhodamine 6G (in excess of 100 000 M^{-1} cm^{-1}), only a minimal amount of label is necessary, which should minimize any adverse effects that might be caused by the dye label.[27]

In principle, chemical modification of rhodamine 6G should allow this biomedically relevant chromophore to be readily incorporated into polymer chains, either as ATRP initiators or as a comonomer. Indeed, we have recently reported using such rhodamine-labeled copolymers for monitoring intracellular uptake of vesicles[28−30] and also for studying vesicle diffusion into tissue-engineered human oral mucosa.[31]

Rhodamine dyes exist in their fluorescent hydroquinone form at neutral/acidic pH and in their nonfluorescent spirolactone form at basic pH (Scheme 1).[32] Amidation of rhodamine esters in the 2′ position has been reported in several recent papers and patents.[10,32−36] Primary amines react directly with the cyclic ester to form secondary amides under mild conditions. For these compounds, conversion to the cyclic spirolactam occurs at a lower solution pH than for the rhodamine ester precursor, and no significant fluorescence was observed above pH 6.[37] Thus, such compounds are not likely to be useful as fluorescence probes within living tissue at pH 7.4. However, they may offer some potential as fluorescent pH indicators.[38,39] A synthetically elegant solution to this problem has been reported, where a rhodamine-based secondary amide was coupled to a fluorescein dye.[37] This fluorophore emits light over a wide pH range at a wavelength that depends on the solution pH. However, this approach requires a multistep synthesis.

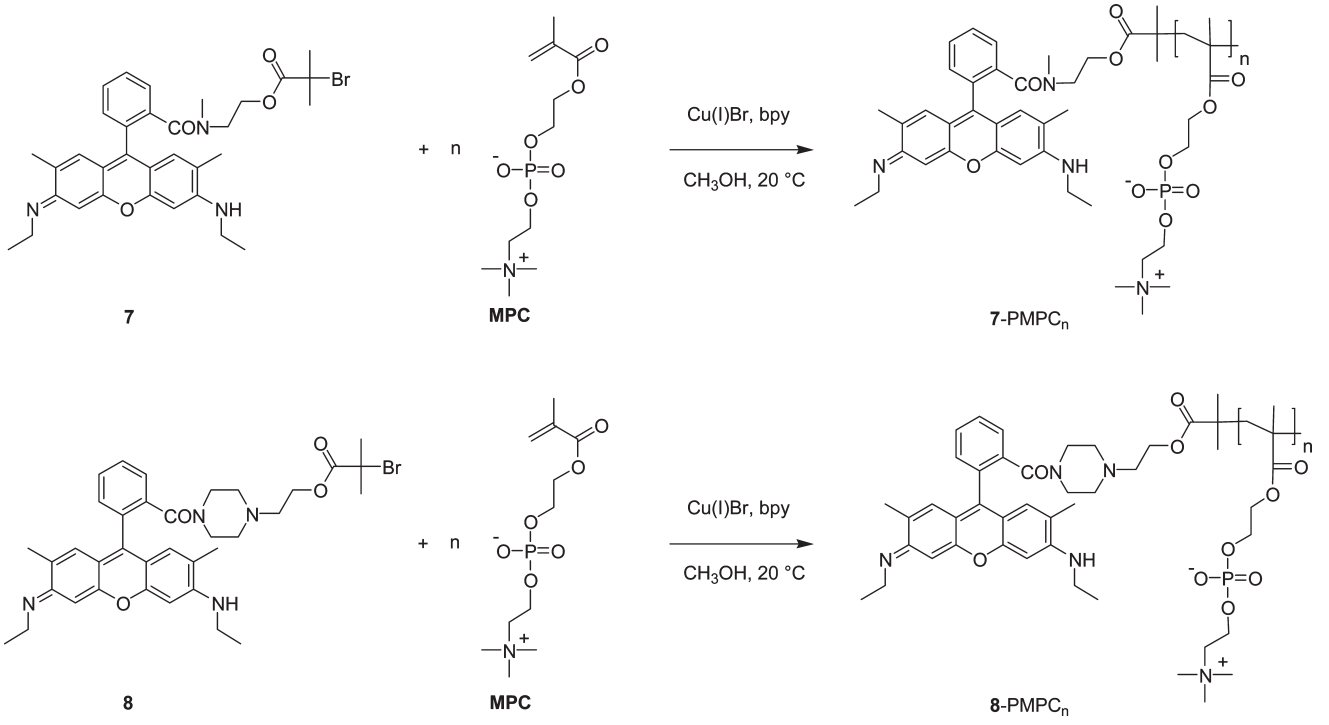
Scheme 3. Esterification of Three Hydroxy-Functional Rhodamine Derivatives to Produce Various Fluorescently-Labeled ATRP Initiators and a Fluorescently-Labeled Methacrylic Monomer



If a tertiary amide rhodamine dye derivative is used instead of a secondary amide, then internal amide formation at high pH is prevented. Thus conjugation is retained and no loss of fluorescence is observed in neutral or alkaline solution.[10] Formation of the tertiary amide does not occur under mild conditions, but it has been reported using either benzotriazole coupling agents[35,36] or highly reactive Lewis acids.[10] In contrast, other commonly used amidation reagents such as carbodiimides afforded only low yields.[10] In addition, the synthesis of rhodamine-based acid halides has been described in the patent literature. These highly reactive compounds have been used to prepare a range of tertiary amide derivatives.[33,34] However, this approach precludes the use of functional amines such as γ-aminoalcohols, unless protecting group chemistry is employed. Therefore, an additional synthetic step is required to prepare hydroxy-functional rhodamine dyes that exhibit pH-independent fluorescence.[10] On the other hand, direct reaction between cyclic lactones and secondary amines has been reported to afford high yields under relatively mild conditions, particularly if a large excess (up to 20 equiv) of the amine is used.[40] In practice, the amine can be used as a reactive solvent. As far as we are aware, this attractive approach has not previously been reported for the preparation of rhodamine-based tertiary amides.

Herein we report a convenient one-step synthesis of a range of hydroxy-functional rhodamine 6G-based dyes with tertiary amide linkages (Scheme 2). In addition, protocols for the esterification of both hydroxy-functional secondary amides and tertiary amides to produce a series of fluorescent 2-bromoisobutyryl esters are described (Scheme 3). These compounds can be used as fluorescent ATRP initiators for the controlled polymerization of methacrylic monomers (Scheme 4). In addition, a rhodamine 6G methacrylic ester has also been synthesized. The labeled initiator was evaluated by preparing a range of fluorescently labeled poly(2-methacryloyloxy)ethyl phosphorylcholine) (PMPC) homopolymers by ATRP. In addition, two pH-responsive, vesicle-forming block copolymers comprising PMPC and poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) were prepared (Scheme 5).[41] One was prepared using the fluorescent ATRP initiator (with the MPC being polymerized first) and therefore had a single rhodamine 6G label at the PMPC terminus. The other was prepared by statistically incorporating the fluorescent methacrylic monomer into the pH-responsive PDPA block. The pH-dependent emission of these two copolymers depended on the spatial location of the fluorophore. Thus, if the labeled initiator was used, self-assembly led to a reduction in fluorescence to approximately half of the original intensity of the initially dissolved copolymer chains. On the other hand, if the labeled comonomer was incorporated into the pH-responsive PDPA block, self-assembly led to complete fluorescence quenching.

Scheme 4. Synthesis of Rhodamine-PMPC$_n$ Homopolymers by ATRP Using Initiator 7 or 8



RESULTS AND DISCUSSION

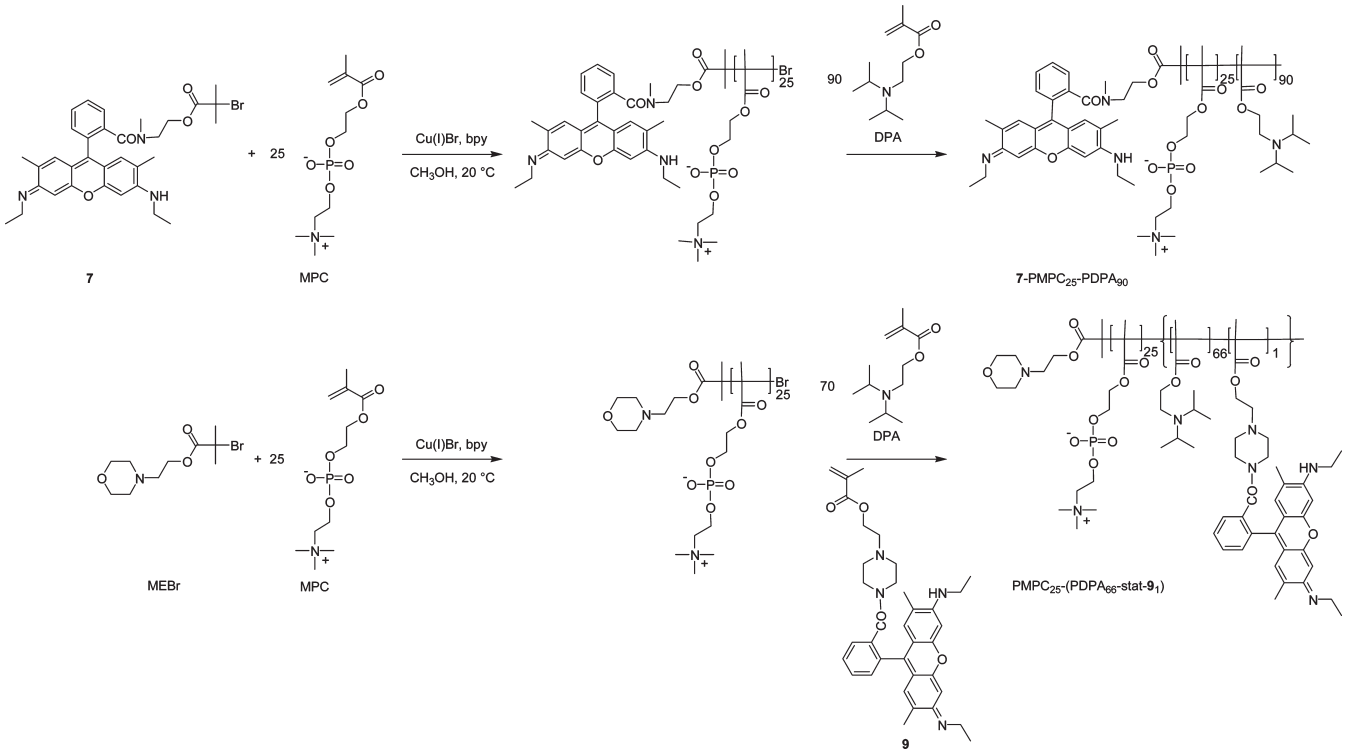
Reaction between Rhodamine 6G and 3-Aminopropan-1-ol. The literature reaction[32,37] between rhodamine 6G and primary amines is shown in Scheme 2a. This reaction was reported to proceed spontaneously at room temperature in DMF, with spirolactam yields ranging from 54 to 92%, depending on the primary amine used. In our hands, replacing DMF with acetonitrile gave an isolated yield of 89% when using 3-aminopropan-1-ol. Because acetonitrile is much more volatile (and hence easier to remove) than DMF, the former solvent was preferred for such reactions. As both reactants are water-soluble, purification was readily achieved by washing the water-insoluble product with excess water. ^{1}H and ^{13}C NMR spectra and ES-MS analysis were all consistent with the target structure (see Supporting Information).

Reaction between Rhodamine 6G and Secondary Amines. The direct reaction between rhodamine 6G and a secondary amine is depicted in Scheme 2b. The secondary amine was used as a reactive solvent in this synthesis, typically using 1.0 g rhodamine 6G dye per gram of amine. Maintaining the reaction mixture at 90 °C for 17−23 h gave the desired tertiary amide in 52−75% yield. The main byproduct was the cyclic lactone, as determined by electrospray mass spectroscopy (ES-MS). Various secondary amines were evaluated, as indicated in Scheme 2. Rhodamine 6G is highly soluble in protic solvents such as alcohols.[42] In this context, it is perhaps noteworthy that the synthesis of a similar hydroxy-terminated rhodamine B derivative has been reported in two steps, with an overall yield of 50%.[10] Thus, our one-step protocol produces comparable or better yields without requiring protecting group chemistry. All products were highly water-soluble, whereas the cyclic lactone byproduct is water-insoluble. Similar aqueous solubility has also been reported for a related rhodamine B-based compound.[10] Thus, the reaction was also attempted using rhodamine B instead of rhodamine 6G. However, little or no tertiary amide was formed with the former dye. The main difference here is that the rhodamine 6G precursor is an ethyl ester, whereas the rhodamine B starting material was present in its free carboxylic acid form. Secondary amines are both more basic and sterically congested than 3-aminopropan-1-ol. Thus, in addition to the desired tertiary amide, there is also some lactone formation due to internal cyclization. Unfortunately, although this byproduct can react with primary amines,[10] it is unreactive toward secondary amines, which therefore reduces the overall yield.

Esterification of Hydroxy-Functional Rhodamine 6G Derivatives. Synthetic routes to various rhodamine 6G-based esters are shown in Scheme 3. The secondary amide, **1**, was isolated in its nonprotonated spirolactam form. Addition of excess 32% aqueous HCl to a suspension of this compound in acetonitrile gave a deep red solution, indicating protonation of the amine groups and formation of the conjugated hydroquinone form. Heating to reflux afforded better solubility and addition of 2-bromoisobutyryl bromide gave the target product in 94% yield within 3 h (Scheme 3). The resulting ATRP initiator, **2**, was isolated in sufficient purity (≥95% by ^{1}H NMR and HPLC) to be used directly for polymer syntheses. Further purification (>99%) could be achieved either by recrystallization from methanol or by preparative reverse phase HPLC.[32,37]

Using the same protocol with tertiary amide **3** as a substrate gave the desired product but in a much lower yield. ES-MS analysis indicated that amide hydrolysis was prevalent in this case and that the acid was the main byproduct. This indicates that the tertiary amide is significantly more prone to acidic hydrolysis than the secondary amide. In addition, it was found by ES-MS that if the amine hydrochloride salt form of the dye was used, a significant amount of the 2-chloroisobutyryl ester was obtained, presumably due to halogen exchange occurring during the

Scheme 5. Synthesis of pH-Responsive Diblock Copolymers 7-PMPC$_{25}$-PDPA$_{90}$ and PMPC$_{25}$-P(DPA$_{66}$-9$_1$) Using Sequential Monomer Addition



reaction. In general, the overall yield of the targeted ester was only around 10%. Several modifications of the reaction conditions were examined. For example, addition of base leads to deprotonation of the secondary aromatic amine. This entity reacts with the acid bromide to give an amide in addition to the desired ester. This route was not synthetically useful, because the resulting products were difficult to separate. On the other hand, use of 2-bromoisobutyric anhydride instead of the 2-bromoisobutyryl bromide afforded 2-bromoisobutyric acid as a byproduct, instead of HBr. Because the former acid is weaker, it caused virtually no amide hydrolysis. Unfortunately, this reaction was very slow in common organic solvents such as acetonitrile and DMF, with only 10−20% conversion being achieved over 4−5 days even at 80−90 °C; this is probably related to the low solubility of the tertiary amide in these aprotic solvents. However, using 2-bromoisobutyric acid as solvent significantly improved the yield. This acid melts at 47 °C, hence it is necessary to work above this temperature. This approach is illustrated in Scheme 3. At 70 °C, a conversion of around 70% was obtained after 24 h for the reaction with **3** (see Supporting Information, Figure S1). Because the resulting ester is highly soluble in dichloromethane after neutralization, purification is relatively straightforward. The deprotonated form of the piperazine-based initiator **8** proved to be less soluble in water than the deprotonated initiator **7**, as judged by the reduced coloration of the aqueous phase in the former case; this is believed to be the main reason for the higher isolated yields of **8**.

Both esters could be purified by recrystallization from THF. On the other hand, attempts to purify these products by silica column chromatography were unsuccessful, because both column adsorption and a significant degree of hydrolysis were observed.

A similar approach was used for the preparation of rhodamine-based methacrylic monomer **9** (Scheme 3). This reaction was conducted at 20 °C, which is above the m.p. of methacrylic acid (16 °C) but sufficiently low to avoid thermal polymerization. Compound **5** had relatively low solubility in pure methacrylic acid, thus, it was necessary to add chloroform as a cosolvent in this particular case. This approach gave a yield of 76%.

Absorption Maxima and Absorption Coefficients Obtained for Various Rhodamine Derivatives. Table S1 shows the wavelengths of maximum absorption and absorption coefficients determined for the various rhodamine 6G derivatives. For the two dyes containing a secondary amide group (**1** and **2**), the maximum absorption wavelength is essentially the same as for rhodamine 6G, both in water and in acidified methanol. The absorption coefficient of **1** in methanol containing 0.1% trifluoroacetic acid (TFA) is similar to that of rhodamine 6G in methanol, whereas it is significantly lower for **2**. The absorption coefficients observed in 0.1 M HCl are significantly lower than those reported for compounds prepared using diamines, which are typically of the order of 60000.[37] On the other hand, a more complex adduct of normetanephrine and rhodamine 6G has an absorption coefficient of 41400 cm^{-1} M^{-1},[32] which is much closer to the value of 34000 ± 2000 cm^{-1} M^{-1} obtained for **2**. Both of these dyes were isolated in their spirolactam form (Scheme 1), which is not directly soluble in water at neutral pH. These dyes only dissolved very slowly in 0.1 M HCl, even with heating and ultrasonic treatment. Dissolution was rapid in 32% HCl, which could then be diluted with water without precipitation. However, the absorption coefficients determined using this protocol were of the order of 5000 M^{-1} cm^{-1}, which is significantly lower than that obtained for rhodamine 6G and also for the rhodamine derivatives prepared with tertiary amides.

Instead, stock solutions for spectroscopic studies were prepared by dissolving these chromophores in methanol containing 0.1% v/v TFA followed by serial dilution using 0.1 M aqueous HCl. These observations suggest that the conversion of each dye to its fluorescent hydroquinone form is relatively slow and may not go to completion in aqueous acid. Moreover, poorer solvent quality is known to reduce the absorption coefficient.[43]

Solvency effects were observed for almost all the rhodamine derivatives in Table S1. Compounds **3** and **4** have absorption coefficients close to those of unmodified rhodamine 6G in methanol, whereas the corresponding values observed in 0.1 M aqueous HCl are generally lower, indicating that water is a poorer solvent than methanol.

The absorption coefficient for compounds **5** and **6** in methanol is around 10% lower than that for rhodamine 6G. This may be due to increased steric congestion or, in the case of compound **6**, it may be due to reduced solvation of the *n*-butyl group in methanol. Similar observations were made in the same solvent for ATRP initiators **7** and **8** and also for the monomer, **9**, which each exhibit absorption coefficients around 80% of that of rhodamine 6G in methanol.

The absorption coefficients of compounds **5**−**7** in 0.1 M HCl is significantly lower than those observed in methanol, which is probably related to its reduced solvation (as for compounds **3** and **4**). Figure 1 shows absorption spectra recorded for **7** dissolved in both 0.1 M HCl and methanol. Despite the differing concentrations, the maximum absorbance is almost identical for these two solutions. However, the relative absorbance at 508 nm is significantly less in methanol than in 0.1 M HCl. This feature is directly related to the aggregation of the dye molecules.[26,44] According to Figure 1, there is a significantly higher degree of dye aggregation in 0.1 M HCl, which confirms that solvent quality influences fluorescent intensity, as expected.

In contrast, the piperazine-based ATRP initiator and monomer (**8** and **9**) exhibit very similar absorption coefficients in 0.1 M HCl and methanol. These compounds have additional amine functionality due to the piperazine moiety. This extra amine becomes protonated at low pH, which enhances the aqueous solubility of such compounds relative to the other derivatives.

pH-Dependence of Absorption and Emission Behavior of Rhodamine 6G Derivatives. Figure 2 shows typical normalized absorption and emission fluorescence spectra obtained for the derivatized rhodamine dyes in acidic aqueous solution. These are similar to those reported for rhodamine 6G.[26,44]

The effect of increasing the solution pH on the emission and absorption spectra of a 10^{-5} M solution of **1** in dilute HCl is shown in Figure 3a. The maximum emission and absorption at 530 nm both increase monotonically from pH 1 to pH 4. This is because pH adjustment involves the addition of a dilute aqueous base (see Supporting Information), which shifts the dye's unimer−dimer equilibrium in favor of the unimers.[26,44] Because dimers act as fluorescence quenchers,[44] increasing the relative unimer concentration leads to an increase in both the absorption at 530 nm and therefore also the emission intensity, provided that the increase in unimer concentration is larger than the dilution factor. Raising the pH leads to precipitation, which increases the background scattering in these absorption spectra. This is due to formation of the water-insoluble nonfluorescent spirolactam form of the dye. Above pH 4, this becomes the dominant factor in the attenuation of the absorption and emission spectra. This effect is also evident in digital

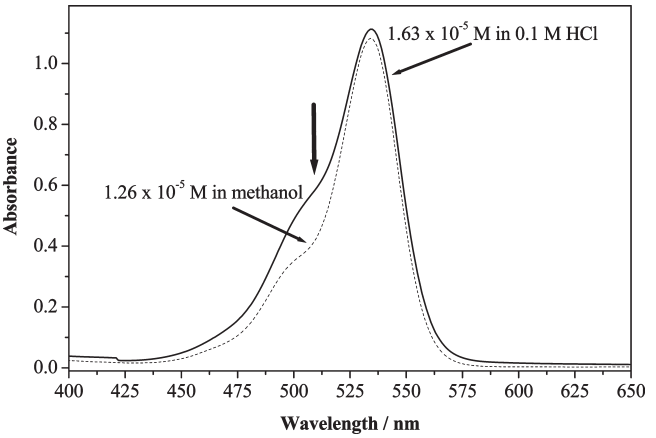


Figure 1. Absorption spectra obtained for **7** in methanol and 0.1 M HCl.

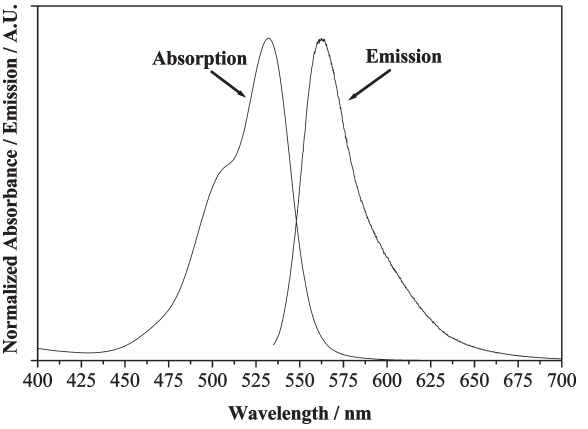


Figure 2. Normalized absorption and emission spectra of **3** in aqueous HCl at pH 2.0. The emission spectrum was recorded using an excitation wavelength of 530 nm.

photographs of the aqueous solutions/suspensions (see Supporting Information, Figure S3). Figure 3b shows the variation of the relative emission and the relative A_{530}/A_{508} ratio as a function of pH for a 10^{-5} M solution of **3**. The shoulder at approximately 508 nm is attributed to dimer formation and is somewhat higher than the reported value of 496 nm for the rhodamine 6G dimer.[44] This spectral shift may be due to substituent effects for these dyes, but overlapping peaks make the precise location of such features rather problematic and the precise determination of the wavelength for dimer absorption is outside the scope of this paper. Nevertheless, the change in the A_{530}/A_{508} ratio corresponds to a change in the unimer/dimer ratio. Increasing the pH from 1.5 to 10 more than doubles the emission intensity, despite concomitant dilution of the solution. This is believed to be due to a shift in the unimer−dimer equilibrium.

Relative quantum yields of the rhodamine 6G derivatives that were soluble at physiological pH were determined using the method described by Fery-Forgues and Lavabre.[10,45] These values are listed in Table S1. In general, the quantum yields observed for the dyes with hydrophilic substituents (i.e., compounds **3**−**6** and **10**) are comparable to those of rhodamine 6G, whereas the quantum yields obtained for dyes with hydrophobic

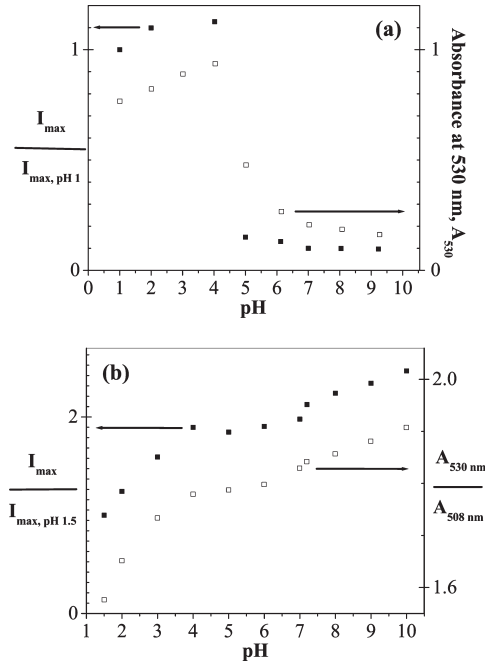


Figure 3. (a) Effect of increasing the solution pH on the maximum emission normalized with respect to pH 1.0 and absorbance at 530 nm for an aqueous solution initially containing 5×10^{-5} M **1**. (b) Effect of increasing the pH on the maximum emission and the absorbance ratio at 530 and 508 nm respectively for an aqueous solution initially containing 1×10^{-5} M of **3**.

substituents (i.e. **7**−**9**) are significantly lower. This indicates that relatively poor solvation adversely affects the relative quantum yield, as expected.

Use of Rhodamine-Labeled ATRP Initiators and a Methacrylic Monomer To Prepare PMPC Homopolymers. Initial experiments with the pH-dependent fluorescent ATRP initiator, **2**, confirmed that this compound efficiently initiated polymerization of MPC. However, because it is only fluorescent below pH 4, it is not particularly relevant for most biological studies, hence it was not explored further. The pH-independent ATRP initiators **7** and **8** were used to prepare PMPC homopolymers via a previously reported protocol, as illustrated in Scheme 4 for initiator **7**.[46,47] Table S2 summarizes the characterization data obtained for the various PMPC homopolymers prepared using the rhodamine-labeled initiators **7** and **8**. The maximum absorption wavelength was red-shifted by 5−10 nm for all molecularly dissolved copolymers relative to that of their corresponding initiators. This indicates a change in the local environment,[43] which is presumably due to the presence of the highly hydrophilic polymer chains.

Both initiators give well-defined homopolymer chains with relatively low polydispersities for target degrees of polymerization up to 100 (Table S2 and Figure S4). However, reduced control is achieved if a degree of polymerization of 200 is targeted, as evidenced by an increase in polydispersity and the appearance of a multimodal GPC trace.

For PMPC homopolymers prepared with a target DP of 20 using initiators **7** and **8**, the actual DP determined by ^{1}H NMR corresponds fairly well to that expected (see Table S2, entries 1 and 5, and Figure S5). In both cases, this experimental DP determined by ^{1}H NMR is slightly higher than that targeted, which suggests that only around 80% of the theoretical amount of rhodamine is attached to the polymer. These homopolymers were purified by dialysis against methanol using membranes with a molecular weight cutoff of 1000. This protocol inevitably removes oligomers, which leads to a higher M_n for the purified homopolymer. However, homopolymers prepared using a target DP of 50 (Table S2, entries 2 and 6) also have lower initiator contents than anticipated, as judged by ^{1}H NMR. Thus around 75% of the aromatic groups are intact for **7**-PMPC$_{50}$, whereas for **8**-PMPC$_{50}$ this value is around 62%. Although the experimental error in the integrated aromatic signals in these longer chains is higher, these results indicate that loss of oligomers during dialysis is not the main reason that the apparent molecular weight is higher than targeted. There are two alternative explanations for this observation. Either the initiator efficiency is less than 100%, that is, not all initiators are incorporated into the chain, or the initiator end groups are partially lost during polymerization and purification. If the initiator efficiency is less than 100%, the molecular weight should increase proportionally, that is, the polymer made from the initiator with the lowest efficiency should be longer. Thus, in principle, this hypothesis can be evaluated by comparing ^{1}H NMR and GPC homopolymers made from either **7** or **8** with the same target degrees of polymerization. For example, entries 1 and 5 (which each had a target DP of 20) or entries 2 and 6 (both target DP of 50) in Table S2 can be compared. The ^{1}H NMR data indicate that the polymers based on initiator **8** (see entries 2 and 6 in Table S2) should have higher molecular weights than those based on initiator **7** (entries 1 and 5 in Table S2). However, the GPC data indicates that, in fact, **8**-PMPC$_{20}$ is shorter than **7**-PMPC$_{20}$ and, similarly, that **8**-PMPC$_{50}$ is shorter than **7**-PMPC$_{50}$. Of course, an imperfect initiator efficiency cannot be excluded on the basis of these results because the GPC data are relative to the poly(ethylene oxide) calibration standards. Indeed, GPC analysis of a PMPC$_{20}$ homopolymer synthesized using a previously reported morpholine-based ATRP initiator[48] gave a lower M_n value (∼12000 g mol^{-1}, data not shown), indicating that the initiator efficiency is around 80%. This corresponds fairly well to the ^{1}H NMR DP, hence, the loss of end groups during polymerization appears to be a relatively minor problem.

In view of the above observations, loss of aromatic initiator signals during polymerization may explain the observed discrepancies for polymers prepared using initiators **7** and **8**. It has previously been shown that tertiary amine methacrylates can undergo partial transesterification in methanol to form methyl methacrylate and the corresponding alcohol.[49] Similar transesterification of the initiator would lead to formation of methyl 2-bromoisobutyrate and the corresponding rhodamine alcohol. However, no evidence for such transesterification side-reactions were observed by either ^{1}H NMR or HPLC in solutions of **7** or **8** in perdeuterated methanol over 24 h (data not shown). The addition of two equivalents of 2,2′-bipyridine had no effect and neither did the addition of both 2,2′-bipyridine and copper(II) bromide (data not shown). However, in the presence of the ATRP catalyst (i.e., 1 equiv of copper(I) bromide and 2 equiv of 2,2′-bipyridine in nitrogen-purged perdeuterated methanol), rapid transesterification was observed (as shown for initiator **8**, Figure S6). Thus, some transesterification is likely to occur during the PMPC polymerization, which would account for the higher DP values that are observed.

In general, the mean DP determined by absorption spectroscopy using the absorption coefficient of the initiator was much higher than the targeted DP values and also somewhat higher

than the values determined by ^{1}H NMR, where applicable (Table S1). This was the case for both initiators, although there was a closer correlation when using **8** than **7** (Table S2, compare entries 1−4 with entries 5−8). Thermogravimetric analyses (see Supporting Information, Figure S7) indicated that these homopolymers contain around 15% water, even after extensive drying under vacuum for 24 h at 90 °C. This is not unexpected, because it is well-known that water binds tenaciously to PMPC.[50] Unfortunately, even allowing for such relatively high water contents cannot account for the high DP values observed. A possible solvent effect was examined by determining the mean DP in methanol for selected polymers. However, this gave an essentially identical DP to that obtained in 0.1 M HCl (data not shown). An alternative explanation may be that the absorption coefficient of the initiator is reduced when conjugated to a polymer chain, due to steric congestion of the fluorophore.[51] If that is the case, initiator **7** should be more affected than initiator **8**, because the latter has a longer spacer group between the conjugated ring system and the initiator moiety. This hypothesis is consistent with the absorption data presented in Table S2.

Use of Rhodamine 6G-Labeled ATRP Initiators and a Methacrylic Monomer To Prepare pH-Responsive Diblock Polymers. Initiator **7** was also used to prepare a pH-responsive diblock copolymer comprising MPC and 2-(diisopropylaminoethyl) methacrylate, DPA, with a targeted composition of 7-$PMPC_{25}$-$PDPA_{90}$ (see Table S2, entry 9). Such diblock copolymers have previously been shown to be molecularly dissolved under acidic conditions but to self-assemble to form polymer vesicles at around physiological pH.[41]

For 7-$PMPC_{25}$-$PDPA_{90}$, the ^{1}H NMR data agrees with the target block composition. It was not possible to determine the amount of rhodamine directly in the final copolymer by this method due to its relatively high DP. Based on the results obtained for the homopolymers discussed above, the amount of rhodamine in the polymer is probably less than one label per chain. This copolymer had a unimodal GPC trace and a narrow polydispersity of 1.22, indicating a well-controlled polymerization. However, it was necessary to apply a different GPC-protocol to that used for the PMPC homopolymers, as the PDPA block does not dissolve in water at neutral pH. Therefore, the GPC results are not directly comparable. The mean DP of 7-$PMPC_{25}$-$PDPA_{90}$ determined by absorption spectroscopy is around 25% higher than that targeted. This is somewhat closer to the target value than that achieved for PMPC homopolymers prepared using this initiator (compare entry 9 with entries 1−4 in Table S2).

A similar diblock copolymer was prepared using monomer **9**. In this case, a nonfluorescent ATRP initiator was used to target a $PMPC_{25}$-P(DPA_{70}-*stat*-$\mathbf{9}_1$) copolymer and a total of 1 equiv of monomer **9** was used per copolymer chain. ^{1}H NMR studies revealed that the actual copolymer composition corresponded to $PMPC_{25}$-P(DPA_{66}-*stat*-$\mathbf{9}_1$) (Table S2, entry 10). This alternative approach to the synthesis of fluorescent diblock copolymers also led to a relatively narrow polydispersity. As with the longer PMPC-based homopolymers discussed above, the exact amount of **9** incorporated into the copolymer could not be accurately determined by ^{1}H NMR due to its relatively low concentration. However, the mean DP estimated from absorption spectroscopy studies is very close to that targeted in this case.

Hydrodynamic Size and Fluorescence Emission of Rhodamine 6G-Labeled PMPC-PDPA Diblock Copolymers as a Function of pH. The pH-dependent behavior of the two PMPC-PDPA diblock copolymers was investigated by dynamic light scattering, fluorescence spectroscopy, and absorption spectroscopy. Figure 4 shows that the hydrodynamic diameter for both diblock copolymers increases by an order of magnitude between pH 6 and 7, indicating vesicle formation as previously reported.[41] The relative fluorescence intensity shows a marked difference between the two copolymers. Up to pH 6, a small reduction is observed for both copolymers, which is probably largely due to dilution effects caused by the addition of base. Between pH 6 and 7, a significant decrease in the relative fluorescence intensity is observed in both cases, which correlates with the increased hydrodynamic diameter. Above pH 7, there is a marked difference between the two copolymers: The 7-$PMPC_{25}$-$PDPA_{90}$ vesicles are still fluorescent, but the relative fluorescence intensity is reduced to approximately half of that observed below pH 6. In contrast, the fluorescence observed for the $PMPC_{25}$-P(DPA_{66}-*stat*-$\mathbf{9}_1$), is completely quenched above pH 7. To understand this observation, absorption spectra were recorded as a function of pH. More specifically, the maximum absorbance of the rhodamine unimer (at approximately 540 nm) was compared to that due to the rhodamine dimer at 508 nm from pH 2 to pH 9 (Figure S8).[44] These results are shown in Figure 4b. From pH 2 to pH 6, the A_{max}/A_{508} ratio does not change, indicating that the unimer−dimer equilibrium is not affected. This was expected, because there is on average one rhodamine per copolymer chain and, below pH 6, these chains are molecularly dissolved, which is why the pH-dependence should be similar to that of the native rhodamine dye (Figure 3b). For 7-$PMPC_{25}$-$PDPA_{90}$, there is a maximum of one fluorophore per chain (due to the initiator fragment). However, for $PMPC_{25}$-P(DPA_{66}-*stat*-$\mathbf{9}_1$), a certain fraction of chains will contain more

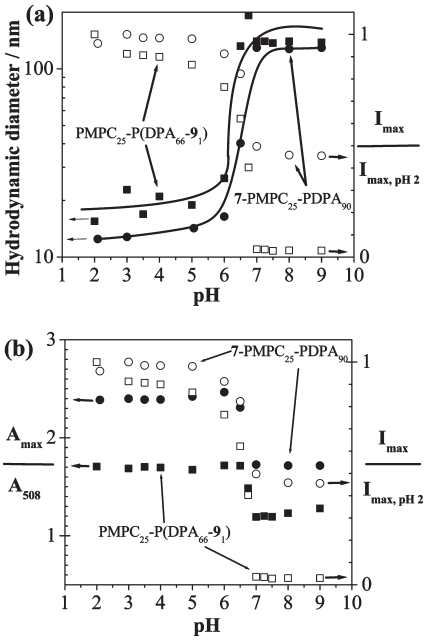


Figure 4. (a) Variation of hydrodynamic diameter and relative fluorescence intensity on increasing the pH of 0.20% w/w aqueous solutions of 7-$PMPC_{25}$-$PDPA_{90}$ and $PMPC_{25}$-($PDPA_{66}$-**9**) with 1 M NaOH. (b) Relative change in the 530 and 508 nm absorption bands compared to the maximum normalized fluorescence intensity and relative fluorescence intensity as a function of pH. The initial volume was 25 mL and the total amount of added base was no more than 2 mL.

than one label, especially if the reactivity ratios of **9** and DPA deviate significantly from unity. In this case, the dye labels may be so close to each other that dimer formation is significant even for the molecularly dissolved chains (Figure S8). This explains why the A_{max}/A_{508} ratio is lower for $PMPC_{25}$-P(DPA_{66}-*stat*-$\mathbf{9}_1$) than for **7**-$PMPC_{25}$-$PDPA_{90}$.

Above pH 7, the A_{max}/A_{508} ratio shifts significantly, indicating dimer formation. This is more pronounced for $PMPC_{25}$-P-(DPA_{66}-*stat*-$\mathbf{9}_1$), whose absorption spectrum is very similar to the literature spectra reported for rhodamine dimers.[44] Therefore, polymer self-assembly above neutral pH, combined with a hydrophobic environment strongly favors formation of dimers and possibly even higher order aggregates.[52] In contrast, when the pH is increased for a dilute aqueous solution of **7**-$PMPC_{25}$-$PDPA_{90}$, the relative distance between chromophores is greater and these labels experience a highly polar environment. Thus, these results indicate that dimer formation due to high local fluorophore concentration and/or decreased solvent quality is the main cause of fluorescence quenching. In addition, there may be some contribution from the tertiary amines in the PDPA block as these species are known to act as fluorescence quenchers.[53]

CONCLUSIONS

A facile one-step protocol has been developed to prepare hydroxy-functional rhodamine derivatives. Esterification of these protonated precursors using 2-bromoisobutyryl bromide or 2-bromoisobutyric anhydride afforded three new rhodamine 6G-based ATRP initiators in good yields. One initiator exhibited fluorescence below pH 4 but was nonfluorescent at higher pH, while two initiators proved to be highly fluorescent over a wide pH range (from pH 1 to pH 10). A new permanently fluorescent rhodamine 6G-based methacrylic monomer was also synthesized using a similar approach. All compounds exhibited similar absorption characteristics to rhodamine 6G. In addition, the quantum yields estimated for the hydrophilic labels were similar to those obtained for rhodamine 6G in PBS at pH 7, whereas the hydrophobic labels had somewhat lower quantum yields. The two initiators were used to prepare reasonably well-defined, rhodamine-labeled poly(2-(methacryloyloxy)ethyl phosphorylcholine) via ATRP in methanol at 20 °C. For both initiators, mean degrees of polymerization calculated for these homopolymers using absorption spectroscopy were significantly higher than those estimated by ^{1}H NMR spectroscopy. In general, end-group analyses calculated from absorption spectroscopy correlated more closely with ^{1}H NMR data for initiator **8** than for initiator **7**. The former has a longer spacer connecting the chromophore to the polymer chain, which may lead to less perturbation of the intrinsic chromophore signature.

In addition, two rhodamine 6G-labeled vesicle-forming diblock copolymers were synthesized. One copolymer was prepared using a rhodamine initiator and therefore contained a maximum of one *terminal* fluorophore per chain. The other copolymer was synthesized using a methacrylic rhodamine monomer, which was statistically incorporated into the tunably hydrophobic PDPA block. In both cases, increasing the solution pH above 6 led to vesicle formation, which led to a significant reduction in fluorescence. However, if the fluorophore was attached to the terminus of the hydrophilic chains, only *partial* reduction in fluorescence was observed, whereas statistical incorporation of the fluorophore into the pH-responsive membrane-forming block led to *complete* quenching.

In related work with collaborators, we have already demonstrated that these rhodamine 6G-labeled copolymers have the appropriate molecular characteristics for a range of biological studies, such as various intracellular delivery experiments.[28−31]

ASSOCIATED CONTENT

Supporting Information. Full experimental protocols and characterization data for all reported compounds, transesterification kinetic data, additional absorption spectra, digital photographs, TGA curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

Biocompatibles UK Ltd. is thanked for CASE support of J.M. and N.J.W., for supplying the MPC monomer, and for permission to publish this work. The University of Sheffield is thanked for a Ph.D. studentship to support J.M. BBSRC is thanked for a Ph.D. studentship to support N.J.W. Dr. J. A. Balmer and Mr. L. A. Fielding are acknowledged for assistance with the TGA measurements. The three reviewers of this manuscript are acknowledged for their useful comments.

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