A Non-toxic Cellular Staining Agent Based on Fluorescently Labeled Poly[2-(methacryloyloxy)ethyl phosphorylcholine]

N. J. Warren[*], I. Canton[**], J. Madsen[*], S. P. Armes[*], G. Battaglia[**] and A. L. Lewis[***]

[*] Dainton Building, Department of Chemistry, The University of Sheffield, Brook Hill, South Yorkshire, S3 7HF, UK

[**] Department of Biomedical Science, The University of Sheffield, Western Bank, Sheffield, South Yorkshire, S10 2TN, UK.

[***] Biocompatibles UK Ltd., Chapman House, Farnham Business Park, Weydon Lane, Farnham, Surrey, GU9 8QL, UK.

ABSTRACT

In this study we show that poly[2-(methacryloyloxy)ethyl phosphorylcholine] (PMPC) can be used as a carrier for small molecules by covalently attaching a fluorescent label. ATRP was used to prepare various fluorescent PMPC homopolymers or statistical copolymers of varying molecular weights. Results indicate that the nature of the fluorescent tag does not have any detrimental effect on the polymerization and the resulting (co)polymers exhibitied the expected absorption/emission profiles that are characteristic of the fluorescent label. Cellular viability assays indicated negligible cytotoxicity after incubation of HDF cells for 24 hours in the presence of the (co)polymers.

Keywords: Phosphorylcholine, ATRP, Biocompatible, cell uptake

1 INTRODUCTION

2-(Methacryloyloxy)ethyl phosphorylcholine) [MPC] is a biomimetic monomer that confers excellent biocompatibility when copolymerized with other methacrylic monomers. Thus, despite its relatively high cost, MPC-based copolymers are currently used in a range of commercial applications, including surface coatings for coronary stents, ear grommets, low irritation soft contact lenses and certain cosmetic formulations. MPC can be polymerized via Atom Transfer Radical Polymerisation (ATRP) with good control over both the molecular weight distribution and the target degree of polymerisation.[1,2] Previously, our group has shown that PMPC-b-poly(2 (diisopropylamino)ethyl methacrylate) (PMPC-PDPA) polymersomes are preferentially internalized by cells versus equivalent PEO-PDPA polymersomes.[3,4] This is due to the stealthy nature of the PMPC corona conferred by its superior hydration leading to favourable interactions with phospholipid cell membranes. Recently, Madsen et al developed and optimised a facile two-step synthesis of both a rhodamine 6G ATRP initiator and methcrylic monomer from cheap and readily available materials.[5]

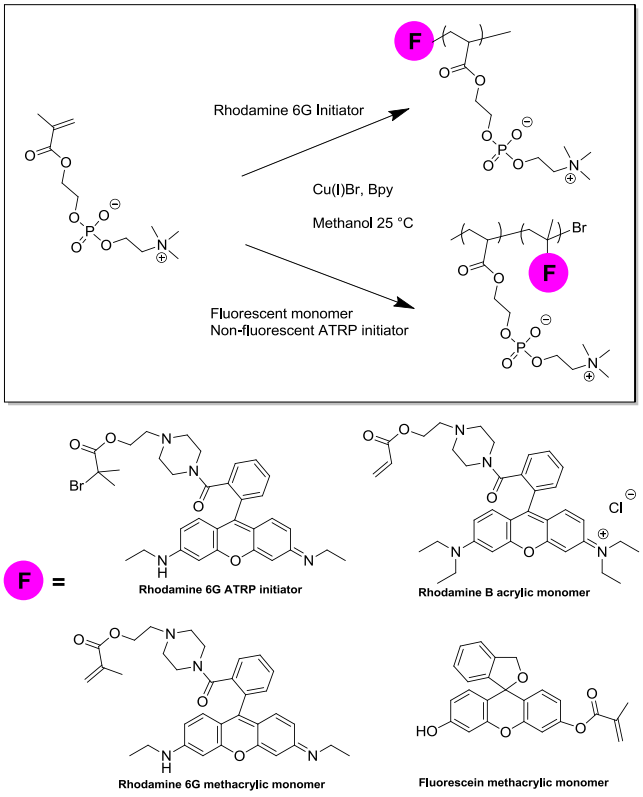

Figure 1: Reaction scheme for the synthesis of fluorescent PMPC homopolymers and statistical copolymers using either a rhodamine 6G derivatized fluorescent ATRP initiator or rhodamine or fluorescein cent comonomers.

Labeling of the PMPC-PDPA diblock copolymer made it possible to track the block copolymer vesicles to be tracked through tissue-engineered human oral mucosa.[6]

Herein we extend our previous work by preparing a series of well-defined, low polydispersity fluorescently-labeled PMPC homopolymers and statistical copolymers by atom transfer radical polymerization (ATRP). Fluorescent labeling is achieved via two strategies: (i) terminal labeling

Target composition	^{1}H NMR		Aqueous GPC		UV/Vis
	Conversion	M_n	M_n	M_w/M_n	M_n
Rh-PMPC$_{50}$	100	21,100	10,200	1.23	26,000
Rh-PMPC$_{100}$	100	31,400	26,000	1.49	50,900
PMPC$_{25}$-*stat*-RhMA$_1$	96	7,400	5,800	1.21	14,900
P(MPC$_{50}$-*stat*-RhMA$_1$)	100	16,500	9,500	1.28	26,500
PMPC$_{100}$-*stat*-RhMA$_1$	100	-	16,000	1.29	42,100
P(MPC$_{50}$-*stat*-FlMA$_1$)	100	14,500	10,500	1.26	17,800
PMPC$_{100}$-*stat*-FlMA $_1$	100	-	18,000	1.28	22,200
PMPC$_{100}$-*stat*-RhBA$_1$	98		32,600	2.03	-

Table 1: . Summary of monomer conversions and molecular weight data obtained for various fluorescently-labeled PMPC (co)polymers. GPC analyses were conducted using 0.05 M Trisma buffer with 0.2 M sodium nitrate (pH 7) as the eluent and a series of near-monodisperse poly(ethylene oxide) calibration standards.

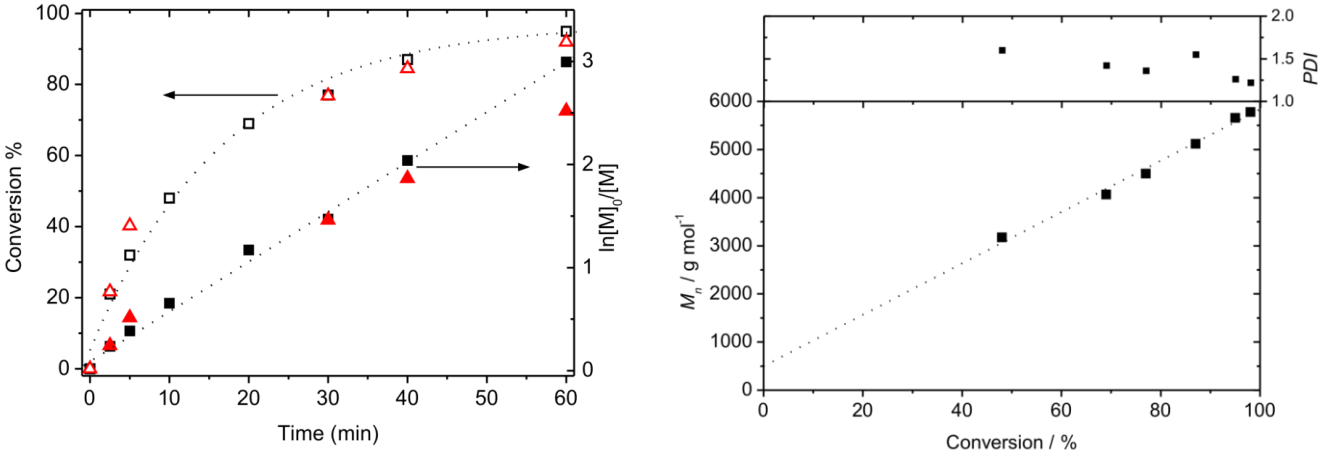

Figure 2: Left: Kinetic data obtained for Evolution of comonomer conversion with time and the corresponding semi-logarithmic plot for the statistical copolymerization of MPC (□ and ■) with RhMA (△ and ▲) at 20°C in methanol for 1 h. Right: Evolution of M_n and polydispersity with conversion for the statistical copolymerization of MPC with RhMA. Reaction was carried out in methanol at 25°C where [MPC]: [initiator]: RhMA]: [CuCl]: [bpy] = 50: 1: 1: 1: 2

using a rhodamine 6G-based ATRP initiator; (ii) statistically placing one fluorescent chromophore within the polymer chains by using fluorescent comonomers (Figure 1). The basic photophysical properties PMPC (co)polymers as well as the effects on HDF cells when exposed to the (co)polymers for 24 hours is investigated.

2 RESULTS AND DISCUSSION

On immediate inspection, the presence of two secondary amines and two tertiary amines within the rhodamine methacrylate molecule suggests that it may act as a ligand to the copper(I) during ATRP, thus competing with the bipyridine. To ensure there was no resulting effect on the ATRP process, a kinetics study on the copolymerisation of MPC with the RhMA was carried out. A DP of 25 for the MPC was targeted along with one RhMA per chain. The monomer conversion versus time plots in Figure 2 indicate that almost full conversion of both rhodamine 6G methacrylate (measured by HPLC) and MPC monomer (measured by ^{1}H NMR spectroscopy) was achieved after 60 minutes with no apparent preference between monomers during the reaction. This suggests that there is minimal presence of any short poly(RhMA) oligomers. The semi logarithmic plot indicates that the reaction proceeds with first order kinetics which is typical of ATRP. The living character of the polymerisation was confirmed by a linear increase in molecular weight versus conversion indicating that there were minimal chain terminations occurring during the reaction. Moreover, the resulting PMPC$_{25}$-*stat*-RhMA$_1$ copolymer had a low polydispersityty (M_w/M_n = 1.21). These data confirm that there are no detrimental effects on the ATRP process due to the presence of rhodamine methacrylate.

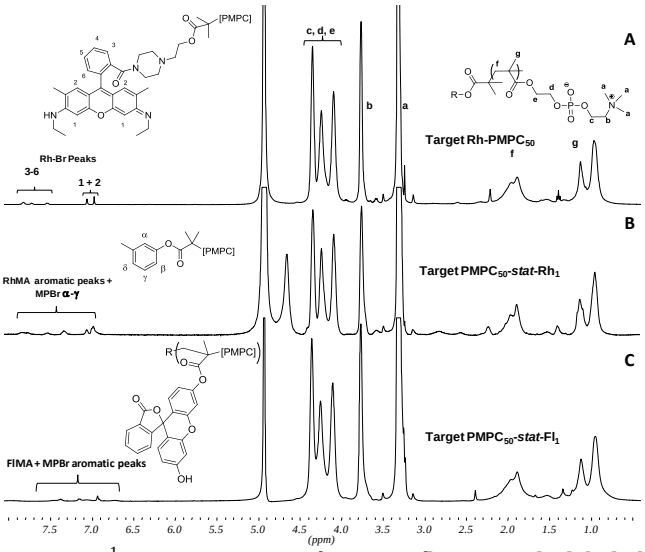

Figure 3: ^{1}H NMR spectra of various fluorescently-labeled PMPC homopolymers and statistical copolymers. recorded in CD_3OD.

Representative ^{1}H NMR spectra recorded for fluorescent PMPC (co)polymers are shown in Figure 3 and all show the expected peaks resulting from the MPC units. The lack of vinyl peaks indicates the successful purification of each (co)polymer. As well as the MPC signals, spectrum A has peaks resulting from the rhodamine 6G end group, these peaks are sharp since the rhodamine groups are all present in the same environment (at the terminus) on the polymer chains. The sharpness of these peaks as well as their location on the spectrum allows the M_n to be determined. According to the spectra, these polymers have a larger M_n than originally targeted, equating to 70% and 84% efficiency for the Rh-PMPC$_{50}$ and Rh-PMPC$_{100}$ respectively. Spectrum B is similar to A in that there are also peaks resulting from rhodamine 6G, however, in this case they are more broad since the location within the copolymer chains in this case is random. Additionally in spectrum B, the peaks resulting from the non fluroescent 2-methylphenyl bromoisobutyrate ATRP initiator are present. These also allow an estimation of the M_n of the copolymer to be made. Otherwise the RhMA peaks were too broad to be used to determine the M_n as the signal to noise ratio was too high. Spectrum C shows the spectrum of the PMPC copolymerised with FlMA. In these copolymers, like the RhMA labeled copolymer, the peaks are broad and tend to be overcome by the signal to noise ratio. The peaks are, however visible, indicating that the FlMA has been incorporated into the copolymer.

Visible absorbtion and fluorescent emission spectroscopy was carried out on PMPC (co)polymers labeled with either the rhodamine 6G, rhodamine B or fluorescein derived labels. The normalised absorbance spectra show that the characteristic properties of each chromophore is maintained when incorporated into the PMPC chains (Figure 4) with maximum absorbances being observed at 488 nm for the PMPC$_{100}$-*stat*-FlMA$_1$, 542 nm for the PMPC$_{100}$-*stat*-RhMA$_1$ and 576 nm for the PMPC$_{100}$-*stat*-RhBA$_1$. The fluorescence spectra were also characteristic of the fluorescent chromophores showing maximum fluorescent emission at 513 nm, 575 nm and 610 nm respectively. These results indicate that, in each case, the photophysical properties of the relevant fluroescent label is retained when incorporated into the PMPC chains.

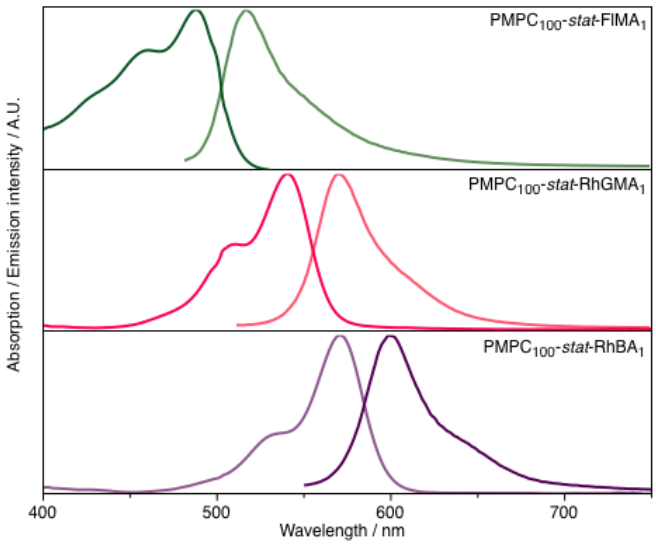

Figure 4: Visible absorption and fluorescent emission spectra recorded for various fluorescently labeled PMPC (co)polymers with target degrees of polymerization of 100. Spectra were recorded in 0.1 M PBS at pH 7.4.

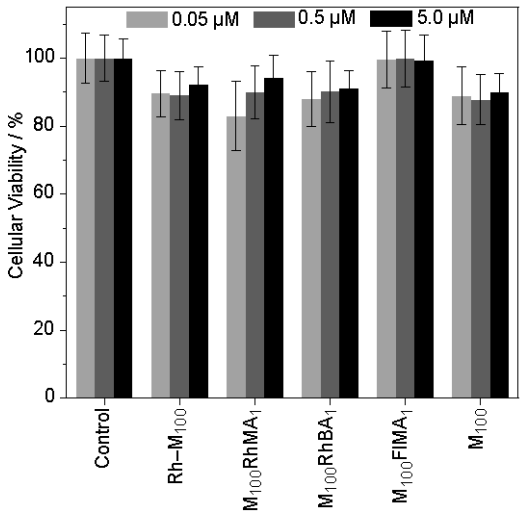

Figure 5: MTT -ESTA results showing the relative cytotoxicities to HDF cells of PMPC$_{100}$ (co)polymers labeled with either the rhodamine 6G ATRP initiator (Rh-M_{100}), rhodamine 6G methacrylate (M_{100}RhMA$_1$) rhodamine B acrylate (M_{100}RhBA$_1$), fluorescein methacrylate (M_{100}FlMA$_1$) as well as an unlabeled PMPC$_{100}$ homopolymer. Cells were incubated for 24 hours at 0.05 μM, 0.5 μM, 5.0 μM (co)polymer concentrations in media.

Cell cytotoxicity studies were carried out on human dermal fibroblast cells. After 24 h incubation with each of the (co)polymer solutions at three concentrations, only very weak cytotoxicity was observed in each case (Figure 5). Concentration dependence was observed for the $PMPC_{100}$-*stat*-$RhMA_1$ copolymer ranging from 82 % for the lowest concentration to 91 % for the highest concentration. This is likely to be due to the higher metabolic activity required to internalize and subsequently digest the copolymer. The fact that equivalent toxicity was observed for the unlabeled $PMPC_{100}$ homopolymer as the rhodamine (6G or B) labeled (co)polymers indicates that there is very little negative effect of the presence of the fluorescent label in this case. The statistical copolymer labeled with FlMA however, showed no toxicity whatsoever, being almost identical to the control. This indicates that the nature of the fluorescent label does have some influence on the fate of the co)polymers.

3 CONCLUSIONS

A series of fluroescently labeled PMPC polymers were prepared by using either a rhodamine 6G derivatized ATRP initiator, or by copolymerization of the MPC with fluorescent comonomers. The resulting homopolymers and statistical copolymers were all narrow polydispersity indicating good control of the polymerization. Copolymerization of MPC with the rhodamine B acrylic comonomer produced a relatively polydisperse copolymer. This is due to the poor monomer compatibility of methacrylates and acrylates. The photophysical properties of the fluorescent chromophores was retained after incorporation into the PMPC chains as judged by both visible absorption and fluroescent emission spectroscopy. Cytotoxicity studies indicated that minimal toxicity was observed when HDF cells were treated with (co)polymer solutions at three different concentrations.

REFERENCES

(1)Ma, I. Y.; Lobb, E. J.; Billingham, N. C.; Armes, S. P.; Lewis, A. L.; Lloyd, A. W.; Salvage, J. *Macromolecules* **2002**, *35*, 9306.

(2)Ma, Y.; Tang, Y.; Billingham, N. C.; Armes, S. P.; Lewis, A. L.; Lloyd, A. W.; Salvage, J. P. *Macromolecules* **2003**, *36*, 3475.

(3)LoPresti, C.; Massignani, M.; Fernyhough, C.; Blanazs, A.; Ryan, A. J.; Madsen, J.; Warren, N. J.; Armes, S. P.; Lewis, A. L.; Chirasatitsin, S.; Engler, A. J.; Battaglia, G. *ACS Nano* **2011**, in press.

(4)Massignani, M.; LoPresti, C.; Blanazs, A.; Madsen, J.; Armes, S. P.; Lewis, A. L.; Battaglia, G. *Small* **2009**, *5*, 2424.

(5) Madesn, J.; Warren, N. J.; Armes, S. P.; Lewis, A. L.; *Biomacromolecules*, **2011** accepted for publication

(6)Hearnden, V.; Lomas, H.; MacNeil, S.; Thornhill, M.; Murdoch, C.; Lewis, A.; Madsen, J.; Blanazs, A.; Armes, S.; Battaglia, G. *Pharmaceutical Research* **2009**, *26*, 1718.