Blood compatibility of hydrophilic polyphosphoesters

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ABSTRACT

Polyphosphoesters (PPEs) are a class of versatile degradable polymers. Despite the high potential of this class of polymer in biomedical applications, little is known about their blood interaction and compatibility. We evaluated the hemocompatibility of water-soluble PPEs (with different hydrophilicity and molar masses) and PPE-coated model nanocarriers. Overall, we identified high hemocompatibility of PPEs, comparable to poly(ethylene glycol) (PEG), a well-known water-soluble and biocompatible polymer. Hydrophilic PPEs caused no significant changes in the blood coagulation, negligible platelet activation, the absence of red blood cells lysis or aggregation. However, when a more hydrophobic copolymer was studied, some changes in the whole blood clot strength at the highest concentration were detected, but only at concentrations above that are typically used for biomedical applications. Also, the PPE-coated model nanocarriers showed high

hemocompatibility. These results contribute to define hydrophilic PPEs as a promising platform for degradable and biocompatible materials in the biomedical field.

KEYWORDS: blood compatibility, poly(ethylene glycol), biodegradable polymer, hemocompatibility.

MAIN TEXT

Polyphosphoesters (PPEs) belong to the few polymer classes that enable the synthesis of well-defined and degradable water-soluble polymers.[1,2] The presence of a pentavalent phosphorous in the main chain allows to tune the polymer properties (e.g. hydrophilicity, crystallinity, degradability, thermal stability, etc.) by varying the lateral group, providing to the polymers high versatility, interesting for biomedical applications, e.g. as biodegradable substitutes for poly(ethylene glycol) (PEG). To date, several studies reported the hydrolytic and enzymatic degradation of PPEs *in vitro*.[3,4] Besides, PPE-containing (co)polymers were reported as non-cytotoxic against different cell lines even at high concentrations.[1] Hydrophilic PPE-coated nanocarriers exhibit a so-called "stealth effect", similar to PEG but their cellular uptake could be controlled depending on the hydrophilicity of the respective PPE.[5] Based on such results, PPEs have been proposed for different biomedical applications, e.g. protein-polymer conjugates,[6–8] nanocarriers loaded with drugs,[9] antimicrobial agents,[10] or gene vectors,[11] or hydrogels for tissue engineering.[12] The evaluation of PPEs blood compatibility is an important step to translate these *in vitro* results to the clinics. In a study, Murgia and co-workers evaluated the hemolysis and the complement system activation by cubosomes, i.e., lyotropic nanoparticles with cubic internal nanostructure, that were self-assembled from poly(propylene oxide)-block-poly(methyl ethylene phosphate). A lower cytotoxicity compared to the commonly used Pluronic F127 analogues was detected.[13] A systematic blood compatibility study of different PPEs has not been reported to date. Here, we report the hemocompatibility of a set of PPEs with various molar masses and different hydrophilicity and PPE-coated model nanocarriers. The results were compared to PEG, a well-known hemocompatible polymer.[14]

A set of PPEs (Figure 1) was synthesized via ring-opening polymerization of cyclic phosphates or phosphonates and the polymers were characterized for their purity, molar mass, and molar mass dispersity. The PPE coated nanocarriers were prepared by aza-Michael addition between the amino group on the polystyrene surface and poly(ethyl ethylene phosphonate) (PEtEP), which was previously ω-functionalized with commercial 4-(maleinimido)phenyl isocyanate (Figure 1). Hemocompatibility assays were performed on the samples using fresh blood collected from consented donors at the University of British Columbia. More details on the experimental procedures are reported in the Supporting Information, section 1. The samples' main features are shown in Table 1.

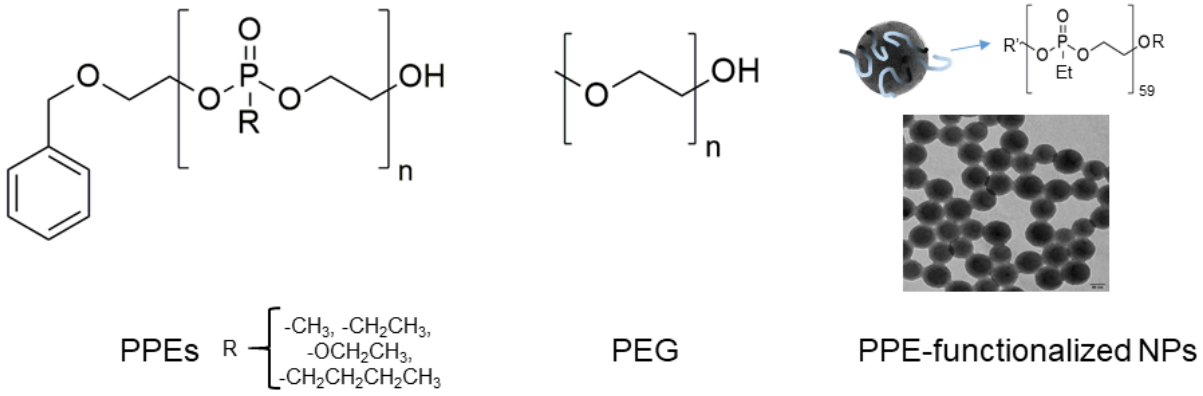


Figure 1. Set of samples analysed in this work.

Table 1. Features of samples analysed in this work.

Type of sample	Sample	Name	Features
PPEs	**1**	Poly(methyl ethylene phosphonate (PMeEP)	R= -CH_3; M_n: 10kDa; DP_n: 75
	2a	Poly(ethyl ethylene phosphonate) (PEtEP)	R= -CH_2CH_3; M_n: 7 kDa; DP_n: 50
	2b	Poly(ethyl ethylene phosphonate) (PEtEP)	R= -CH_2CH_3; M_n: 10 kDa; DP_n: 75

	2c	Poly(ethyl ethylene phosphonate) (PEtEP)	R= -CH_2CH_3; M_n: 26 kDa; DP_n: 190
	3	Poly(ethyl ethylene phosphate) (PEEP)	R= -OCH_2CH_3; M_n: 7.2 kDa; DP_n: 50
	4	Poly (ethyl ethylene-co butyl ethylene phosphonate) (PEtBuEP)	R= -CH_2CH_3, -$CH_2CH_2CH_2CH_3$ M_n: 10 kDa; DP_n: 71; 36 (-CH_2CH_3) and 31 (-$CH_2CH_2CH_2CH_3$)
PEG	**5a**	Polyethylene glycol (PEG)	M_n: 8 kDa; DP_n: 133
	5b	Polyethylene glycol (PEG)	M_n: 20 kDa; DP_n: 333
	5c	Polyethylene glycol (PEG)	M_n: 35 kDa; DP_n: 583
PPE-functionalised PS-NPs	**6**	PS nanoparticles functionalised with PEtEP	PEtEP: R= -CH_2CH_3; M_n: 8.4 kDa; DP_n: 59; number of polymers attached to each particle: 4000

The influence of PPEs on the blood coagulation was investigated by a number of assays including the measurement of plasma clotting times and whole blood clotting in human blood. The pro- or anticoagulant nature of the PPE polymers was measured using two clinical coagulation assays: prothrombin time (PT) (Figure 2a) and activated partial thromboplastin time (APTT) (Figure 2b) in human plasma. The data showed no significant changes in the blood coagulation behaviour at any of the concentrations tested (0.1 to 1 mg/mL final concentration) with respect to the buffer incubated plasma control, differently from the positive control (heparin, UFH), which showed an anticoagulant effect in the APTT assay.

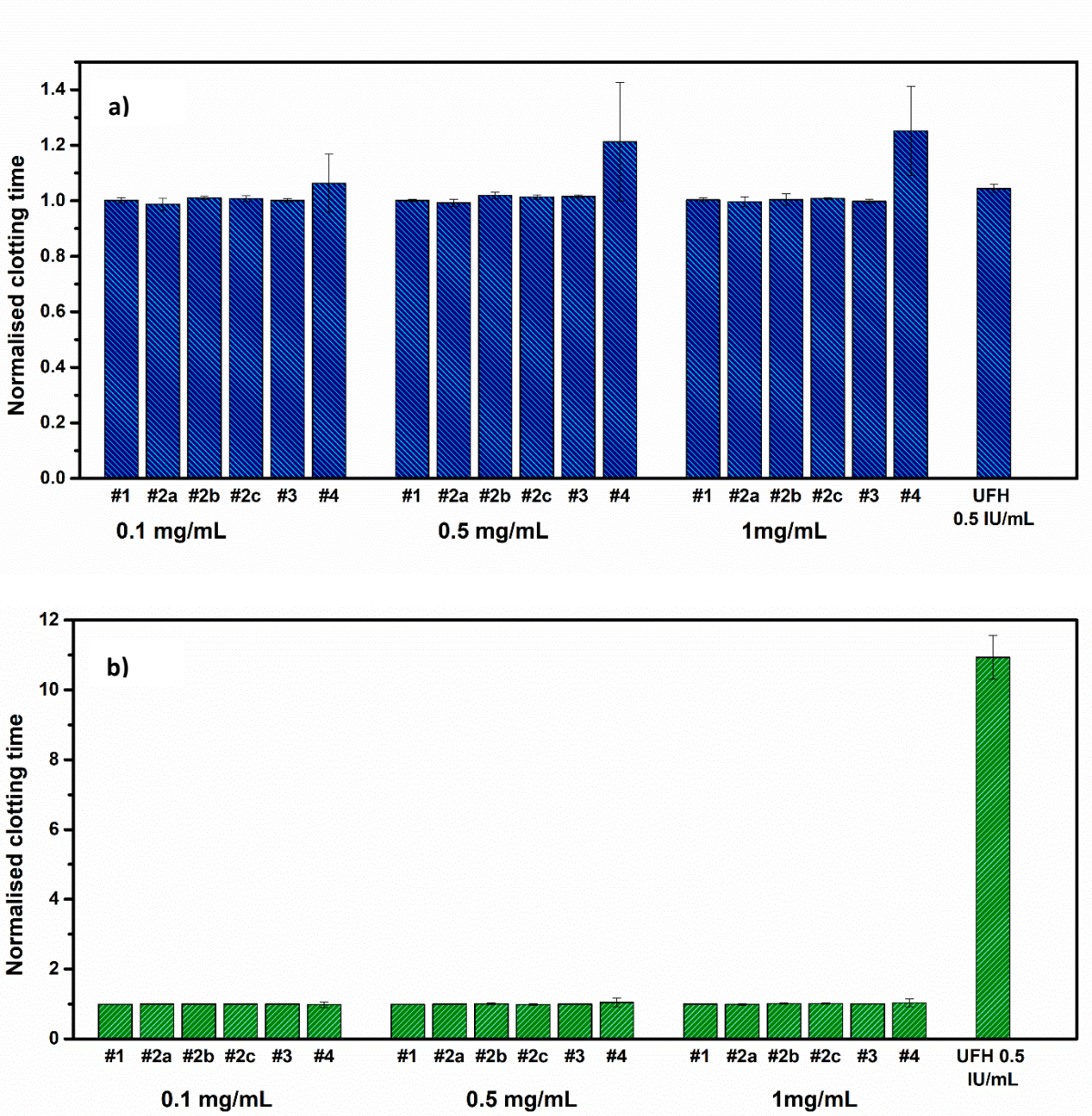
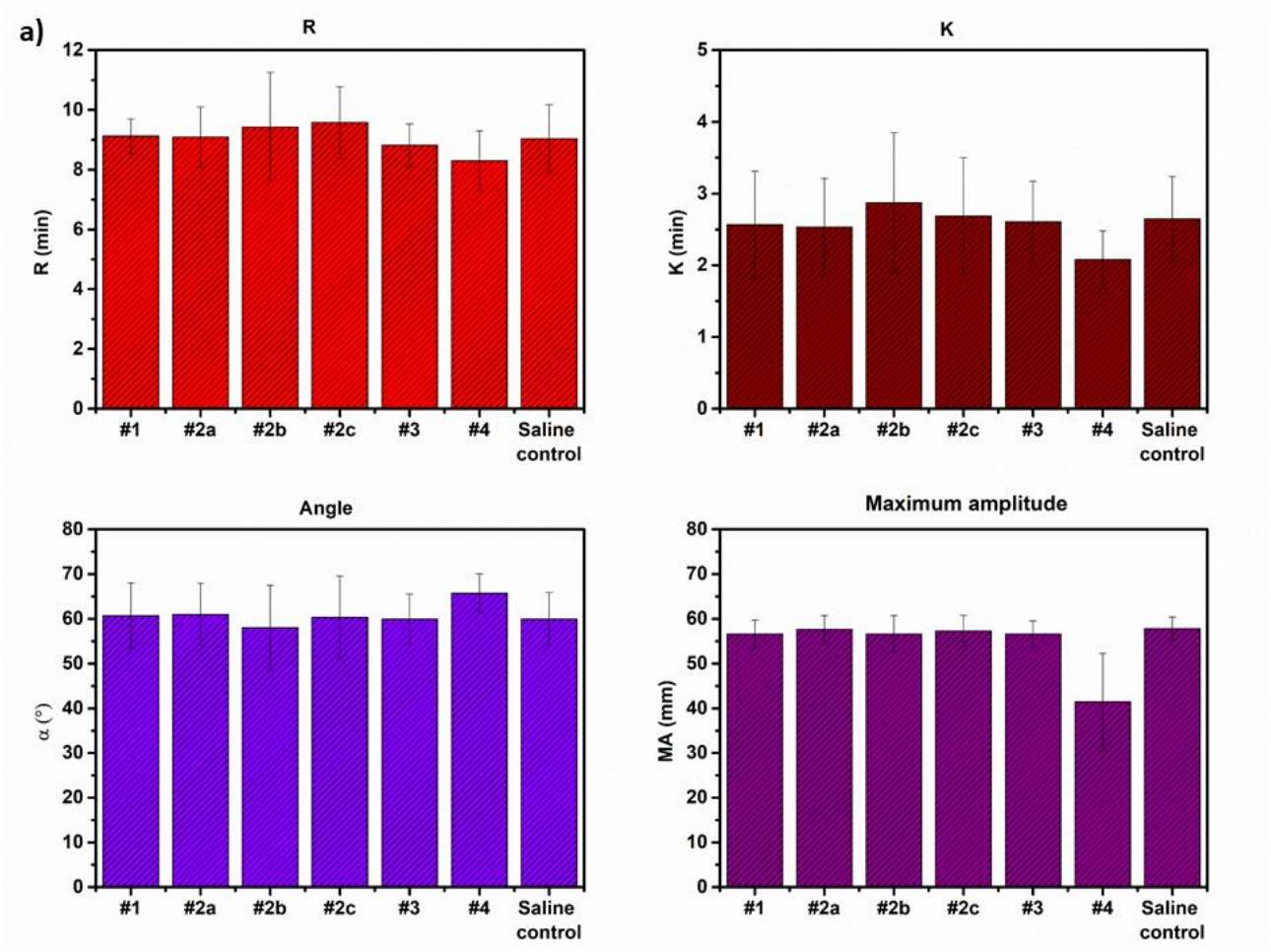


Figure 2. Effect of PPE polymers on a) prothrombin time (PT) and b) activated partial thromboplastin time (APTT) at different concentrations (0.1, 0.5, 1 mg/mL). The clotting time was normalized for the saline control run for each sample (33.2±2.8 seconds for APTT and 9.8±1.1 seconds for PT analysis). Unfractionated heparin (UFH) was used as a control. The data have been obtained by analyzing the blood of three different donors (N=3).

To further investigate the influence of PPEs on the whole blood coagulation, rotational thromboelastometry analysis (ROTEM) was used.[15] Whole blood samples (N=3, independent

donors) were incubated with different PPEs at 1 mg/mL concentration, and the clotting parameters were measured, including the 'R' time, 'K' values, Angles and maximum clot firmness (MA) (Figure 3a). We also measured the influence of PPEs incubated with buffer for 2 weeks and then used for ROTEM measurements to understand whether polymer degradation influence blood coagulation. The ROTEM profile of sample #1 is shown in Figure 3b and the others are reported in SI, Fig S2. As shown in the figure, the PPEs did not modify any of the ROTEM parameters, even in their degraded form (measurements were repeated after 2 weeks of incubation) suggesting that the PPEs are not influencing the whole blood clot formation. Only sample #4 at a concentration of 1 mg/mL showed an unusual ROTEM profile (SI Figure S2), similar to that shown by amphiphilic polymers such as poly (*N*-isopropylacrylamide).[16] It is important to note that this sample at lower concentrations did not modify the blood clotting profile.



b)

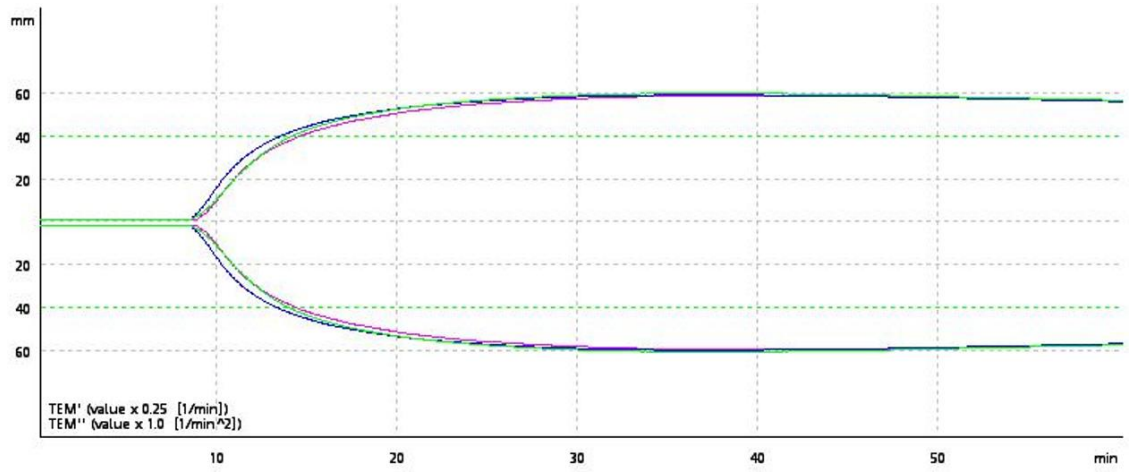


Figure 3. Whole blood coagulation studied using rotational thromboelastography (ROTEM) in presence of PPEs. a) Reaction time (R), time for first significant clot formation, K (achievement of certain clot firmness, Angle (kinetics of clot development) and MA (maximum amplitude-maximum strength of clot). b) ROTEM profile of sample #1 at 1 mg/mL (as made (blue), two weeks (green) and saline control (pink)).

Next, we investigated interaction of platelets with PPEs by measuring the platelet activation by flow cytometer in platelet rich plasma. The platelet activation in presence of the PPEs was detected by measuring the expression of the activation marker CD62P on the surface of platelets using anti-CD62P antibody. In support of the normal whole blood clotting profile previously shown, PPEs did not show any significant platelet activation at any concentration (SI Fig S3a) suggesting that PPEs did not interact unfavourably with the platelets. Further, we measured the interaction of PPEs with red blood cells (RBC) by measuring their lysis in whole blood as well as the RBC aggregation.[17] Linear hydrophilic polymers are known to aggregate RBCs by adsorbing to RBC surface and form bridge between the cells.[18] PPEs did not induce any lysis of the cells as shown by the data given in (SI Fig. S3b) at different concentrations. Besides, the RBC aggregation was measured by optical microscopy. Representative optical micrographs are shown in Figure 4; in agreement with the other results, we did not detect any RBC aggregation or crenation in the samples, except for sample #4 at 1 mg/mL concentration, which showed slight aggregation (SI Fig S4).

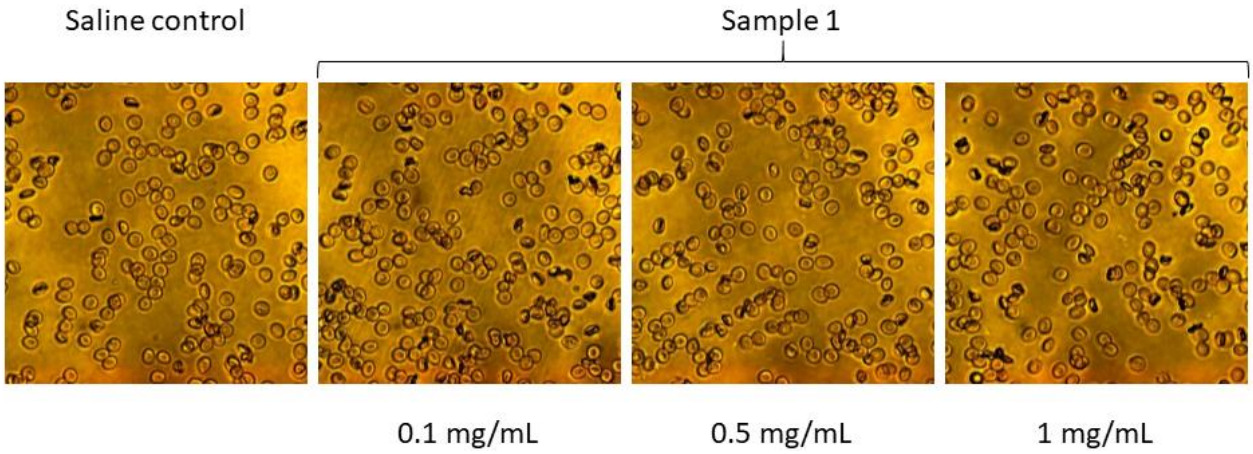


Figure 4. RBC aggregation of sample #1 observed by optical microscopy (magnification: 40x). The sample does not show any detectable aggregation at all the concentrations tested (0.1, 0.5, 1mg/mL), in comparison with the saline control shown on the left. The analysis made on the other samples are reported in SI, Fig S4.

Having obtained promising results for soluble PPEs, we also investigated polystyrene NPs functionalised with PPEs as model nanocarriers for biomedical applications. Such model nanocarriers were studied before with respect to cellular uptake and protein adsorption and indicated a similar behavior to PEGylated nanocarriers.[19] For the PPEylated nanocarriers satisfactory hemocompatibility data was (reported in SI, Figures S5-6), except for light crenation observed in the blood cells in presence of functionalised nanoparticles (visible from optical images, Figure S4).

Overall, we identified high hemocompatibility of hydrophilic PPEs (samples #1-4), comparable to PEG (samples #5a-c). In particular, changes in the molar mass (samples #2a-c) did not influence the polymer hemocompatibility. The more hydrophobic copolymer (sample #4) presented some changes in the whole blood clot strength at the highest concentration, nonetheless it was highly compatible at the concentrations usually needed for biomedical applications. The conjugation of PPEs to model nanocarriers also did not alter their hemocompatibility. These results underline that hydrophilic PPEs are promising, biodegradable substitutes to currently used PEG, which might cause side effects in some patients.[20,21] We believe that the data presented herein will further stimulate the use of PPEs in

biomedical and translational studies for fully degradable drug formulations without negative immune responses.

ACKNOWLWDGEMENTS

C.P. and F.W thank Angelika Manhart, Katia Klein, and Renate Vanbrandwijkr from the Max Plank Institute for Polymer Research (Mainz) for synthetic assistance and the endotoxin test performed on the samples. JNK acknowledges funding by the Canadian Institutes of Health Research and the Natural Sciences and Engineering Council of Canada (NSERC). The infrastructure facility is supported by the Canada Foundation for Innovation (CFI) and the British Columbia Knowledge Development Fund (BCKDF).

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