Characterisation of the binding interaction between poly(L-lysine) and DNA using the fluorescamine assay in the preparation of non-viral gene delivery vectors

Martin L. Read[a], Thomas Etrych[b], Karel Ulbrich[b], Leonard W. Seymour[a,*]

[a] *CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TT, UK*
[b] *Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague 6, Czech Republic*

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Abstract A major factor limiting the development of non-viral gene delivery systems is the poor characterisation of polyelectrolyte complexes formed between cationic polymers and DNA. The present study uses the fluorescamine reagent to improve characterisation of poly(L-lysine) (pLL)/DNA complexes post-modified with a multivalent hydrophilic polymer by determining the availability of free amino groups. The results show that the fluorescamine reagent can be used to monitor the self-assembly reaction between pLL and DNA and the degree of surface modification of the resultant complexes with a hydrophilic polymer. This experimental approach should enable the preparation of fully defined complexes whose properties can be better related to their biological activity.

Key words: DNA; Gene delivery; Gene therapy; Fluorescamine; Polyelectrolyte complex

1. Introduction

Inter-polyelectrolyte complexes (also known as IPECs) formed by the spontaneous self-assembly of DNA with cationic polymers are widely used as simple and effective vectors for the delivery of DNA into cells [1–3]. Despite their usefulness in vitro, these vectors are rapidly cleared from the bloodstream upon intravenous administration, which greatly limits their potential use as a systemic delivery system for DNA in vivo [4]. In an attempt to improve their biocompatibility and extend blood circulation times, hydrophilic polymers such as poly(ethylene glycol) (pEG) have been incorporated onto the surface of IPECs formed with synthetic polymers such as poly(L-lysine) (pLL) or polyethylenimine [5–7]. However, at present, none of the DNA delivery systems based on modified IPEC designs is capable of prolonged plasma half-lives of up to 20 h as exhibited by Stealth liposomes in mice [8,9].

A major factor limiting the development of IPECs modified with hydrophilic polymers is the lack of techniques available to properly characterise their formation. The preparation of IPECs with fully defined characteristics would enable the relationship with their biological activity in vivo to be better understood. There are two stages in the formation of modified IPECs that require a better definition. The initial binding interaction between the cationic polymer and DNA is influenced by several factors. These include the molar ratio of cationic polymer to DNA, pH conditions and the molecular weight and type of cationic polymer used. Secondly, the degree of modification of IPECs with hydrophilic polymers is affected by the length of the reaction time with the polymer, pH conditions and the amount and type of polymer used.

The aim of the present study was to improve the characterisation of IPECs by determining the availability of free amino groups in IPECs formed with pLL. Each of the lysine residues in pLL contains an epsilon amino group that forms a ionic interaction with DNA. A method based on fluorescamine, a reagent that specifically reacts with amino groups, was used to monitor the self-assembly reaction between DNA and pLL. Previous studies have shown that fluorescamine cannot react with amino groups once they are involved in stable salt linkages with DNA [10]. Using this approach, the influence of pH conditions and the molecular weight of pLL on the formation of IPECs was examined. This method was also used to monitor the post-modification of IPECs with a synthetic hydrophilic polymer bearing several 4-nitrophenyl ester groups that is designed to react with amino groups and hence to crosslink the surface of the IPEC.

2. Materials and methods

2.1. Chemicals and preparation of IPECs

Calf thymus DNA (CT DNA, approximately 8 kb) was obtained from Sigma Chemical (Poole, UK) and purified using phenol/chloroform extraction and ethanol precipitation. pLL of average molecular weights, 3.4, 20 and 459 kDa, were purchased from Sigma Chemical (Poole, UK). Fluorescamine was purchased from Cambridge Bioscience (Cambridge, UK). IPECs were formed by adding a small volume of pLL (1 mg/ml) to CT DNA in 100 mM boric acid-NaOH at pH 7.0–9.5. The complexes were then gently inverted three times and allowed to form for 10 min at room temperature prior to use. The amount of pLL to DNA used for each IPEC is represented as the molar ratio of pLL nitrogen to DNA phosphate (N:P ratio).

2.2. Fluorescamine assay

Free primary amino groups in pLL were determined using the fluorescamine assay as previously described [11]. One-hundred μl aliquots of each sample were diluted in 1.4 ml assay buffer (100 mM boric acid-NaOH, pH 7.0–9.5) and 500 μl 0.01% fluorescamine (prepared in acetone) was rapidly added. Samples were rapidly inverted 4–5 times and incubated at room temperature for 10 min. Fluorescence was measured using a Perkin-Elmer LS 50B fluorimeter using λ_{ex} 392 nm, λ_{em} 480 nm with 5–10 nm slit widths. Background fluorescence was measured using assay buffer alone.

2.3. Ethidium bromide exclusion assay

The ability of pLL to form complexes with DNA was determined by measuring changes in ethidium bromide-DNA fluorescence [12]. DNA solutions (20 μg/ml) were mixed with ethidium bromide (400

*Corresponding author. Fax: (44) (121) 414-3263.
E-mail: l.seymour@bham.ac.uk

Abbreviations: IPEC, inter-polyelectrolyte complex; pLL, poly(L-lysine); pEG, poly(ethylene glycol); pHPMA-ONp, poly(*N*-(2-hydroxypropyl)methacrylamide); CT DNA, calf thymus DNA

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ng/ml) in cuvettes in a Perkin-Elmer LS 50B fluorimeter. Fluorescence was measured at λ_{ex} 510 nm, λ_{em} 595 nm and calibrated to 100%. Following stepwise addition of pLL, samples were inverted three times and readings were taken after 1–2 min. Background fluorescence was set to 0% using ethidium bromide (400 ng/ml) alone.

2.4. Synthesis of multivalent hydrophilic polymers

Hydrophilic polymers were synthesised based on poly(*N*-(2-hydroxypropyl)methacrylamide) (pHPMA-ONp) bearing pendent tetrapeptide (Gly-Phe-Leu-Gly, 8.6 mol%) or dipeptide (Gly-Gly, 10.3 mol%) sidechains terminated in reactive 4-nitrophenyl ester groups, as previously described [13]. These materials were characterised by FPLC (calibrated with pHPMA standards) and showed an average molecular weight of 35 000 and 21 500 Da, respectively.

3. Results

The rate of reaction between pLL and fluorescamine was examined under different pH conditions and it emerged that the reaction proceeded more quickly at pH 9.0–9.5, where it was completed within 1 min (Fig. 1A). At pH 7.0–8.0, the reaction took longer to reach completion and a steady signal was generated only after approximately 5–10 min. Therefore, in all subsequent studies, the fluorescence readings were measured precisely 10 min after addition of fluorescamine to the sample. Determination of pLL using the fluorescamine assay was found to be linear up to 5 μg/ml under all pH conditions used (Fig. 1B). The actual level of fluorescence generated was approximately 2–3-fold greater at pH 9.0–9.5 than at pH 7.0, which probably reflects deprotonation of more pLL epsilon amino groups under higher pH conditions. No fluorescence signal was produced when fluorescamine was added to DNA alone (Fig. 1B).

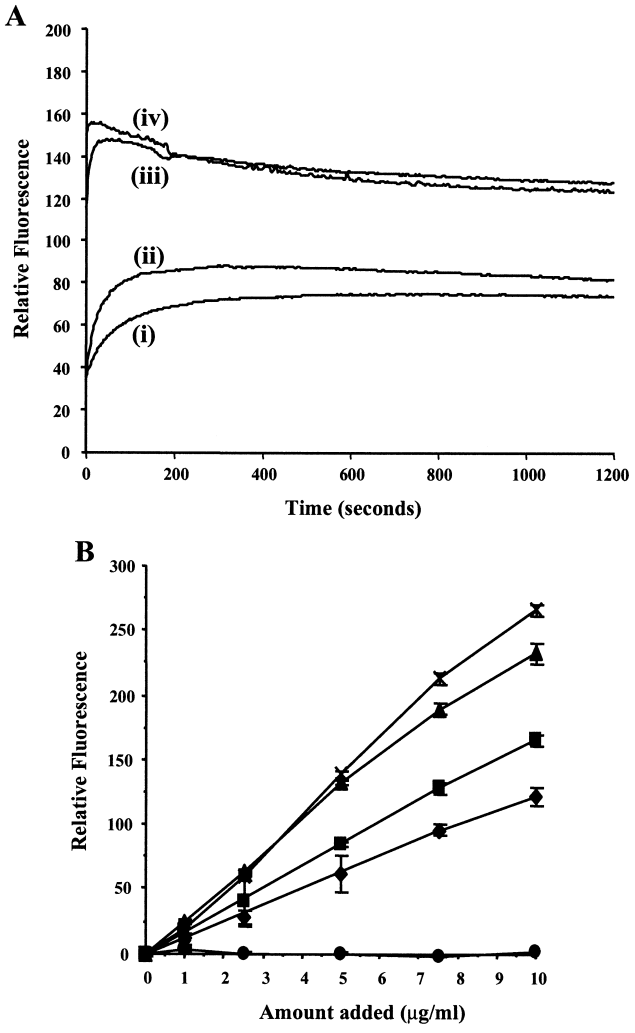

Fig. 1. (A) Rate of reaction between fluorescamine and pLL under different pH conditions. Fluorescamine was added to pLL (5 μg/ml) in 100 mM boric acid-NaOH at pH 7.0 (i), pH 8.0 (ii), pH 9.0 (iii) and pH 9.5 (iv) and the fluorescence signal (λ_{ex} 392 nm, λ_{em} 480 nm) was recorded over 1200 s. (B) Linearity of the fluorescamine reaction with pLL. Fluorescamine was added to pLL in 100 mM boric acid-NaOH at pH 7.0 (diamonds), pH 8.0 (squares), pH 9.0 (triangles) and pH 9.5 (crosses) and to CT DNA in 100 mM boric acid-NaOH at pH 9.5 (circles) at the indicated concentrations. Data represent the means of triplicate samples ± S.D.

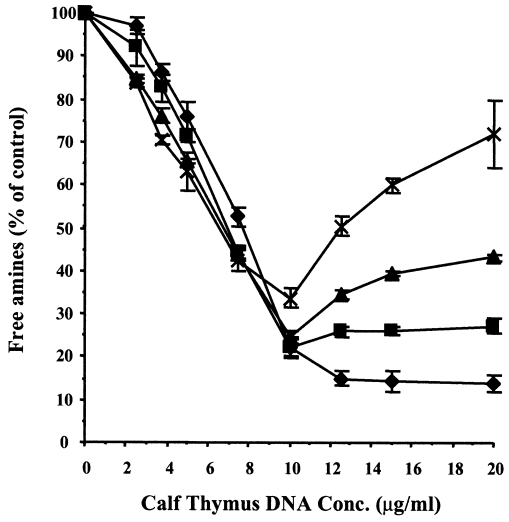

Fig. 2. Characterisation of the binding interaction between pLL and DNA. pLL (5 μg/ml) was incubated with CT DNA for 10 min at the indicated concentrations in 100 mM boric acid-NaOH at pH 7.0 (diamonds), pH 8.0 (squares), pH 9.0 (triangles) and pH 9.5 (crosses) prior to the analysis of free amino groups using the fluorescamine assay. The control was pLL alone at 5 μg/ml at the corresponding pH. Data represent the means of triplicate samples ± S.D.

IPECs are formed by charge-neutralised microdomains between pLL and DNA, resulting in the collapse of the DNA molecule into a compacted structure of 50–100 nm in diameter [1]. The binding reaction between pLL and DNA was characterised by following the loss of amino groups upon the addition of pLL to DNA under different pH conditions using the fluorescamine assay. An inter-polyelectrolyte binding reaction between CT DNA and pLL was indicated by a decrease in the available amino groups (Fig. 2), with the lowest fluorescence signal observed after the addition of 10–12.5 μg/ml CT DNA to 5 μg/ml pLL (corresponding to a N:P ratio of 0.6–0.8). There was relatively little influence of the pH on amino group availability when pLL was mixed with 0–10 μg/ml CT DNA (corresponding to N:P ratios ≥0.8). In contrast, at higher DNA concentrations up to 20 μg/ml, there was a significant increase in free amino groups at pH 9.0–9.5 (Fig. 2). These results suggest that pLL in the presence of excess DNA is less tightly bound than it is within more charge-neutralised environments (i.e. N:P ratios ≥0.8) and it is particularly weakly bound at a high pH.

A widely used method for monitoring the interaction between DNA and cationic polymers follows the loss of DNA

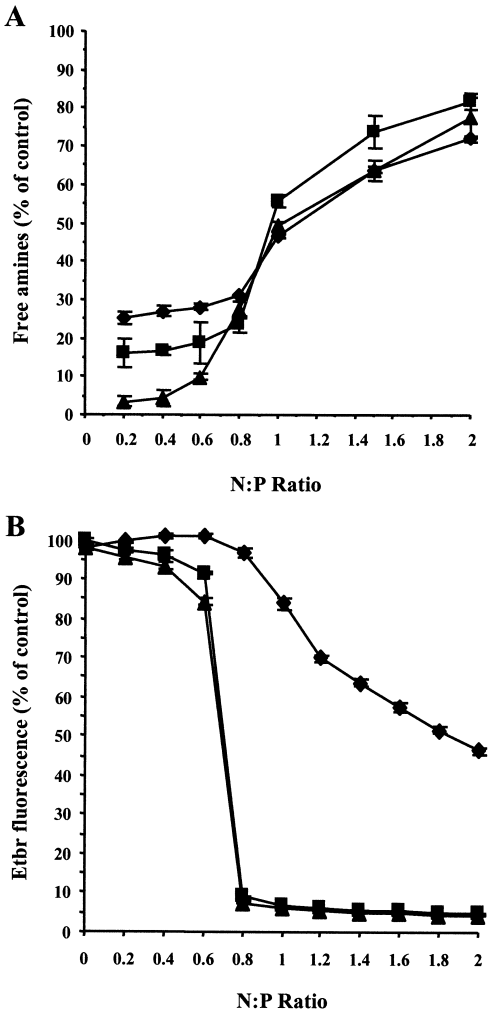

Fig. 3. Effect of the molecular weight of pLL on amino group availability. (A) pLL of average molecular weights, 3.4 kDa (diamonds), 20 kDa (squares) and 459 kDa (triangles) were incubated with CT DNA for 10 min at the indicated concentrations in 100 mM boric acid-NaOH, pH 7.0, prior to the analysis of free amino groups using the fluorescamine assay. The control was pLL alone at 5 μg/ml at pH 7.0. (B) Profiles of inhibition of CT DNA/ethidium bromide fluorescence by pLL of average molecular weights 3.4 kDa (diamonds), 20 kDa (squares) and 459 kDa (triangles) in 100 mM boric acid-NaOH, pH 7.0. The control was CT DNA alone at 20 μg/ml. Data represent the means of triplicate samples ± S.D.

fluorescence with ethidium bromide when DNA is condensed into IPECs by interaction with polycations [12]. To investigate the influence of polymer size on the interaction of pLL with DNA, pLL of a different molecular weight was added to CT DNA and the resultant binding reaction was examined using the fluorescamine and ethidium bromide exclusion assays. At N:P ratios less than 0.8, a low level of fluorescence was observed with 459 kDa pLL using the fluorescamine assay, indicating that high molecular weight pLL binds very efficiently to DNA (Fig. 3A). In contrast, 3.4 kDa pLL bound less efficiently to DNA with a 25–30% higher level of fluorescence observed at N:P ratios of 0.2–0.6 as compared to 459 kDa pLL (Fig. 3A). At N:P ratios greater than 0.8, there was no significant difference in the amino group availability with different sized pLL. Using the ethidium bromide exclusion assay, a dramatic loss in the fluorescent signal was observed at a N:P ratio of 0.8 with both 20 and 459 kDa pLL (Fig. 3B), indicating the efficient formation of IPECs. In contrast, 3.4 kDa pLL produced a gradual decrease in fluorescence that only fell below 50% of the original level at a N:P ratio of 2.0. These results suggest that lower molecular weight pLL binds less efficiently to DNA, which may contribute towards poor condensation of the DNA molecule.

Modification of the surface of IPECs with the multivalent hydrophilic polymer pHPMA-ONp has led to the recent development of a DNA delivery system with promising characteristics for use in vivo [14]. Attachment of the multivalent polymer to the IPEC involves a covalent linkage between the reactive esters of the polymer and free amino groups on the

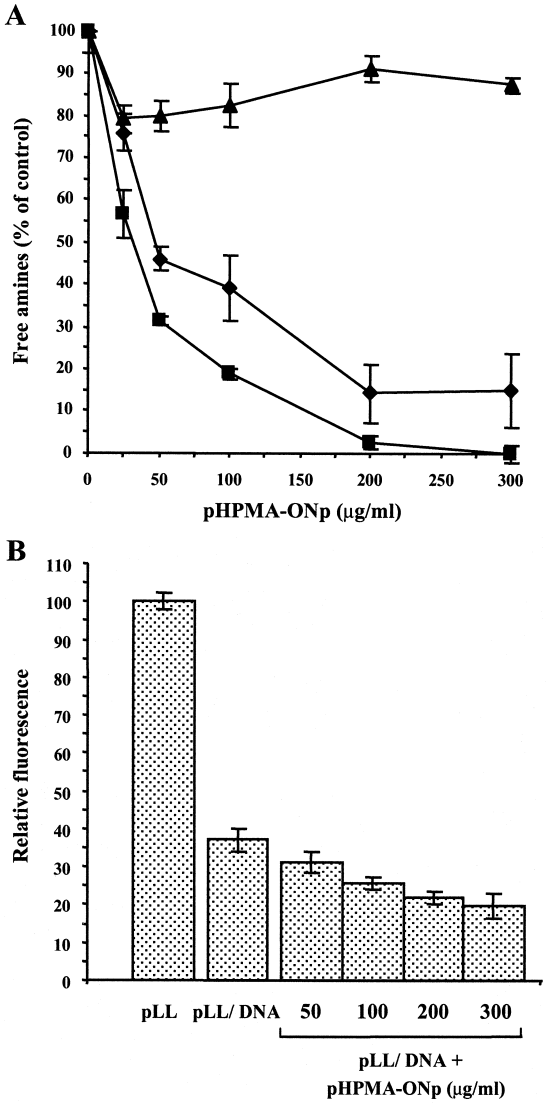

Fig. 4. Modification of IPECs with the hydrophilic polymer. (A) pLL 20 kDa (5 μg/ml) was incubated for 72 h in 100 mM boric acid-NaOH, pH 8.0, at room temperature with pHPMA-ONp containing tetrapeptide (diamonds) or dipeptide (squares) side-chains and pre-hydrolysed pHPMA-ONp (triangles). Pre-hydrolysed polymer was prepared by incubating pHPMA-ONp overnight in buffer at pH 9.5. (B) pLL/DNA complexes were formed at a N:P ratio of 1.0 and incubated overnight with the indicated concentrations of pHPMA-ONp at pH 8.0. The control was pLL alone at 5 μg/ml at pH 8.0. Free amino groups were analysed using the fluorescamine assay. Data represent the means of triplicate samples ± S.D.

surface of the IPEC. Fluorescamine assays were performed to characterise the interaction between pHPMA-ONp and pLL. The reaction between pHPMA-ONp and pLL was indicated by an 85% decrease in the fluorescence signal following the addition of 200 μg/ml pHPMA-ONp (Fig. 4A). A more efficient reaction with pLL was indicated by a 98% loss of signal at the same concentration of polymer using a modified form of the pHPMA-ONp polymer containing a dipeptide (Gly-Gly) side-chain instead of the tetrapeptide (Gly-Phe-Leu-Gly) side-chain (Fig. 4A). Partial masking of amino groups on pLL by electrostatic and not by covalent interactions with pHPMA-ONp was also indicated by a 10–20% fall in the fluorescence signal upon addition of the pre-hydrolysed form of the polymer (Fig. 4A). Together, these results indicate that both the nature of the peptide side-chain and hydrolysis of the reactive ester group are factors that contribute towards the interaction of the pHPMA polymer to free amino groups in pLL.

To characterise the interaction of the pHPMA-ONp polymer with free amino groups on the surface of IPECs, pHPMA-ONp was added to pLL/DNA complexes formed at a N:P ratio of 1.0 and the free amino groups were determined using the fluorescamine assay. The level of detectable free amino groups gradually decreased following the addition of 50–300 μg/ml pHPMA-ONp (Fig. 4B). An approximate 50% decrease in the fluorescence signal was observed following the addition of 300 μg/ml pHPMA-ONp to the IPEC. This result indicates that a significant proportion of free amino groups associated with the pLL/DNA complexes was modified by the hydrophilic polymer.

4. Discussion

Previous studies have used an assay based on the fluorescamine reagent to monitor the incorporation of pEG onto the mouse IgG antibody [15] and for analysing cationic lipid-nucleic acid complexes [16]. In this study, the reaction of fluorescamine with amino groups was used to characterise non-viral DNA delivery vectors modified with a hydrophilic polymer. The results show that the fluorescamine assay can be used to probe the binding interaction between pLL and DNA in the formation of IPECs. It is clear, for example, that binding of pLL to DNA is pH dependent at N:P ratios less than 0.8 and that higher molecular weight pLL binds more efficiently to DNA. Furthermore, the degree of incorporation of a hydrophilic polymer onto the surface of IPECs can also be monitored using the fluorescamine assay.

One factor that appears to be important in determining the efficiency of the binding interaction between pLL and DNA is the molecular weight of pLL used. Higher molecular weight pLL bound more completely to DNA at low N:P ratios with few residual amino groups than low molecular weight pLL. The relatively high avidity achieved with 459 kDa pLL is most likely caused by the simultaneous attachment of many positive charges (in this case, up to 1000) to the DNA. The binding reaction might be rendered essentially irreversible under these conditions, leading to small amounts of free residual amino groups. A 3.4 kDa pLL, on the other hand, might be able to dissociate from the DNA producing a binding equilibrium with higher amounts of free amino groups. One consequence of this lower avidity for DNA is that 3.4 kDa pLL will be less efficient at neutralising the negative charges of the phosphate groups along the DNA backbone resulting in the poor condensation of DNA observed with the polycation.

pLL is a weak polybase and under high pH conditions, it is likely that the interaction with DNA will be unfavourable since a greater proportion of the amino groups will be in the deprotonated state. Indeed, at N:P ratios less than 0.8, the degree of interaction between pLL and DNA was dependent on pH conditions. However, at higher N:P ratios, the binding of pLL to DNA was no longer pH dependent. The increased stability of the interaction between pLL and DNA is most likely the result of the formation of condensed structures that are typically observed at N:P ratios of 0.8 or more [1]. In contrast, when DNA is in excess, it is likely that IPECs will be formed with an extended structure and increased separation of pLL molecules from each other. In this case, the co-operative effects of charge-neutralised microdomains involving several pLL molecules will be lost. Hence, the increased availability of amino groups at a high pH probably reflects the weak binding of individual pLL molecules to DNA.

The long circulatory properties of pEG-containing liposomes (or Stealth liposomes) has enabled their use as carriers for cancer drugs in the treatment of tumours. The pEG headgroup on the liposome promotes blood circulation by interfering with the absorption of plasma proteins and reducing interaction with the reticuloendothelial system [9]. Hydrophilic polymers such as pEG have also been incorporated into DNA delivery vectors based on modified IPECs designed for use in gene therapy protocols [5–7]. However, at present, none of these vectors circulates in vivo with a plasma half-life of greater than 30 min [7]. It is envisaged that an IPEC capable of prolonged circulation needs to be completely enshrouded in a hydrophilic shield. Hence, it is important to properly characterise the incorporation of the hydrophilic polymer onto IPECs to ensure that there is sufficient polymer to provide a steric barrier against the interaction of plasma proteins and the reticuloendothelial system.

Unfortunately, there are few techniques available to characterise the formation of polymer-coated IPECs and therefore optimise conditions for attachment of the hydrophilic polymer. We have shown that the fluorescamine assay can be used to directly monitor the modification of an IPEC with a multivalent hydrophilic polymer. It was possible, for example, to significantly reduce the number of available amino groups on IPECs using the pHPMA polymer, indicating a high level of surface modification. This experimental approach will be useful in determining the amount of polymer required to completely modify the surface of IPECs with a corresponding minimum number of free amino groups. The preparation of fully polymer-coated IPECs will facilitate the development of vectors capable of prolonged systemic circulation.

The use of the fluorescamine assay should also enable the rational design and testing of new hydrophilic polymers to optimise the coating reaction with IPECs. In the present study, for example, the tetrapeptide (Gly-Phe-Leu-Gly) sequence attached to the pHPMA polymer was chosen as it can act as a substrate for cathepsin B [17], which should facilitate release of the DNA molecule within the cell. However, the pHPMA polymer containing the shorter dipeptide (Gly-Gly) side-chain was more reactive towards free amino groups in pLL and may be better suited for coating of the IPEC. A possible explanation for this observation is that the hydrophobic amino acids might cause partial micellisation of

the pHPMA-(Gly-Phe-Leu-Gly)-ONp polymer, making some ester groups unavailable for reaction with pLL.

In summary, the use of the fluorescamine reagent enables better characterisation of IPECs modified with hydrophilic polymers, which in turn will facilitate the correlation between the physicochemical properties of IPECs with their biological activity. Work is currently ongoing to use this approach in the systematic development of DNA delivery vectors capable of prolonged circulation in vivo.

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