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Hyperbranched PEGmethacrylate linear pDMAEMA block copolymer as an efficient non-viral gene delivery vector

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A B S T R A C T

A unique hyperbranched polymeric system with a linear poly-2-dimethylaminoethyl methacrylate (pDMAEMA) block and a hyperbranched polyethylene glycol methyl ether methacrylate (PEGMEMA) and ethylene dimethacrylate (EGDMA) block was designed and synthesized via deactivation enhanced atom transfer radical polymerisation (DE-ATRP) for efficient gene delivery. Using this unique structure, with a linear pDMAEMA block, which efficiently binds to plasmid DNA (pDNA) and hyperbranched polyethylene glycol (PEG) based block as a protective shell, we were able to maintain high transfection levels without sacrificing cellular viability even at high doses. The transfection capability and cytotoxicity of the polymers over a range of pDNA concentration were analysed and the results were compared to commercially available transfection vectors such as polyethylene imine (branched PEI, 25 kDa), partially degraded poly(amide amine) dendrimer (dPAMAM; commercial name: SuperFect®) in fibroblasts and adipose tissue derived stem cells (ADSCs).

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1. Introduction

Gene therapy introduced in the early 1970’s (Aposhian, 1970; Rogers, 1971; Osterman et al., 1971), is a promising approach for the treatment of genetic disorders, mutation and intractable diseases, and its success relies on the transfection capabilities of its vectors. Three main types of gene delivery systems have been described so far: viral and non-viral vectors and the direct injection of genetic materials into the tissues using “gene guns” (Goverdhan et al., 2005; Verma and Weitzman, 2005; Young et al., 2006; Lee et al., 2008; Itaka and Kataoka, 2009; Maurer, 2010). Viral vectors show high transfection efficiency. However, their application is limited by their toxicity, difficulty in pharmaceutical processing and scale-up as well as the possibility of the reversion of an engineered virus to a wild type (Young et al., 2006). Even though the idea of using non-viral cationic vectors as transfection agents was introduced in 1973 (Henner et al., 1973), this area was not fully explored until the mid to late 1990’s (Abdallah et al., 1995, Frederiksen et al., 1999). Non-viral vectors have increasingly drawn attention due to their large scale manufacture, transport, storage, reduced immunogenic response and the possibility of modifications and their capacity to carry large inserts (Romano et al., 2000). In this regard, cationic polymers such as polyethyleneimine (PEI), poly-L-lysine (PLL), poly[β-amino esters], poly-2-dimethylaminoethyl methacrylate (pDMAEMA) have been utilized largely because of their ability to condense DNA and form complexes (polyplex) for more efficient uptake through endocytosis. pDMAEMA is widely used as nonviral gene delivery vector (Trubetskoy et al., 1992; Bousif et al., 1995; Wu et al., 2005; Lin et al., 2008). At physiological pH pDMAEMA is partially protonated and behaves as a proton sponge and can transfect cells efficiently (Jones et al., 2004). However, there are limits to its application due to the cationic property of these polyplexes which leads to high levels of toxicity. Several researchers have attempted to reduce this toxicity while maintaining high transfection efficiency (Breunig et al., 2007). Poly- (ethylene glycol) (PEG) modified polymers generally exhibit good solubility and also provide a steric shield to the polyplexes from interaction with blood components (Funhoff et al., 2005). These characteristics enable PEG modified polymers to circulate in the blood without aggregation for an extended period of time in the body (van Vlerken et al., 2007). PEG modified polymers also show reduced toxicity levels. However, the maximum dosage of the polyplex which maintains high transfection with minimum toxicity is still an issue.

The synthesis of polymers with well-defined composition, architecture and functionality is absolutely crucial in determining the efficiency of the polymer as a gene delivery vector. Lack of control over the polymerisation to synthesize a well defined polymer is a major limitation of the conventional radical polymerisation (Brauncker and Matyjaszewski, 2007). With the recent
progress in polymerisation methods, it has become possible to
design and prepare well-defined polymers by controlled/living
polymatisations (Goto and Fukuda, 2004). Atom transfer radical
polymerisation (ATRP) has been widely employed for the prepara-
tion of well-defined biomaterials (Zhang et al., 2007). Wang et al.
(2007) introduced the deactivation enhanced atom transfer radical
polymerisation (DE-ATRP) approach for the synthesis of hyper-
branched copolymers. In this approach a halogen-Cu\textsuperscript{II}/halogen-Cu\textsuperscript{II}
mixture was used to enhance the deactivation of the polymerisa-
tion process which leads to a slow polymer growth. This method
is advantageous for synthesis of soluble hyperbranched polymer
instead of gelation.

Herein a new hyperbranched polymeric system with a lin-
ear pDMAEMA block and a hyperbranched polyethylene glycol
methyl ether methacrylate (PDGEMA) and ethylene dimethacry-
late (EGDMA) block was synthesized via DE-ATRP technique and
analysed for transfection capability and cytotoxicity over a range
of plasmid DNA (pDNA) dosage. The rationale behind the synthe-
sis and use of this new polymer is that the polymer structure
with a linear pDMAEMA unit can efficiently bind pDNA whereas
the PEG based hyperbranched unit will form the micelle-like
structure in the water solution because of its amphiphilicity to
protect the polyplex and enhance its viability thereby reducing
the cytotoxicity while maintaining its transfection efficiency at
high dosages. To prove this concept, two hyperbranched polymers
one with a high PEG content (termed IpD-b-b-P/E 1) and another
with low PEG content (termed IpD-b-P/E 2) were synthesized and
compared with commercially available transfection vectors such
as branched PEI (25 kDa) (gold standard for non viral gene
delivery vector), partially degraded poly(amide amine) dendrimer
(dpDAM; commercial name: SuperFect\textsuperscript{R}), and also with linear
pDMAEMA. These polymers were tested on fibroblast cells which
are generally easy to transfect and also on a primary cell line:
adipose tissue derived stem cells (ADSCs) which are generally con-
sidered as “difficult to transfect cells” (Wang et al., 2008; Uchida
et al., 2009).

2. Materials and methods

2.1. Chemicals

All reagents including HPLC and analytical solvents, synthe-
sis and cell culture reagents were obtained from Sigma–Aldrich
Chemical Co. unless otherwise stated. These reagents were used
with a purity of more than 98%, and were used without further purification. pDNA, Gausia Luciferase kit was purchased from New
England BioLabs and Picogreen\textsuperscript{TM} kit from Invitrogen. Dialysis membrane (MWCO 8000) was used as received from Spectraor.
dpDAM was purchased from QiAGEN.

2.2. Synthesis of poly(2-dimethyl-aminomethylmethacrylate)
(pDMAEMA)

pDMAEMA was synthesized via ATRP as follows: DMAEMA
(15 g, 9.54 × 10\textsuperscript{-2} mol), ethyl-\textalpha- bromoisoobutyrate (EBiB) (0.146 g, 7.51 × 10\textsuperscript{-4} mol), N,N,N,N,N\textprime- pentamethyldiethylenetriamine (PMDETA) (0.0649 g, 3.75 × 10\textsuperscript{-4} mol) and tetrahydrofuran (THF)
(15 ml) was added to a two-necked round bottom flask. After sealing
the reaction flask, the mixture was purged with nitrogen for
20 min. Copper chloride(l) (0.0371 g, 3.75 × 10\textsuperscript{-4} mol) was imme-
diately added. The reaction flask was then sealed and immersed
in an oil bath at 50 °C. The reaction was stopped after 2.5 h at
a monomer conversion of 78%. The polymer was precipitated in
hexane and was then dried under vacuum.

2.3. Synthesis of poly(2-dimethyl-aminomethylmethacrylate)
(pDMAEMA)-block-polyethylene glycol methyl ether
methacrylate (PEGEMA)/ethylene dimethacrylate (EDGMA)

pDMAEMA-b-PEGEMA/EDGMA was synthesized via deacti-
vation enhanced atom transfer radical polymerisation, with feed
ratio 1:90:10, pDMAEMA (7.51 × 10\textsuperscript{-4} mol) was dissolved in THF
(40 ml) in a two-necked round bottom flask. PEGEMA (28.538 g, 6.008 × 10\textsuperscript{-2} mol), EDGMA (2.9779 g, 1.502 × 10\textsuperscript{-2} mol), PMDETA
(0.0324 g, 1.87 × 10\textsuperscript{-4} mol) were added thereafter. After sealing the reaction flask, the mixture was purged with nitrogen for 20 min.
Ascorbic acid (0.0066 g, 3.75 × 10\textsuperscript{-5} mol) was immediately added.
The reaction flask was then sealed and immersed in an oil bath at
50 °C. The reaction was stopped at 60% conversion for IpD-b-P/E 1 and
28% conversion for IpD-b-P/E 2. The polymer was precipitated in
hexane and was dried under vacuum. Copolymer was purified using
membrane dialysis for three days against distilled water and
was then freeze dried and stored at −20 °C until use.

2.4. Gel permeation chromatography (GPC) characterisation

Number average molecular weight (M\textsubscript{n}), weight average molec-
ular weight (M\textsubscript{w}) and polydispersity (M\textsubscript{w}/M\textsubscript{n}) were obtained by
gel permeation chromatography (Varian 920-LC) with a RI detect-
or. The columns (30 cm PolargelM, two in series) were eluted
by dimethyl formamide (DMF) and calibrated with polyvinyl methacrylate standards. All calibrations and analyses were per-
formed at 40 °C at a flow rate of 1 ml/min. All samples were
dissolved in DMF, and passed through 0.2 μm filter before injection
to demonstrate the absence of gelation.

2.5. Nuclear magnetic resonance (NMR) analysis

\textsuperscript{1}H NMR was carried out on a 300 MHz Bruker DPX300 and was
analysed using MestReC\textsuperscript{TM} processing software. The chemical shifts
were referenced to the lock deuterated chloroform (CDCl\textsubscript{3}). The
final polymer composition was calculated by the integral values
provided by the \textsuperscript{1}H NMR.

2.6. Agarose gel electrophoresis

pDNA binding ability of the hyperbranched polymers was exam-
ined by agarose gel electrophoresis. The polymer/pDNA complexes
containing luciferase plasmid were prepared at varying polymer to
pDNA weight ratios (1:1–15:1). 10 μg of pDNA (50 μl) was added
to the polymer solutions (in phosphate buffered saline (PBS)) of
different concentration (final volume 100 μl). The mixtures were then
allowed to incubate at room temperature for 30 min. Thereafter,
the complexes were loaded into individual wells of 0.7% agarose \times TAE
gel containing 10 μl Sybersafe\textsuperscript{TM} and were electrophoresed at 100 V
for 90 min. Naked pDNA was used as the control. The gel was then
visualized on a UV transilluminator (Syngene).

2.7. Particle size and zeta potential analysis

Polymer/pDNA complexes were prepared as described previ-
ously, with weight ratios (1:1–15:1). After 30 min of incubation,
the complex solutions were diluted 11 times in PBS (1 ×). Prior
to analysis, the diluted complex solutions were allowed to stabi-
lize for 30 min. The particle size and charge of the polymer/pDNA
complexes were measured using Zetasizer Nano-ZS90 (Malvern
Instrument Ltd., UK). The particle size and zeta potential measure-
ments were repeated for five runs for each sample, and the data
were reported as the average of five readings.
2.8. Cell culture experiments

The transfection ability and effect of polymers on cellular metabolic activity of the synthesized polymer was evaluated on two different cell types. Human Fetal Foreskin Fibroblasts were purchased from Centre of Applied Microbiology and Research (CAMR). Adipose derived stem cells (ADSCs) were extracted from rabbit adipose tissue as follows. Adipose tissue was digested by collagenase type I at 0.025% for 1 h under agitation at 37 °C. The enzymatic reaction was stopped by addition of complete medium (Dulbecco’s Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Penicillin Streptomycin (P/S)). The stromal fraction was collected by centrifugation 5 min at 1200 rpm, resuspended and filtered on a cell strainer 70 mm (Fisher). After 24 h incubation at 37 °C in a humidified atmosphere of 5% CO2, cells were washed to eliminate the contaminant cells (blood cells and adipocytes). Medium was changed every two to three days and cells were maintained subconfluent. Adipogenic, chondrogenic and osteogenic differentiation assays were conducted to confirm the nature of the extracted cells.

2.9. Cell Metabolic activity: alamarBlueTM assay

Cellular metabolic activity was assessed by the alamarBlueTM assay, wherein dye reduction was considered proportional to the metabolic activity of the cells. Cells were seeded with a cell density of 6500 cells/well for fibroblasts, 6000 cells/well for ADSC cells in a 96-well tissue culture plate and grown overnight. Cells were washed and polymers with different concentrations (5, 25, 50, 100 µg/ml) were loaded on the cell and incubated for 48 h in medium containing serum. alamarBlueTM assay was conducted according to the manufacturer’s instructions. After 3-h incubation, absorbance at 550 and 595 nm was determined on a microplate reader (VarioskanFlash-4.00.53) and the percentage reduction of the dye was calculated. alamarBlueTM assay was also performed on fibroblasts (6000 cells/well) and ADSCs (6500 cells/well) after transfection.

2.10. In vitro gene transfection

The in vitro gene transfection ability of the polymer/pDNA complexes was assessed via G-luciferase activity. Under usual cell culture sterile conditions, cells were seeded in a 96-well plate with a cell density of 6500 cells/well for fibroblasts and 6000 cells/well for ADSCs for 24 h before the transfection experiment. pDNA (1 µg per well) was mixed with 50 µl polymer solutions with different weight ratios from 1:1 to 20:1. The mixture was then incubated for 30 min at room temperature to allow the formation of polymer/pDNA complexes, and 150 µl of serum medium was added to the polyplex solution. The cells were washed with HBSS. Complexes were then added into the well and incubated with the cells for 48 h at 37 °C. After 48 h at 37 °C, the cells were analysed for G luciferase activity.

2.11. Statistics

Results are expressed as mean ± standard deviation. Statistical significance was assessed using the analysis of variance (ANOVA). p values of <0.05 were considered statistically significant. In all studies, the minimum sample size was three.

Fig. 1. GPC trace showing the controlled growth of lpD-b-P/E 1 over time (2, 3, 6, 10 h).

3. Results and discussion

Linear pDMAEMA was synthesized via normal ATRP (Scheme 1a). The molecular weights of the polymers were characterized by GPC. The molecular weight of linear pDMAEMA block was restricted to approximately 8000 Da. Then, this linear pDMAEMA was used as a macro initiator for DE-ATRP of PEGMEMA and EGDMA to produce a PEG based hybrid polymer with a hyperbranched structure (Scheme 1b). Two hyperbranched polymers, one with a high PEG content (lpD-b-P/E 1), and another with low PEG content (lpD-b-P/E 2), were synthesized. A small amount of reducing agent was added, thus being different from DE-ATRP technique introduced by Wang et al. (2007). This was to facilitate a controlled polymer growth without gelation over a period of time (Cao et al., 2011). Ascorbic acid was used as a reducing agent for the production of active CuI species, while Cu1+ remaining in the system controls growth of the polymer chain. GPC clearly demonstrated the controlled polymer growth of lpD-b-P/E 1 over a period of time (Fig. 1). The molecular weight lpD-b-P/E 1 was around 38,000 Da and that of lpD-b-P/E 2 was around 14,000 Da. Low polydispersity index (PDI) in both cases demonstrates the controlled growth of the polymer chain (Table 1). The chemical structure of final hyperbranched polymer was determined using 1H NMR (Fig. 2). The characteristic peaks at chemical shifts of 6.1 and 5.6 ppm are attributed to the vinyl functional groups in the hyperbranched polymer. The degree of branching within the polymer structure was analysed via the following equations (Table 1):

\[
\text{DMAEMA} = \text{integrals of } j/6
\]

\[
\text{PEGMEMA} = \text{integrals of } l/30
\]

\[
\text{Linear EGDMA} = \text{integrals of } d + e
\]

The molar ratio of pDMAEMA to PEGMEMA to EGDMA for lpD-b-P/E 1 was found to be 1:12.215:1.104 with 1.054% of branched EGDMA (Fig. 2a) and 1:0.879:1.12 with 33.3% of branched EGDMA for lpD-b-P/E 2 (Fig. 2b). At the early stage of the reaction, EGDMA adds up to the polymer block leading to high branching ratio. Both lpD-b-P/E 1 and lpD-b-P/E 2 are able to form complexes with pDNA via electrostatic interactions (Scheme 1c). G luciferase (Gluc) pDNA binding ability of the hyperbranched polymers was examined by agarose gel electrophoresis and was compared with controls at their optimum weight ratios, e.g. PEI and dPAMAM to pDNA at 2:1 and 8:1 (w/w ratio) respectively. It was seen...
Table 1

<table>
<thead>
<tr>
<th>Polymer</th>
<th>(M_w) (Da)</th>
<th>(M_n) (Da)</th>
<th>PDI</th>
<th>DMAEMA:PEGMAEMA:EGDMA (molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 1 (pDMAEMA)</td>
<td>8000</td>
<td>7500</td>
<td>1.07</td>
<td>1</td>
</tr>
<tr>
<td>Block 2 (lpD-b-P/E 1)</td>
<td>37,900</td>
<td>44,300</td>
<td>1.17</td>
<td>1:12.21:5:1.104</td>
</tr>
<tr>
<td>Block 2 (lpD-b-P/E 2)</td>
<td>13,700</td>
<td>16,300</td>
<td>1.19</td>
<td>1:0.879:1:1.12</td>
</tr>
</tbody>
</table>

Scheme 1. Schematic diagram of deactivation enhanced – ATRP showing the activated and deactivated routes, controlling the formation of a hyperbranched structure (A and B) and polypelex formation (C).
pDMAEMA (Fig. 4c) increased with increasing weight ratio demonstrating increased electrostatic interaction between the polymer and the plasmid leading to a stronger bond formation.

The size of the polyplexes decreased as the polymer to pDNA weight ratio increased because the higher the w/w ratio the stronger the bond (Fig. 4). The size of linear pDMAEMA polyplexes was much larger than lpD-b-P/E 1 and lpD-b-P/E 2. In contrast to linear pDMAEMA, hyperbranched polymers (lpD-b-P/E 1 and lpD-b-P/E 2), forms micelle like structure when complexed with a plasmid (Scheme 1c). This is the reason why hyperbranched polymers showed reduced size when compared to linear pDMAEMA. Fig. s3a demonstrates the decrease in size of the polyplexes as the ratio increases. At 10:1 (w/w ratio), the size of lpD-b-P/E 1 was around 200–250 nm, while the size of lpD-b-P/E 2 was found to be around 50–80 nm (Fig. s3b), which also supports the results from Zetasizer.

The effect of polymers on cellular metabolic activity of fibroblasts and ADSCs was analysed via alamarBlue™ reduction method and compared to PEI and dPAMAM (Fig. 5). It was seen that at all concentrations both the hyperbranched polymers showed a significantly higher cellular metabolic activity when compared to PEI and at higher concentration (100 μg/ml), and significantly higher than dPAMAM. However, cellular metabolic activity of lpD-b-P/E 2 was significantly reduced at a higher concentration (100 μg/ml) (Fig. 5) when compared to lpD-b-P/E 1, which indicates that high PEGylation for the polymer can significantly decrease the cytotoxicity of the polymer, and was further quantified via cell proliferation
assay (Picogreen™) (Fig. s4). The results show that both lpD-b-P/E 1 and lpD-b-P/E 2 had no impact on cell proliferation in either of the cell lines. Both PEI and dPAMAM significantly reduced the DNA concentration above 5 μg/ml concentration when compared to control cells with no treatment. lpD-b-P/E 1 and lpD-b-P/E by contrast had less impact on DNA concentration levels compared to PEI and dPAMAM suggesting that they had less impact on cell proliferation.

Transfection capability of the hyperbranched polymers was assessed using fibroblast cells and ADSCs. To analyse transfection efficiency for the polymer, G-luc transfection assay kit was used as per protocol. A wide range of weight ratios of polyplexes was analysed for each polymer and the optimum weight ratio for each polymer was determined (Fig. s5). This step is crucial as the optimum weight ratio of the polymer is different on different cells. PEI showed its optimal transfection at 2:1 (w/w ratio) for fibroblasts and 1:1 (w/w ratio) for ADSCs, while dPAMAM showed its optimum transfection at 8:1 (both in fibroblast and ADSCs). lpD-b-P/E 1 showed its optimal transfection at 8:1 (w/w ratio) in fibroblasts and 10:1 (w/w ratio) in ADSCs. lpD-b-P/E 2 had its optimal transfection at 10:1 (w/w ratio) in both cell lines. Linear pDMAEMA showed its optimal transfection at 20:1 (w/w ratio) (in both cell lines) (data not shown). Fluorescent microscopic images of ADSCs also confirmed transfection with lpD-b-P/E 1 and lpD-b-P/E 2 (Fig. s6).

In order to determine the maximum amount of pDNA that the polymer could deliver for a specific number of cells without affecting both transfection and viability, the optimal weight ratio was selected for each polymer, and the transfection capability and cellular metabolic activity was determined as a function of increasing pDNA concentration (Fig. 6). lpD-b-P/E 1 showed no toxic effects at higher pDNA concentration in either cell lines without compromising transfection efficiency. lpD-b-P/E 1 showed at least a fifteen fold higher cellular metabolic activity when compared to PEI, two fold higher when compared to dPAMAM, and six fold higher than linear pDMAEMA. On the other hand, lpD-b-P/E 2 showed a significantly higher level of toxicity (p < 0.05) when compared to vehicle treated cells, but nine fold lower than PEI, 1.5 fold lower than dPAMAM and four to eight fold lower than linear pDMAEMA. PEI is the gold standard among the polymeric genes reported. dPAMAM, is widely used as a transfecting agent. Both these polymers showed toxicity issues at high doses. PEI showed less than 30% cellular metabolic activity above 10 μg pDNA concentration, which resulted in no transfection at these doses. dPAMAM showed less than 50% cellular metabolic activity at 50 μg pDNA concentration. Linear pDMAEMA with no modification showed minimal transfection at all pDNA concentrations. Large polyplex size of linear pDMAEMA (Fig. 4c) (around 400 nm at its optimal transfection w/w ratio i.e. 20:1), makes it difficult to internalize efficiently leading to low transfection. Also, linear pDMAEMA showed less than 30% cellular metabolic activity above 25 μg pDNA concentration, which also resulted in reduced transfection level. Both hyperbranched polymers showed above 70% cellular metabolic activity even at 50 μg pDNA concentration. This might be due to this unique structure i.e. with a linear pDMAEMA unit and a PEG based hyperbranched unit. The linear pDMAEMA with the tertiary amine group could easily bind to the phosphate groups of pDNA and form a strong complex which will lead to high transfection efficiency. PEG based hyperbranched unit, by contrast, enhanced the cellular metabolic activity thereby reducing the cytotoxicity while maintaining its transfection efficiency at high doses.

The transfection efficiency of PEI and dPAMAM complexes does not increase proportionally with increasing pDNA dose. These polymers increase toxicity with increasing dose, which indirectly affects their transfection ability. Both lpD-b-P/E 1 and lpD-b-P/E 2 complexes do not affect the toxicity at high doses as compared to PEI.
and D-PAMAM, and therefore lP-D-b-P/E 1 and lP-D-b-P/E2 maintain their transfection efficiency even at a high dose. This polymeric system can prove favourable for sustained gene therapy applications where long-term and a high DNA dose is required. High doses of polyplex can be loaded into nanospheres carriers for controlled release.

4. Conclusion

A unique structure comprising of a linear poly(MNAEMA block and hyperbranched PEGMEMA and EGDMA block was synthesized using DE-ATRP approach. This is the first time that a polymer of this structure has been synthesized where the poly(MNAEMA unit was able to bind pDNA very efficiently while the hyperbranched unit acted as a shield. This enabled the polymer to maintain high transfection levels without sacrificing cellular viability. Furthermore, unlike the situation with commercially available transfection agents, the cellular was not affected at high doses. This polymeric system can prove favourable for sustained gene therapy applications, where long-term and a high DNA dose is required. Moreover, the free vinyl groups present could be utilized to bind specific antibodies for targeted delivery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.05.010.

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