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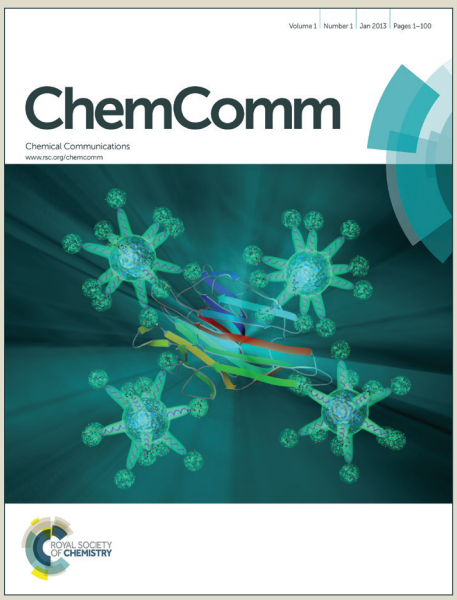

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Hyperosmotic polydixylitol for crossing blood brain barrier and efficient nucleic acid delivery†

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Here, we introduce a polydixylitol based highly osmotic polymer that not only transmigrate BBB by intra-arterial infusion of osmotic polyol but also triggers cellular uptake via modulation of caveolae mediated endocytosis

Brain represents a particularly inaccessible organ for the delivery of therapeutic molecules due to the presence of compact blood-brain-barrier (BBB), which excludes the brain-specific delivery of 100% of large molecules and more than 98% of small therapeutic molecules[1]. The architecture of BBB, which is formed primarily of tight junctions between the cerebral capillary endothelium and surrounding perivascular elements, restricts the influx of molecules from blood stream into the brain [2] and makes it impermeable to most therapeutic molecules, including nucleic acids [3]. Similarly, a compact mass of tumour cells composed of approximately 50% tumour and stromal cells hinders the delivery of cancer therapeutics to deep-seated cancer cells [4]. This compactness of biological tissues precludes pharmacotherapy or requires the use of invasive procedures to bypass the barrier [5]. Therefore, clinical translation of various competent drugs and nucleic acid therapeutics has been obstructed by these biological barriers[6]. For instance, to deliver genes to neurons, viral vectors have been directly injected into selected regions of the brain because BBB impedes their entrance into the brain when delivered systemically [7]. However, using this invasive technique, transduction is limited to the injection site [8]. Therefore, the search for ways to overcome these biological barriers to reach the specific site of action has been the major driving force for the significant development of delivery vectors [9] that will not only protect the therapeutic molecules from degradation in the biological milieu but also steer their cellular entry[1b].

Biological barriers can be globally disrupted by the intra-arterial infusion of osmotic agents, such as mannitol solution, to increase their permeability [10]. A customarily applied method for penetration across biological barriers is the use of these hyperosmotic solutions, which elevates the blood plasma osmolality, resulting in an enhanced flow of water from tissues and thereby loosening the highly compact cell mass and facilitating the vector to cross the barriers and deliver the therapeutic molecules to cells, previously difficult to transfect [10]. Hence, the concept of combining osmotic BBB-opening with vectors to achieve CNS gene-expression was proposed [11]. In these studies, tissues were pre-treated with hyperosmotic mannitol solution, which loosens tight junctions between cells of BBB, and were then treated with various gene/drug delivery vehicles[12]. However, the effect of mannitol solution is often temporary, vanishing after ~ 30 min, sometimes even before the drug or DNA crosses the barrier [13]. Moreover, even after overcoming the BBB, cell membrane barrier and endosomal trapping of the vector still remains challenging towards the successful delivery in brain cells. Immunotherapy[14], virotherapy[15] and non-viral gene transfer[16] are promising approaches but because of safety concerns and ease of fabrication, non-viral gene transfer projects a better alternative for gene therapy clinical trials. Recent data of worldwide gene therapy clinical trials shows that non-viral vectors have occupied 24% of all the clinical trials so far[17].

The development of an ingenious delivery vehicle may provide an important solution to overcome multiple barriers of cellular delivery. Our group recently proposed the use of mannitol and sorbitol -based gene transporters for enhanced transfection efficiency in cancer cells due to the presence of osmotically active hydroxyl groups [18 19]. The number and stereochemistry of the hydroxyl groups affects the intracellular behaviour of gene transporters [20]. Therefore, it is expected that increasing the number of hydroxyl groups in the polymer backbone of gene transporter, would increase its intrinsic osmotic properties that could then facilitate their application in crossing the highly compact BBB. Because sugar alcohols possessing eight hydroxyl groups are not commercially available, here we synthesized the highly hyperosmotic xylitol dimer as an analog of an octamer using the method presented in scheme 1. The current report showcases polydixylitol-based polymer (PdXYP), containing polyol groups as an integral part of the gene delivery vector capable of intra-arterial infusion of osmotically active nanoplexes across the blood-brain-barrier. Moreover, the mechanistic observation indicated directed cellular uptake due to the stimulation of specific caveolae- mediated endocytosis pathways, resulting in a higher level of gene transfection efficiency.

In this manuscript, we propose a rational design for a non-viral gene delivery vector using a combination of appropriate polymer components that not only allows overcoming biological barriers but also enhances the cellular uptake for high transfection efficiency. We propose that (i) the polyol backbone of PdXYP can overcome BBB without affecting transendothelial electrical resistance (TEER) between blood and the brain, (ii) extracellular disturbances caused due to hyperosmotic dixylitol component can modulate the cellular uptake overcoming the cell membrane barrier and enhancing their cellular bioavailability. And finally (iii) the LMW bPEI component can synergistically overcome the endosomal barrier due to high

buffering tendency, leading to the endosomal escape of PdXYP/DNA nanoplexes and improving the transfection efficiency.

Highly hyperosmotic gene delivery vector, PdXYP was synthesized in three steps. (i) Dixylitol (dXY) containing eight hydroxyl groups was synthesized by xylitol dimerization for which xylitol and acetone were condensed into crystalline xylitol diacetone (Xy-dAc). The terminal hydroxyl groups of Xy-dAc were then reacted with trifluoromethyl suphonyl chloride to make trifluoromethylsulphonyl xylitol diacetone (TMSXD). TMSXD was reacted with equal molar ratio of Xy-dAc in presence of dry THF to form xylitol diacetone dimer (dXy-dAc) [26]. dXy-dAc was finally converted into dXY by opening the rings in HCl/MeOH solution. ^{1}H NMR of dXY showed peaks at 3.6-4.2 ppm representing –OH groups (Fig. S1). (ii) Thereafter, primary –OH groups of dXY were reacted with acryloyl chloride to form 1,8-disubstituted dixylitol diacrylate (dXYdA) monomer (iii) In the last step dXYdA-PEI diblock copolymer, PdXYP was synthesized through the copolymerization of dXYdA and low-molecular-weight (LMW) bPEI (1.2 kDa) via the Michael addition reaction [18a, 21] (scheme 1). ^{1}H NMR spectra of the final product showed the disappearance of allyl proton peaks confirming the successful synthesis of PdXYP (Fig. S2). MALDI-TOF-MS: m/z for dXYDA [M^+] = 394.2 and PdXYP [M^+] = 11516.89 (Fig. S3, S4). The composition of dixylitol in PdXYP estimated by ^{1}H NMR was about 42 mol% and the MW measured by GPC was 12-13 kDa (PDI 1.43), which suggests appropriate size of the polymer for effective binding with DNA (Fig. S5A) and forming uniform, stable polyplexes (Fig. 1A). PdXYP was also shown to protect the complexed DNA, suggestive of therapeutic DNA protection against intracellular DNase degradation (Fig. S5B).

Particle size measurements of polyplexes using TEM and DLS indicated that PEI25k/DNA formed loose polyplexes with an average size of 250 nm. On the contrary, PdXYP/DNA formed compact and spherical polyplexes with a relatively homogeneous diameter of approximately 100 nm (Fig. 1A, B, and S6), an optimal size for cellular internalization. Moreover, PdXYP/DNA resulted in ~95% cell viability compared with the high toxicity of PEI25k/DNA (standard transfection agent) and lipofectamine®/DNA polyplexes (Fig. 1C, S7). The lower cytotoxicity of PdXYP is due to the biodegradable ester linkages between polydixylitol and LMW bPEI (Scheme 1) and its lower charge density (Fig. 1B) because of the formation of intra-molecular hydrogen bonds by hydroxyl groups that shield the high surface charge of complexes [18a]. In addition, the degradable ester linkages in PdXYP backbone ensured a gradual disappearance of PdXYP after 3h, 2, 3, 5, and 7 days of transfection (Fig. S8) by hydrolyzing into smaller degradation products that can be exocytosed. Therefore, the occurrence of vesicles was observed to increase with time (maximum on day 5). This further increases the cell viability of PdXYP complexes (Fig. S8) making the vector innocuous for nucleic acid delivery. Further, the transfection efficiency of PdXYP/DNA, as measured in three cancer cell lines (HeLa, HepG2 and A549 cells) and primary rat astrocytes, was markedly higher (25-50 fold) than those of PEI1.2k, PEI25k/DNA and lipofectamine®/DNA (Fig. 1D, S9B,C). FACS also recorded 47% transfection efficiency of PdXYP over 14% of PEI25k (Fig. S9A).

Because the biological barriers are major impediment toward the non-invasive delivery of therapeutic agents, we used an *in vitro* BBB model[1a, 22] to determine the ability of hyperosmotic PdXYP/DNA polyplexes to transmigrate BBB (Fig. 2A). Our results show that after 3 h of incubation of PdXYP/tGFP^F polyplexes in the upper chamber (blood side) of the BBB model, nearly 73% of the original fluorescence was recovered in the lower chamber (brain side). Using similar conditions with naked tGFP^F, only 13% of the original fluorescence was recovered (Fig. 2C). This result shows that the transmigration efficiency of DNA across the BBB increases significantly following its complexation with PdXYP. Furthermore, an immediate depression in TEER values within 15 min of PdXYP polyplexes addition indicates the loosening of BBB compactness due to hyperosmotic stress allowing the polyplexes to pass through the barrier. However, after 3 h the original TEER was resumed to indicate the functional integrity of BBB and that no damage was caused by PdXYP polyplexes while traversing through this barrier (Fig. 2B). PEI25k polyplexes, on the other hand showed decrease in TEER measurements since their addition which was never resumed to indicate heavy functional damage to BBB due to cytotoxicity and therefore, was excluded in subsequent experiments to avoid false results. The penetration of PdXYP/DNA polyplexes through the brain endothelial and pericytes layer of BBB in a blood-to-brain direction was calculated as the apparent permeability coefficient Papp (10^{-6} cm/s) based on Fick's law using the equation;

P_{app} (10^{-6} cm/s) = $V_A/A \times [C]_L \times \Delta[C]_A/\Delta t$

Where V_A: volume of abluminal chamber (cm^3), A: membrane surface area (0.33 cm^2), $[C]_L$: initial luminal tracer concentration (ng/ml), $[C]_A$: abluminal tracer concentration (ng/ml), Δt: time of experiment (s).

PdXYP showed a good permeability (Papp: 17) through the BBB as compared to low permeability of free DNA (Papp: 8) (Fig. 2D). In subsequent experiments, we evaluated the efficiency of PdXYP/tGFP polyplexes, added in upper chamber of BBB kit, to transfect astrocytes cells present in the lower chamber across the BBB. After 48 h, FACS and western blot analysis indicated that PdXYP polyplexes retains its function after BBB transmigration and showed 18.5% transfection in brain astrocytes (Fig. S10). In vivo luciferase expression analysis of PdXYP/pGL3 polyplexes after intravenous injection in six week old mice showed gene (luciferase) expression in various organs, including prominent expression in the brain, compared with that of PEI25k/DNA polyplexes (Fig. S11). *In vivo* bioimaging (Fig. 3) represents the luciferase expression in brain (red arrow), indicating the effectiveness of hyperosmotic behavior of PdXYP in crossing BBB.

Similar to the BBB, the compact tumour mass is also impermeable to most therapeutics due to compactness of the tissue and stromal cells. We designed an *in vivo* experiment to assess the transfection ability of PdXYP into a grown tumour of xenograft mouse. One week after the localized injection of PdXYP/pGL3 polyplexes in tumour, a very high luciferase expression was observed compared to that obtained with naked DNA and PEI25k (Fig. S12). The results show the permeability of PdXYP to uniformly transfect in to the deeper locations in compact tumour tissues.

Table 1. Osmolarity of aqueous solutions of pure mannitol, sorbitol, dixylitol and PdXYP/DNA polyplexes at various concentrations measured as mOsm, calculated from the depression in freezing point of solutions.

Conc[a]	Mannitol	Sorbitol	Dixylitol	PdXYP/DNA
2%	144	149	204	177
5%	391	385	462	523
10%	788	803	1021	899

[a] Aqueous solution

The incorporation of osmolytes into the vector backbone quenches their osmotic activity compared to their pure forms. However, due to the presence of eight –OH groups in xylitol dimer, PdXYP backbone retains its osmolarity equivalent to that of clinically applied pure mannitol (Table 1) required for loosening of BBB. Moreover, this hyperosmotic PdXYP backbone delivers osmotic shocks to the cells, thereby assisting polyplexes not only to penetrate through the biological barriers but also enhance their uptake in astrocytes across BBB (Fig. S10). Further investigations revealed that the extracellular hyperosmotic environment led to the upregulation of caveolin-1 expression (Fig. 4B) present in the membrane lipid rafts. The confocal microscopic images clearly elucidated the co-localization (yellow) of PdXYP/DNA^F polyplexes (green) with the caveolin rich membrane lipid rafts (orange) (Fig. 4A) to suggest enhanced cellular uptake by hyperosmotically induced caveolae-mediated endocytosis. This was further confirmed by the inhibition study of caveolae-mediated endocytosis using methyl betacyclodextrin (BMC). A drastic decrease in the transfection efficiency of PdXYP polyplexes was observed on the addition of BMC (Fig. S13A). In addition, inhibition of endosomal H^+-ATPases by bafilomycin A1 showed a 1000-fold decrease in PdXYP transfection, suggesting the ability of PdXYP to escape endosomal degradation (Fig. S13B).

In conclusion, PdXYP presents a hybrid polymer that possesses hyperosmotic property of dixylitol as an integral part of the vector capable of traversing the compact biological barriers (BBB or dense tumour tissue), thereby significantly enhancing the transfection efficiency in vivo. No cytotoxicity or damage to BBB was observed as indicated by unaltered TEER values after 3 h of transfection, suggesting its innocuous profile for in vivo application. The polydixylitol backbone through its hydroxyl groups corresponds to better DNA complexation and polyplex stability by forming hydrogen bonds resulting in ~ 100 nm particle sizes appropriate for cellular delivery. Mechanistic studies revealed that PdXYP generates a strong hyperosmotic extracellular environment. This hyperosmotic stimulus selectively induces caveolin-1 expression resulting in enhanced cellular uptake by caveolae-mediated endocytosis and therefore PdXYP polyplexes were found co-localized with caveolin rich lipid rafts in cell membrane. This study illustrtates increased cellular uptake and thus higher transfection efficiency in astrocyte across the BBB by transiently opening the BBB due to the hyperosmotic effect of PdXYP. With further success in mice model, we offer an efficient gene delivery vector to hyperosmotically transmigrate the biological barriers and increase the overall transfection efficiency for its application in delivering competent brain-specific and cancer drugs/therapeutic siRNA non-invasively.

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