PABA Nanoparticles as Effective Delivery Agents of siRNAs into Biological Systems

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Abstract

Organic nanomaterials hold promises as new-age drug delivery vehicles *in vivo* and in live cells. Authors have successfully synthesised several fluorescently labelled, self-assembled PABA (*p*-aminobenzoic acid) nanoparticles using various acid side chain combinations. *p*-aminobenzoic acid (PABA), a structural moiety of many commercial drugs, is self-assembled with linker alkyl side chains to form tubular nano structures. Generated molecules demonstrate high level uptake, intercellular delivery, biocompatibility and minimal cytotoxicity, both in human cancer cell lines, mouse embryonic stem cells as well as *in vivo* in *Drosphila* animal model.

Flurophores attached to nanostructures help in rapid in vivo screening and tracking through complex tissues. The sub-cellular inter-nalization mechanism of the conjugates was determined. Physio-chemical parameters of engineered nanoskeletons critical for preferred uptake in multiple organs of live *Drosophila* were also defined. Uptake and inter-cellular delivery of the conjugated nanotubes into cell lines was demonstrated by fluorescence imaging and flow cytometry.

Accumulation of nanotubes had no adverse effects and abnormalities on cell morphology and proliferation rate. The variability of side chains alter size, shape and surface texture of each nanomaterial leading to differential uptake in human and insect cells and different internal organs in live *Drosophila* via energy dependent endocytosis. Our results

showed that physical and chemical properties of C-11 and C-16 acid chain are best fitted for delivery to complex organs in *Drosophila*. Despite differential uptake and clearance from multiple live tissues, the use ofself-assembled nanotubes can add new dimensions to the development oral carriers for the fulfilment of many biological purposes.

Keywords: nano-assemblies, PABA, cell viability, cytotoxicity, endocytosis, fluorescence, flow cytometry.

1. Introduction

In recent years, considerable effort has been channelled into innovation and development of novel therapeutic techniques. An amalgamation of organic nanomaterials and biological delivery is one such recent technological breakthrough. Specially, design of nano delivery vehicles carrying a cargo of biomolecules, biosensors and drugs holds enormous promises[1,2]. Inorganic nanomaterials of various shapes and sizes (nanoparticles, nanorods, nanowires, nanofibres andnanotubes) have long been used as delivery vehicles[3,4]. However the two pervading problems impeding the practice of these vectors –namely, targeted release of the biomolecules and rapid clearance of the carriers[5] have recently spiked the demand for organic nanoparticles.

1.1. 4-*N*-pyridin-2-yl-benzamide(PABA) Nanotubes are Biological-system Compatible Drug Delivery Vehicles.

The attractive hallmarks of organic carriers in biosystems include:chemical flexibility, surface configuration better issue recognition and cell uptake ability[6]. In general, basic cell physiology and cell surveillancedo not allow easy accessibility of foreign particles inside the cells. Exhaustive efforts are being carried out forengineering smooth delivery vehicles, synthesized from biocompatible and biodegradable materials. Though use of nano-materials has been successful in *in vitro* cultured cells[7], *in vivo* the progress is still limited by limited self-life, delivery hurdles, and compatibility to fragile cell environment and potentimmunogenicity[8].

Our approach to combat the potential hurdle to cross the cell membrane barrier involves use of our previously validated,long chain alkyl 4-N-pyridin-2-yl-benzamides (PABA) derivatives, capable of "bottom-up" self-assembly to furnish nanomaterials and accomplish oral delivery in *in vivo* models-mouse stem cells and *in vivo Drosophila*[9]. Our major lookouts were to design a system where variation of side chains inPABA conjugates would achieve minimal absorption in body fluids systemic spreading and the reduction of immunogeneticsymptoms. We also worked onoptimising cellular internalizationmechanisms and targeting to internal organs in*in vivo* models like *Drosophila.*

1.2. Side Chain Variant Derivatives of PNTs(PABA nano-tubes) Optimized for Sub-cellular Intake and Organ Specific Oral Delivery.

Here we usedp-aminobenzoic acid(PABA) as skeletal moiety and self assembled with different acid side chains to produce a library of fluorescentorganic PABA nano-particles having different shapes and sizes and determined their mode of live cellentry. We identified nanoparticles that discriminateamong different physiological environments of humancells and insect cells. Simultaneously, we observed manyphysico-chemical properties of PABA nanoparticles and their uptake mechanism that facilitates targeted organ delivery via oral consumption.

2. Results

2.1. Synthesis and Characterisation of PABA nanoparticles.

Nanoparticles with different side chain variations were synthesized as shown in Fig. 1. The synthesis involved amide formation with 2-aminopyridine followed by reduction of the nitro functionalityusing Pd/C under hydrogen atmosphere as there ducing agent. The free amine functionality present inbenzamide was coupled with different acid chlorides as depicted above. Only seven compounds were subjected to self-assembly of conjugated

nanoparticle formation. To obtain self-assembled nanostructure in each case, 1 mg compound (1-7) was added to 2 ml methanol and heated at 60°C till it dissolved completely. Subsequently, 2 ml deionised water was mixed slowlyat the same temperature to obtain a pure white solution, which on slow cooling at room temperature formed cotton dust-like white aggregates These were isolated using centrifuge at 4,500 rpm for 20 min, followed by overnight drying at 60°C toafford 0.5 mg of final nano-materials. The PABA nanomaterials thus obtained from compound 1-7 were named as C-11, C-11U, C-12,C-14, C-16, C-18, C-18U respectively, based on thelength of the side chains and unsaturated moieties coupled during synthesis.

Laser confocal and scanning electron microscopic (SEM) images showed that the shape and size of each self assembled benzamide structure differs based on the length of the acid side chain (Fig. 2). The saturated acid side chains mainly form tubularshape structure with a hollow space inside, while unsaturated acid chlorides produced cube shapedparticles.TEM and SEM images of halftubes and tubular structure with hollow space inside support the model (Fig. 1C).

Dynamic Light-Scattering (DLS) studies from fresh preparations estimated an average size in therange of 100 to 200 nm but prolonged storage leads to the formation of submicron-sized structures. The average height of each nanoparticleas measured by 3 D reconstituted AFM images is 3-5 nm.

A

Acids	Conditions	Final products	Yields (%)	Fluorescence
	THF, Reflux, 4 Hrs		77	Green Fluorescence
	THF, Reflux, 4 Hrs		75	Non Fluorescence
	THF, Reflux, 6 Hrs		70	Non Fluorescence
	THF, Reflux, 6 Hrs		70	Non Fluorescence
	THF, Reflux, 8 Hrs		68	Green Fluorescence
	THF, Reflux, 14 Hrs		65	Green Fluorescence
	THF, Reflux, 14 Hrs		65	Non Fluorescence

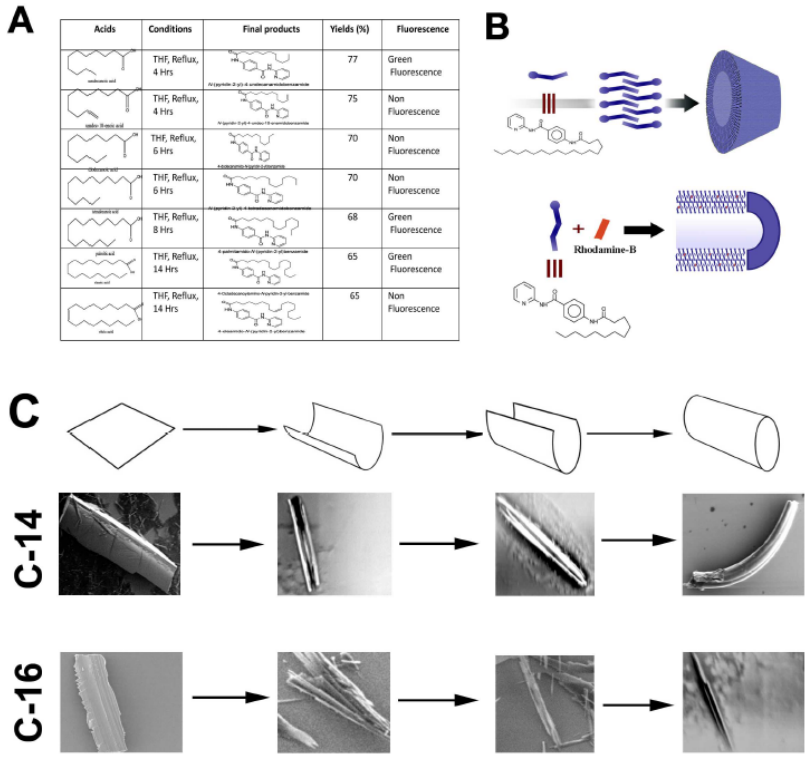

Fig. 1. Design and synthesis of nanomaterials.A.Chemical structure of acid side chains, final self assembled product reaction condition, percentage of yield, fluorescent dyes summarized in a table. B. Schematic diagram showing formation of two nanoparticles (C12 and C18).C.Cartoon diagram and compatible SEM images showing rollover mechanism of two nanomaterial (C-14 and C16) formation[9].

2.2. Relative Uptake of Nanomaterials in Insect and Human Celllines

Three different cell lines : *Drosophila* S2 (Fig. 2), neoplastic HeLa cells and nonneoplastic Human Embryonic Kidney (HEK-293) were cultured in media containing different concentrations of all the nanomaterial;10 µg/ml, 30 µg/ml and 60 µg/ml in 0.01%DMSO. In all cases, nanomaterial containing media to a final concentration 60 µg/ml in 0.01% DMSO showed no adverse effect on cell physiology. Indeed, nanoparticles that emit green

fluorescence (C-11, C-16 and C-18) accumulate almost equally in all three cell types despite the differences in the length of carbon side chains. These results suggest that the tubular shape of all three nanostructures is more important than the length of the acid chains for cellentry.

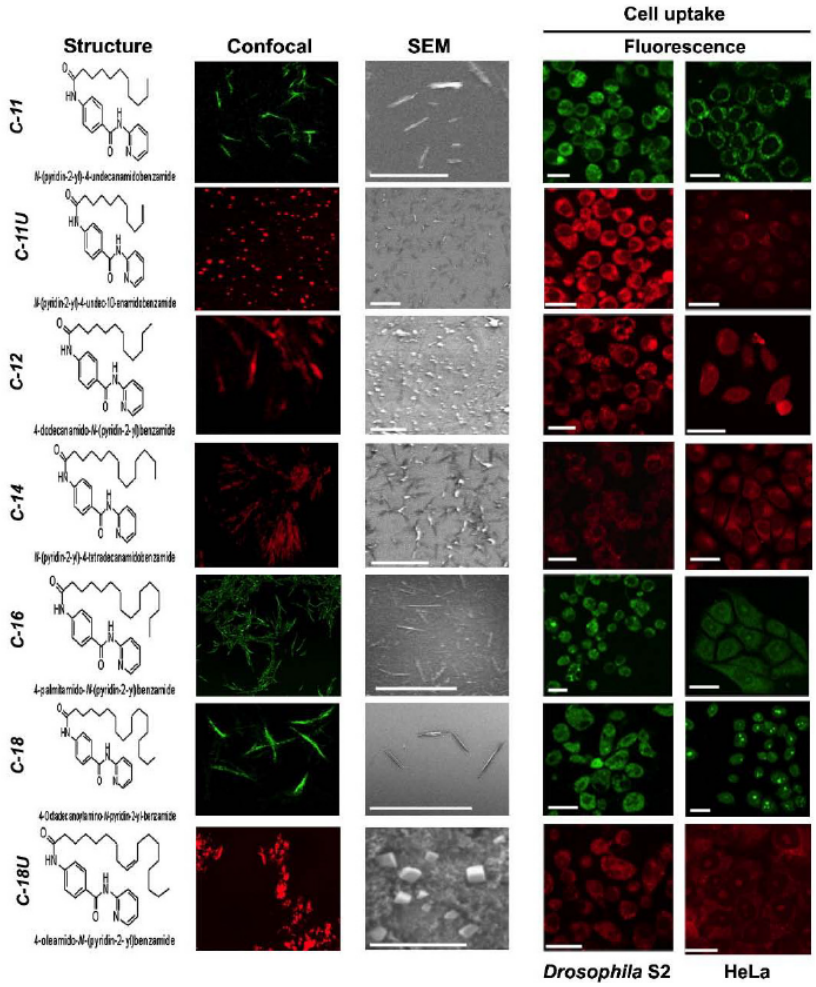

Fig. 2. Physico-chemical properties and microscopic views of 7 PABA nanomaterials. Uptake of several nanomaterials in insect (*Drosophila* S2) and human tumour cells (HeLa) are shown. The differences in chemical structure, shape and surface texture of nanomaterials leads to a variation in cell uptake.Scale- 250 nm (SEM), 50 µm (cells) [9].

2.3. Effect of Nanoparticles on Cell Viability and Cytotoxicity

Uptake of nanoparticles in all cell types does not disturb normal cell propagation andshowed more than 90% cell viability even at the highernanomaterial concentration (120 µg/ml) relative toDMSO treated cells in a colorimetricassay was performed using 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.
Further, flow-cytometry measurements revealed that therelative progression of cells from G1 to S phase in three separate cultures containing 0 µg/ml, 30 µg/ml, 60 µg/ml nanoparticles, were normal based on the incubation time (Fig. 3).

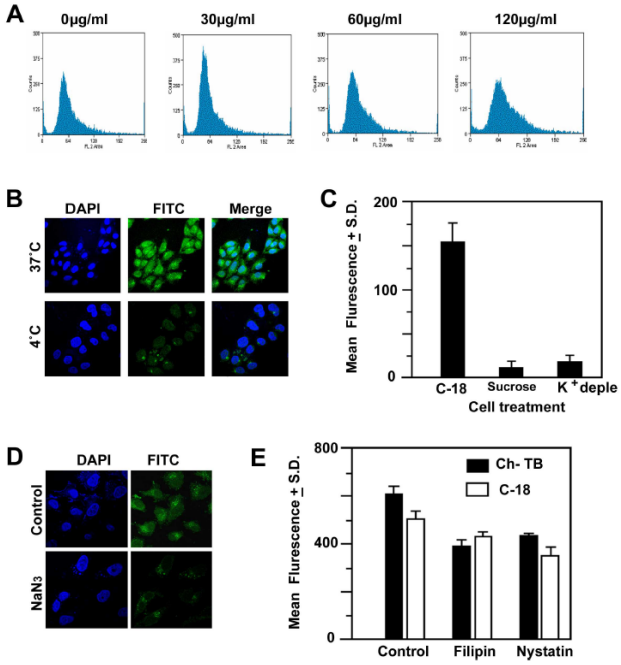

Fig. 3. Sub-cellular internalization of nanomaterials in human cultured cells.A.Cell-cycle arrest and cell viability were tested by flow cell cytometry data of HEK-293 cells obtained after incubation in culture media containing different concentration of nanoparticles C-16. B.The confocalimages of HeLa cells after incubation at 37°C and 4°C in nanoparticles (C-16 containing media).C. Cells pre-treated with 0.45 M sucrose and K+ -depleted medium, D. after pre-treatment with NaN3 respectively.E.Flow cytometry data of HeLa cells with no pre-treatment and pre-treated withfilipin and nystatin were presented in a bar diagram. Cholera toxin B (Black) and C-18 nanoparticle (blank) uptake was shown. Scale 50 µm (Cells).

2.4. Mode of Uptake of PABA Nanomaterials

Cells incubated in media containing nanoparticles were either cultured at 4°C orpretreated with NaN3 for inhibiting the production ofATP, thereby hampering the endocytosis process. The level of fluorescent intensity in the cytosol of each cultured cells was reduced dramatically relative to cells cultured in regular standard conditions(Fig. 3).This reduction determines that PABA conjugates enter in the sub-cellular compartment of cultured cells via endocytosis. Internalization often occurs when the clathrin coat on the plasma membrane forms conspicuous invagination in the cell membrane leading to the budding of clathrin-coatedvesicles. As a result, extracellular species located on the cell membrane are trapped within the vesicles and invaginated inside the cells[10,11]. To disrupt the formation of clathrin coated vesicles on the cell membranes, cells were pre-incubated in sucrose (hypertonic) soluton orK+-depleted media before treatment with all seven nanoparticles. Data showed a drastic reduction in PABA nanoparticle uptake (Fig 3), which suggests that a clathrindependent endocytosis process is involved in entrymechanism.

2.5. Oral uptake of Variable PABA Nanomaterials in *Drosophila*

Different sets of larvae, pupae and adult flies were grown with sole feeding of nanoparticle containing media. The accumulation to various tissues, selective organ uptake and their clearance was alsomonitored by imaging the fluorescence signals during the stages of development in *Drosophila*. In live insects, oral feeding of nanomaterials causes systemic spreading of signals through the gut by peristaltic movement to cross the cell membrane barrier. In general, majority of the nanoparticles carrying unsaturated side chains (C-11U, C-18U) showed a low level of incorporation in all stages of *Drosophila* life cycle although C-18U showed a

comparatively high level of incorporation in two different life stages, larvae and pupae.

2.6. Efficiency of Organ Specific Delivery of PABA Nanomaterials by Side Chain Variation in *Drosophila*

A clear contrast was observed in the delivery of nanomaterials in the salivary glands. C-14 and C-18 containing nanostructures incorporatedat a massive level in the glands but shows an intermediate level of incorporation in both neural tissues and organism itself (Fig. 4). Nanoparticle entry showed a clear variation in rapidly dividing cells of mature larval imaginal discs.

2.7. Transgene Silencing in *Drosophila* Through Delivery of Nanotube-conjugated siRN Aassemblies.

Injection of dsRNA bearing nanoparticles in the preblastoderm stage of *Drosophila* allow disruption of target gene function in all developmental stages. (Fig. 5).To dissect gene function in the adult stage, the intra-abdominal injection technique seems superior to egg injection as it gives a much higher penetrance, it is much simpler, and it makes it possible to address genes that are also expressed in the embryonic, larval or pupal stages.

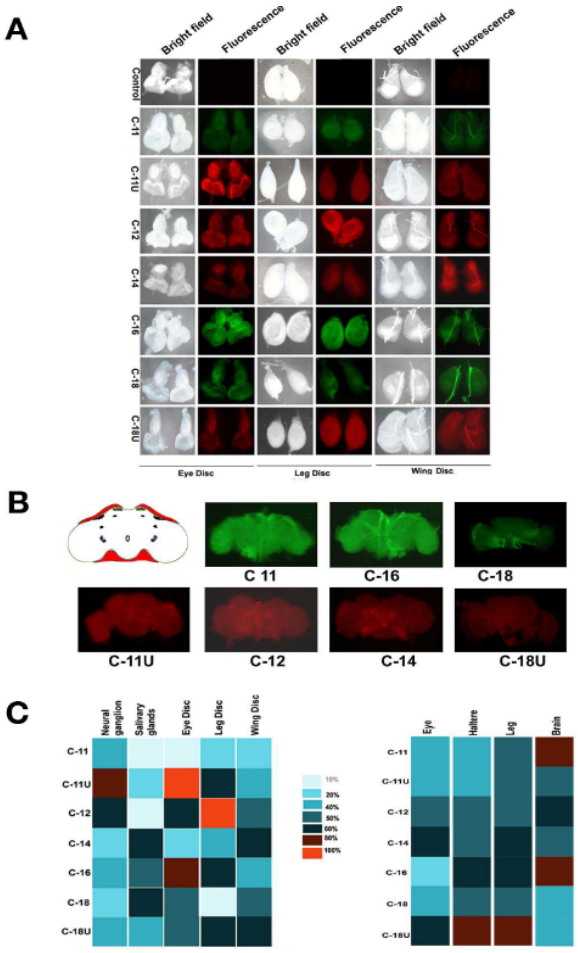

Fig. 4. Uptake and accumulation of orally deliverable 7 PABA nanomaterials. A. Uptake and accumulation of nanoparticles in eye, legand wing imaginal discs. B. adult brain were shown. C. Heat and intensity map representing larval discs specific uptake in complex adult tissues, eyes, halters, legs and brains of 7 PABA nanoparticles were presented. The different colour represents intensity of nanoparticles uptake(noted at the top). Each column represents mean values from six different experiments. The whitish blue refers to the lowest percentage of uptake (10%) and red refers to the highest accumulation (100%).

RNAi injections in live *Drosophila*was able to persistently suppress eGFP expression in transgenics. Knockdown of eGFP was observed at 1 week, still persisted after 10 days and in

embryos, could also be achieved by injection of lentiviral shRNA vector-conjugated nano vehicle 1 week after injection of eGFP had occurred.

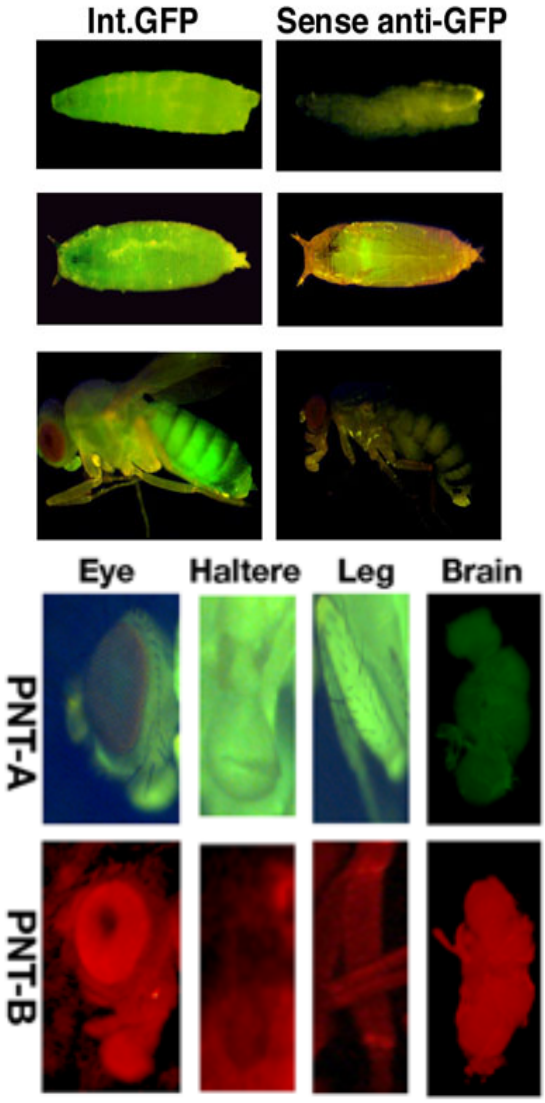

Fig. 5. A. Significant knockdown of eGFP through delivery of nanoparticle conjugated siRNA into life cycle stages of *Drosophila.*B.Successful uptake of synthesised fluorphore conjugated PABA nanotubes into various organs in adult fly.

3. Conclusion and Discussion.

Authors have shown that C-11 and C-16 group of acid side chain forms tubular nanomaterials that are best fitted for oral delivery in complex multiorgans. The cellular uptake mechanism is energy dependent endocytosis. The detailed endocytosis pathways for nano PABA structure is operated thorough clathrin-coated pitsrather than caveolae or lipid rafts. *In vivo* screening ofPABA nanomaterials produced by different acid side chain select the compatible nanostructure ideal for oral delivery and establish energy dependent entry mechanismis of fundamental importance that will facilitate future developments of PABA nanoparticle transporters for biological delivery application.

Our results clearly demonstrate that the properties of each acid side chain together with common PABA moiety influences size, shape and surface texture of nanomaterials that lead to differential uptake and specificity in live cell delivery. The physio-chemical modifications of organic nano carriers also affect cellinternalization mechanisms in sub-cellular organelles as found by distinct accumulation pattern of each nanomaterials following same energy dependent endocytosis.

A systematic screening of PABA conjugated library provides sufficient evidences to support the followingstatements:1)Two nanomaterials carrying C-11and C16 acid side chains are best suited for optimalentry in cells and multiple organs. 2) In live tissues, an internal environment might be a useful barrier for improving nanoparticles delivery in multiple organs.3)Cellular internalization or uptake mechanism of nanomaterials might unravel the clues for smooth entryin human cells and efficient delivery and 4) finally screening of PABA conjugates determine a functional relationship between energy dependent endocytosis and nanomaterial structure for each organ specific targeting.

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