Synthesis and cellular uptake of cell delivering 2,6-pyridinediylbisalkanamide submicron-sized sheets in HeLa cells†

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2,6-Pyridinediylbisalkanamides were synthesized, using diaminopyridine (DAP) as a linker and alkyl chains of varying lengths, that upon self-assembly form submicron-sized sheets and their uptake into the cytoplasm of HeLa cells was studied by confocal microscopy.

There is tremendous interest in the use of self-assembly processes to develop complex molecules, due to their potential in the generation of novel materials at a scale and complexity not possible using covalent methods.[1,2] In recent years research efforts have been directed toward the application of nanosystems as "transporters" to deliver molecular "cargo" to targets within the cytoplasm and the nucleus of the cells.[3,4] We have initiated research programs to develop molecules which self-assemble into nanometric structures with appropriate size, structure and other biological parameters.[5]

In this study we show that the alkanamide synthetic systems formed by combining long alkyl chains, using 2,6-diaminopyridine (DAP) linker, which self-organize in solution to form micro/nano architectures with hydrophobic domains gain entry into the cells. Since the sheets appear to be nontoxic to the cells, they can be considered as "transporters" to deliver pharmacologically important molecules and have applications in nanomedicine. Previous studies using the DAP linker in self-assembly, involved monosaccharides as the head group to form nanotubes for molecular recognition.[6] Here, we focus on five compounds in which the alkyl chain lengths connected to DAP were varied and were evaluated for their capability for cell entry.

In a typical synthesis of the lipid micro/nano structures, 0.2 mg of purified *N*,*N*′-2,6-pyridinediylbisalkanamides [Fig. 1: Schemes 1 and 2 (all identified using NMR, MS)][7] was dissolved in methanol (1.4 ml) and heated to 50 °C for ½ h to obtain a clear solution, and then water (0.6 ml) was slowly added dropwise (solution becomes milky), and the solution was again heated to 60–70 °C for 1 h until it became transparent. The resulting clear solution was allowed to cool to room temperature under ambient conditions over night and afforded white cotton-like aggregates at the bottom

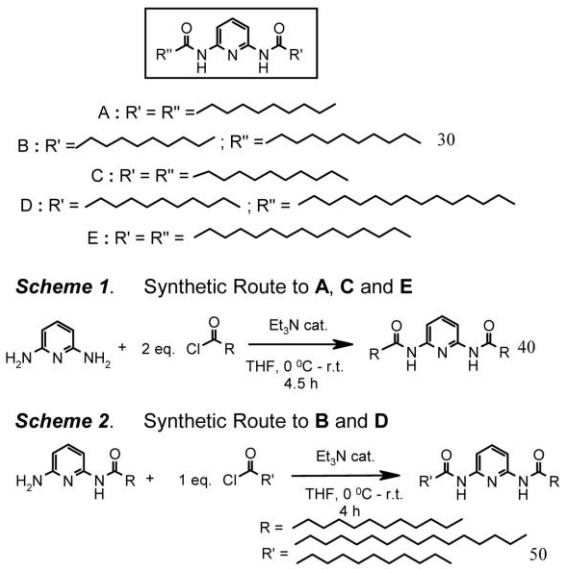

Fig. 1 Chemical structures of the five DAP alkanamides and the synthesis routes.

of the sample tube showing the formation of submicron-sized sheets. These aggregates were filtered, washed thoroughly and dried in a desiccator for 2–3 days.

The structures of the aggregates **A–E** were characterized by the imaging techniques: Differential Interference Contrast microscopy (DIC), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The TEM and SEM images revealed that in all the cases **A–E** self-assembled into a collection of thin sheet-like structures with various dimensions and clear borders (Fig. 2). The electron microscopy images indicate that the sheets are in the submicron range.

The mechanism and the forces that drive the formation of nanometric morphologies is not clearly understood.[8] Compounds **A–E** possesses the DAP moiety and it is likely that the π–π interactions between the pyridine groups provide the required rigidity and also play a major role in directing the aggregation. FT-IR studies of the films deposited on KBr pellet furnished evidence for the hydrophobic forces between the alkyl chains. The FT-IR spectra of **A–E** studied[7] indicate that the absorption bands of the antisymmetric (ν_{as}) and symmetric (ν_s) CH_2 stretching vibrational modes are observed around 2920 cm^{-1} and 2850 cm^{-1}, respectively. The lack of dependence of both vibrating bands on the odd/even alkyl chains confirm the presence of a highly ordered *trans* structure in the aliphatic region. The absence of a single

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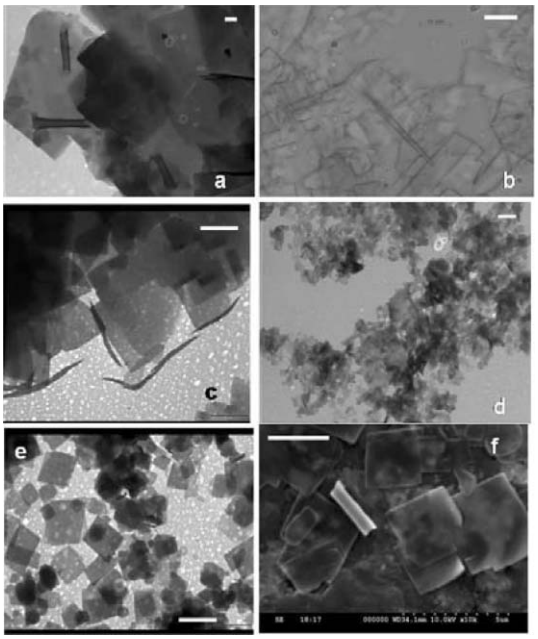

Fig. 2 SEM, TEM and DIC images of **A–E**. (a) **A** TEM, (b) **B** DIC, (c) **C** TEM, (d) **D** TEM (e) **E** TEM and (f) **E** SEM (TEM scale bar represents (a) = 3 μm; (b) = 10 μm; (c) = 5 μm; (d) = 3 μm; (e) = 5 μm; (f) = 3 μm).

strong N–H stretching mode at 3424 cm^{-1} and multiple N–H stretching peaks at 3290–3300 cm^{-1}, which are attributed to a two-point hydrogen bonding[9,10] confirms that the driving forces for the nanomorphologies of **A–E** are π–π interactions and van der Waals interactions of the aliphatic chains. Further evidence of the layered structure came from powder X-ray diffraction (XRD). A single diffraction peak in the small angle region, which shows the presence of long range ordering, characterized the microstructures. Comparison of *d* (obtained from XRD) and *L* the length of the molecule, suggests that when $d \approx L$ the molecules form multilayered structures (Fig. 3) within the self-assemblies with a periodicity (*d*).[7,11]

Diaminopyridine derivatives are known to have a lower tendency to dimerize and can recognize and bind to ligands through hydrogen bonding.[12] Addition of water-soluble thymidine to **A–E** revealed that addition of up to 250 μM thymidine caused

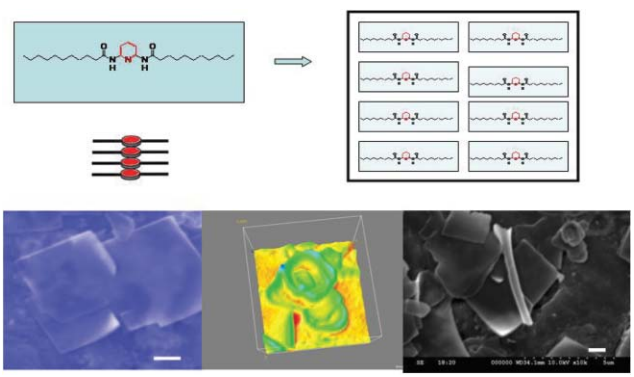

Fig. 3 Suggested packing model for the self-assembled micro/nano-structures. Red disks indicate DAP and their π–π stacking. (a) SEM image of **A** (scale bar = 1 μm); (b) Image analysis of a SEM image of **A**; and (c) SEM image of **D** (scale bar = 2 μm).

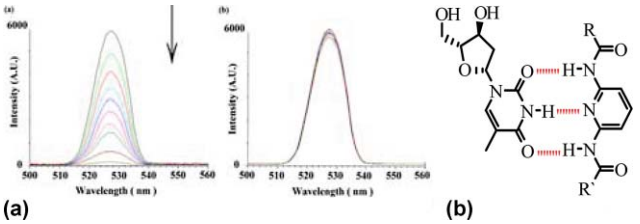

Fig. 4 (a) Fluorescence quenching of **A** by thymidine and the corresponding urea graph. (b) Schematic representation of the possible interaction between microstructures of **A** and thymidine.

immediate quenching of the fluorescence (Fig. 4a). On the contrary, addition of urea (which is capable of undergoing extensive H-bonding) did not quench the fluorescence of the sheets even at concentrations as high as 10 mM.[7] These results imply that the interaction between the assemblies and thymidine occurs through a three-point hydrogen bonded network (Fig. 4b). These results also confirm that the sheets are layered and the DAP based assemblies can act as recognition sites for DNA.

The HeLa (human cervical carcinoma) cell lines were used for the cellular uptake studies of the submicron-sized sheets **A–E**. The cells were grown in small dishes to approximately 80% confluence and subsequently incubated with submicron-sized sheets (60 μg ml^{-1}) dissolved in DMSO for 24 h. The final concentration of DMSO during the incubation was 1% or lower, which has been proven not to effect cell growth.[13] After incubation, the medium was removed and the cells were washed with phosphate buffered saline (PBS) (pH 7.4). The cells were treated with nuclear staining dyes such as acridine orange (100 μg ml^{-1}) and ethidium bromide (100 μg ml^{-1}) simultaneously for 45 s to differentiate viable and dead cells, respectively, followed by three gentle washes with PBS to remove the excess stain from the cells, and were then observed by fluorescence microscope (Olympus microscope IX71). The fluorescence images (Fig. 5) show the differences between the control cells and those incubated with **D**. The submicron-sized sheet **D** facilitates the intracellular localization as shown by the green fluorescence within the cytoplasm.

The initial fluorescence microscopy studies revealed that these submicron assemblies may have ability to cross the cell membrane and this was further confirmed by confocal microscopy. HeLa cells were grown on cover slips and incubated with compound **D** using DMSO as a control. After 24 h of incubation, cells were washed with PBS and fixed in 4% formaldehyde for 15 min. at room temperature. Excess formaldehyde was removed by gentle washing

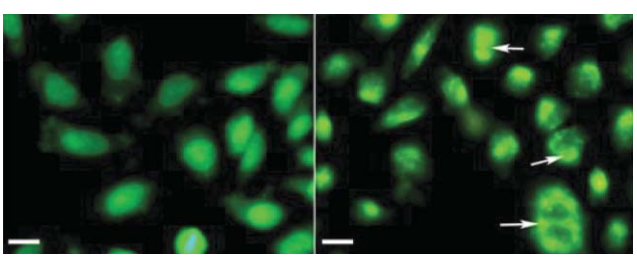

Fig. 5 Cellular uptake study of **D** using HeLa cell line by optical microscopy: (left) control and (right) treated. All scale bars represent 25 μm. Arrows point to submicron-sized sheets that have entered the cells.

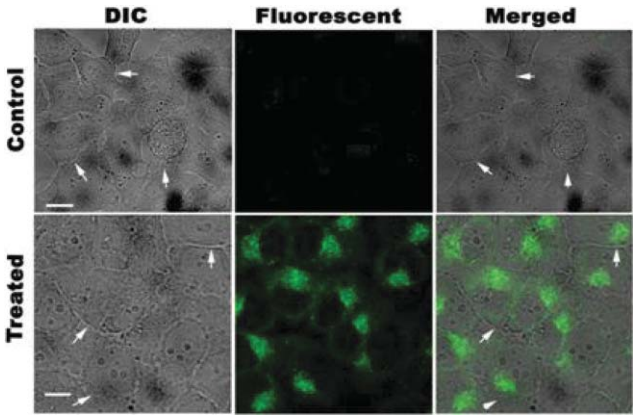

Fig. 6 Confocal images of HeLa cells showing the uptake of submicron-sized sheet **D**. Scale bars are 50 μm. Upper panel control (DMSO) and the lower panel shows the images of cells treated with **D**. The arrows show the cell membrane. Merged figures clearly show the accumulation of fluorescent spots in the cytoplasm.

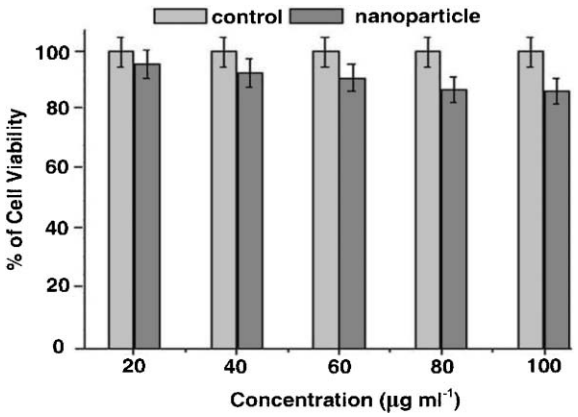

Fig. 7 Cytotoxicity of **D** monitored in HeLa cells. Cell viability was quantified using MTT assay for different submicron-sized sheets concentrations.

with PBS and then the cells were mounted in 70% glycerol. Due to intrinsic fluorescence of the self assemblies, the green fluorescent signals were observed around the nucleus without using any DNA binding dyes. Cells were viewed after taking 12 different Z sections using a LSM 510-meta Zeiss microscope. The projected 3D figures were reconstituted using LSM-FCS (Version 3.2 software) and are shown in Fig. 6.

In depth study with confocal microscopy, showed the presence of clear fluorescence signals throughout the cytoplasm of the cells treated with submicron sheets. The complete absence of fluorescence observed in DMSO treated cells for the same period of time serves as confirmation that the sheets are capable of crossing the cell boundary.

Cell viability was determined by a colorimetric assay described by Plumb *et al.*[14] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This assay depends on reductive capacity to metabolize the tetrazolium salt to blue colored formazone.[15] Briefly, the cells were grown in 96-well plates in DEME medium supplemented with 10% FBS at 37 °C in a CO_2 incubator. After 24 h in culture, the medium in the wells was replaced with fresh medium containing various concentrations (10–100 μg ml^{-1}) of submicron-sized sheets. After an incubation period, cells were added with MTT and the absorbance of colored product was read on a microplate reader (Spectra MAX Plus, Molecular Devices supported by SOFTmax pRO-3.0) at 570 nm. In all the cases tested, the metabolic activity and the proliferation of the HeLa cells treated with submicron sheets **A–E** showed 80–90% cell viability when compared to the control (untreated cells with 100% cell viability) as shown in the Fig. 7. The whole experiment was performed in triplicate under standard laboratory conditions.

In summary, 2,6-pyridinediylbisalkanamides were synthesized using standard organic procedures. The compounds when self-assembled in methanol–water mixture formed submicron-sized sheets. Confocal microscopy studies proved that micro/nano sheets have the ability to penetrate HeLa cells and concentrate in clusters in the cytoplasm. While further studies are required to define the detailed mechanism of the cellular uptake of the submicron assemblies, it is apparent that both the hydrophobic alkyl chain and the DAP linker play important roles in assisting the delivery of the submicron-sized sheets to the interior of the targeted cells. The results reported here offer the opportunity to further tailor the submicron-sized sheets by designing them for use in medicinal therapy.

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Notes and references

1 J. M. Lehn, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 4763.
2 G. M. Whitesides, J. P. Mathias and C. T. Seto, *Science*, 1991, **254**, 1312.
3 W. C. Heiser, *Gene Delivery to Mammalian Cells*, Humana Press, New Jersey, 2004, vol. 1–2.
4 M. Hallbrink, A. Floren, A. Elmquist, M. Pooga, T. Bartfai and U. Langel, *Biochim. Biophys. Acta*, 2001, **1515**, 101.
5 B. Jagannadh, M. S. Reddy, C. L. Rao, A. Parabhakar, B. Jagadeesh and S. Chandrasekhar, *Chem. Commun.*, 2006, 4847.
6 G. John, M. Mason, P. M. Ajayan and J. S. Dordick, *J. Am. Chem. Soc.*, 2004, **126**, 15012.
7 Please see supplementary information†.
8 N. Nandi and B. Bagchi, *J. Am. Chem. Soc.*, 1996, **118**, 11208.
9 A. R. Katrizky and I. Ghiviraga, *J. Chem. Soc., Perkin Trans. 2*, 1995, 1651.
10 O. Uzun, A. Sanyal, H. Nakade, R. J. Thibault and V. M. Rotello, *J. Am. Chem. Soc.*, 2004, **126**, 14773.
11 M. Masuda and T. Shimizu, *Langmuir*, 2004, **20**, 5969.
12 F. H. Beijer, R. P. Sijbesma, J. A. J. M. Vekemans, E. W. Meijer, H. Koojman and A. Spek, *J. Org. Chem.*, 1996, **61**, 6371.
13 A. C. Beekman, H. J. Woerdenbag, H. V. Wiksrom and T. T. Schmidt, *J. Nat. Prod.*, 1997, **60**, 252.
14 J. A. Plumb, R. Milroy and S. B. Kaye, *Cancer Res.*, 1989, **49**, 4435.
15 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.

