The use of fullerene substituted phenylalanine amino acid as a passport for peptides through cell membranes

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We report the formation of a fullerene-peptide conjugate *via* the incorporation of a fullerene substituted phenylalanine derivative, "Bucky amino acid" (Baa), to a cationic peptide, which acts as a passport for intracellular delivery, enabling transport of a range of sequences into HEK-293, HepG2, and neuroblastoma cells where the peptides in the absence of the fullerene amino acid cannot enter the cell. Delivery of the fullerene species to either the cytoplasm or nucleus of the cell is demonstrated. Fullerene peptides based on the nuclear localization sequence (NLS), H-Baa-Lys(FITC)-Lys-Lys-Arg-Lys-Val-OH, can actively cross over the cell membrane and accumulate significantly around the nucleus of HEK-293 and neuroblastoma cells, while H-Baa-Lys(FITC)-Lys_8-OH accumulates in the cytoplasm. Cellular studies show that the uptake for the anionic peptide Baa-Lys(FITC)Glu_4Gly_3Ser-OH is greatly reduced in comparison with the cationic fullerene peptides of the same concentration. The hydrophobic nature of the fullerene assisting peptide transport is suggested by the effect of γ-cyclodextrin (CD) in lowering the efficacy of transport. These data suggest that the incorporation of a fullerene-based amino acid provides a route for the intracellular delivery of peptides and as a consequence the creation of a new class of cell penetrating peptides.

Introduction

Efficient intracellular delivery of a drug can not only reduce non-specific effects and toxicity, but also enhance the effectiveness of drugs incapable of reaching their *in vivo* therapeutic target. Since the major limitation in developing peptide- and nucleic acid-based drugs is their incapability to enter the cell, the conjugation of therapeutic agents to cell penetrating peptides (CPPs) has become a strategy to improve their pharmacological properties.[1–3] CPPs are often short peptides derived from the protein transduction domains of virus proteins, and cannot only be internalized into cells, but also can deliver conjugated species through the cell membrane.[1] Since transport of any species into the nucleus of an intact cell is limited by at least three major membrane barriers, namely the cell membrane, the endosomal membrane, and the nuclear membrane, the delivery of any compound across each given membrane is a significant challenge.

Despite extensive investigation of the mechanistic aspects of CPPs[4] and the increase in the range of CPPs over recent years, there is still limited range of sequences that are available. A further issue concerning the use of CPPs is their toxicity. Although the toxicity of a particular CPP will depend on the amino acid sequence, secondary structure and net charge, some CPPs disrupt the plasma membrane during cell entry through a mechanism similar to the pore forming antimicrobial peptide.[5] We have been interested in the possibility that the membrane translocation properties of a range of peptides could be altered through chemical modification of the peptide such that the resulting conjugate enables the transport of peptides that ordinarily cannot enter the cell.

After the discovery of fullerenes at Rice University in 1985,[6] their potential application in biological and medicinal fields has attracted tremendous interest:[7–10] including their utilization as photodynamic cancer therapy[11] and as DNA transfection agents.[12] With regard the ability of fullerenes to effect membrane translocation, the enhancement of cellular uptake of a CPP through a counter-anion-mediated activation process by the (non-covalent) addition of condensed aromatic rings and anionic fullerenes has been recently reported.[13] This result prompted our investigation of the cell uptake properties of peptides in which one of the amino acids is a fullerene-substituted amino acid. In particular we were interested in the application of the fullerene amino acid derived from phenylalanine, "Bucky amino acid" (Baa, Fig. 1),[14] whose cellular toxicity is demonstrated as low. We have recently reported that at Baa concentrations less than 0.04 mg mL^{-1}, cell viability of human epidermal keratinocytes is maintained.[15] Based upon these results, we have prepared fullerene-derivatized peptides and investigated how the fullerene alters the cellular uptake of low molecular peptides.

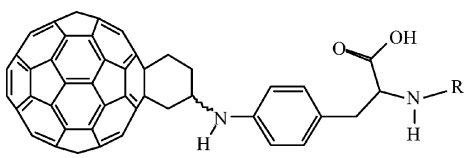

Fig. 1 Schematic representation of Baa (R = H), Boc-Baa (R = C(O)-O^{t}Bu).

A poly-lysine derivative (primary sequence H-Pro-Lys_8-OH) was initially chosen because it is known that oligolysines are not

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cell penetrating peptides. Once inside a cell, an oligo-lysine should show no specific targeting propensity but general uptake in the cytoplasm. To demonstrate selective uptake, the SV-40 T antigen nuclear localization sequence (NLS; primary sequence H-Pro-Lys-Lys-Lys-Arg-Lys-Val-OH) was chosen. This heptapeptide serves as an "address label" for proteins, and leads to their targeting of the cell nucleus. To achieve this goal, the NLS peptide itself has to be inside the cytoplasm, however, it is not ordinarily incorporated into cells. Even if endosomal uptake could happen, the peptide or its conjugate may not be able to be released into cytoplasm and eventually will be excluded from the cells again. In order to ascertain the effects of peptide charge, the anionic peptide (primary sequence H-Pro-Glu_4Gly_3Ser-OH) was also investigated. For each peptide sequence, the fullerene peptide derivatives were prepared and compared to their parent non-fullerene containing sequence. To visualize the fullerene peptide inside the cells, fluorescein isothiocyanate (FITC) was introduced as a label.

We report herein a new general approach to the formation of a cell penetrating peptide in which the combination of a fullerene-based amino acid as a component of a cationic peptide readily allows for the synergistic intracellular delivery of both the peptide and the fullerene component. In this manner, peptides unrelated to protein transduction domains of virus proteins can be converted to cell penetrating peptides by the addition of a fullerene-derived amino acid.

Results

Synthesis and characterization of Baa-peptides

For each of the peptides, the appropriate sequence, including the Lys(Mtt) amino acid (Mtt = 4-methyl trityl) were first synthesized on an automated peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The coupling between Boc-Baa and the appropriate sequence was performed manually using 1*H*-benzotriazol-1-yloxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP)–*N*-hydroxybenzotriazole (HOBt)–diisopropylethylamine (DIPEA) in a three-fold excess. For all peptide sequences, the fluorescent marker was introduced by selective removal of the Mtt protecting group followed by reaction with fluorescein isothiocyanate (FITC). After cleavage from the resin, the peptide solutions were purified by semi-preparative Luna C5 and preparative Dynamax C18 columns for Baa containing and parent peptides, respectively. HPLC analysis, using a 0.1% TFA in H_2O–IPA gradient (see Experimental), showed one major product for all peptides (*e.g.*, Fig. 2).

For all the fullerene-peptides, the isolation of the desired sequence is confirmed by the presence of the M^+ or $M^+ + H$ ion in the MALDI-ToF mass spectrometry. For some of the sequences the observation of the parent ion [M^+] is accompanied with peaks due to the addition of Group 1 metals, *e.g.*, [$M^+ + Na$] or [$M^+ + Na + K$] (*e.g.*, Fig. 3). The MALDI-ToF MS for each of the FITC derivatives also exhibit a peak due to M^+-FITC, see Experimental.

The Baa-functionalized peptides are sufficiently soluble in H_2O to provide a UV–visible spectrum. The observed spectra are consistent with C_{60}, the amide region of the peptide, and where present the FITC. It is important for cell studies that the FITC label retains its fluorescence, *i.e.*, quenching by the C_{60} is not observed. As is seen from Fig. 4, the FITC labeled fullerene peptides do indeed retain their fluorescence. It should be noted, that as with other FITC derivatives, the fluorescence spectrum is dependent on the solvent system employed.

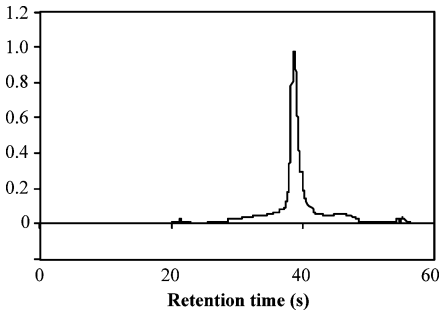

Fig. 2 Typical HPLC chromatogram of Baa-Lys(FITC)-NLS after cleavage from the solid support by washing with TFA–TIPS–H_2O (98 : 1 : 1) and washing with Et_2O.

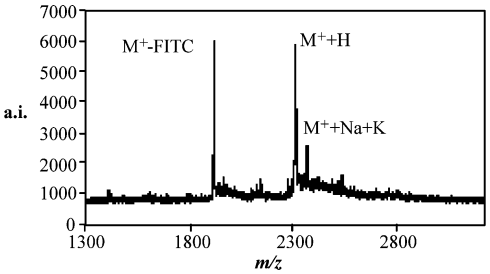

Fig. 3 Typical MALDI-ToF MS of Baa-Lys(FITC)-NLS showing the presence of a fragmentation peak due to loss of FITC as well as the presence of alkali metal ions.

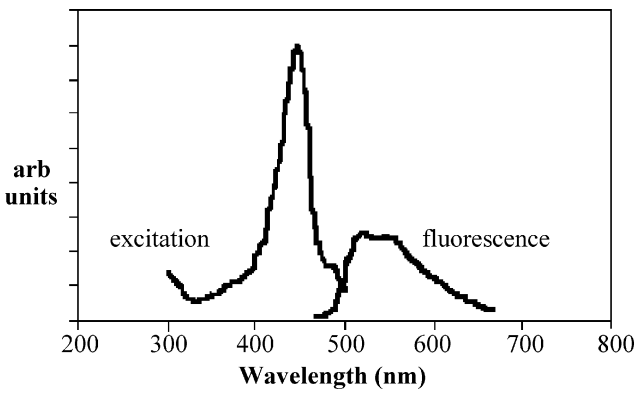

Fig. 4 Fluorescence spectrum of Baa-Lys(FITC)-NLS in H_2O–2-propanol at pH 4.

We have previously observed that the parent amino acid, Baa, aggregates in aqueous solution as a consequence of the presence of both hydrophobic and hydrophilic groups within the same molecule.[16] In order to understand the intracellular transport of the FITC functionalized Baa-peptides, Baa-Lys(FITC)-Lys_8-OH and Baa-Lys(FITC)-NLS, their particle size in solution was determined by dynamic light scattering (DLS). Three series of solutions of each peptide in the range of 0.125–2.0 mg mL^{-1} was prepared by a weighing method. The solution aggregation of Baa-Lys(FITC)-Lys_8-OH was compared with its non-FITC containing analog Baa-Lys_9-OH to determine the effects of the FITC. Each

of the peptides shows aggregation in aqueous solution across the concentration ranges measured. The poly-lysine peptide, Baa-Lys_9-OH, exhibits a single broad aggregate distribution (50–350 nm) with an average size of *ca.* 200 nm. The size of the aggregate is independent of concentration (0.25–2.0 mg mL^{-1}), although the distribution narrows with increased concentration. In contrast, FITC-labeled poly-lysine peptide, Baa-Lys(FITC)-Lys_8-OH, shows two distinct aggregate sizes at concentrations between 0.125 and 1.0 mg mL^{-1}. The most major component (*ca.* 60%) is comparable in size (*ca.* 180 nm) to that seen for Baa-Lys_9-OH. The minor component is a smaller aggregate (*ca.* 10 nm) with a relatively narrow distribution (5–20 nm). At high concentrations (2.0 mg mL^{-1}), a third larger aggregate (*ca.* 1500 nm) is observed at the expense of the both of the other aggregate sizes, see Fig. 5. A similar trend is observed for Baa-Lys(FITC)-NLS (Fig. 6). At 0.5 mg mL^{-1} there appears to be two distinct types of aggregate; the major species (*ca.* 80%) is *ca.* 250 nm while the minor content is again a smaller aggregate (*ca.* 40 nm). As may be seen from Fig. 6, above 1.0 mg mL^{-1} a third distinct aggregate is observed of *ca.* 800 nm. The average size and distribution of each type of aggregate does not change significantly with concentration.

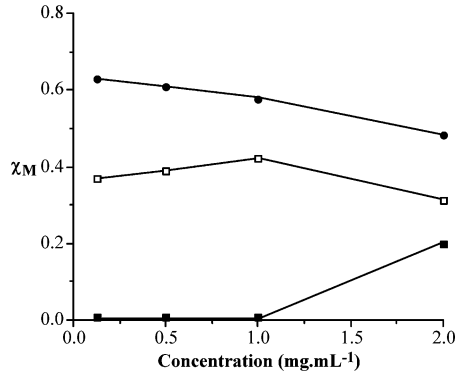

Fig. 5 Plot of the fraction of aggregates for Baa-Lys(FITC)-Lys_8-OH as a function of solution concentration. Average aggregate size = 10 nm (□), 180 nm (●), and 1500 nm (■).

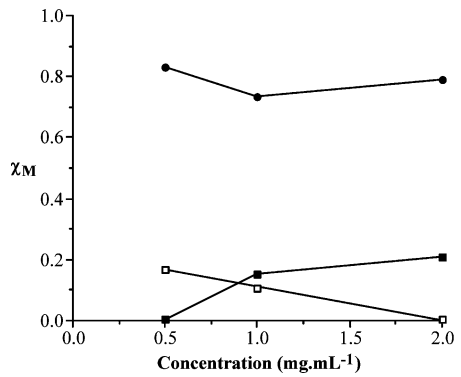

Fig. 6 Plot of the fraction of aggregates for Baa-Lys(FITC)-NLS as a function of solution concentration. Average aggregate size = 40 nm (□), 250 nm (●), and 800 nm (■).

To further examine the actual aggregate size and morphology, cryo-TEM experiments were performed for both Baa-Lys(FITC)-Lys_8-OH and Baa-Lys(FITC)-NLS. Both peptides form spherical and ellipsoidal clusters, with an average aggregate size of 40–80 nm for Baa-Lys(FITC)-Lys_8-OH and 50–150 nm for Baa-Lys(FITC)-NLS, which are generally smaller than the diameters observed by DLS. Consistent with the DLS study, Baa-Lys(FITC)-Lys_8-OH is more uniform in size than Baa-Lys(FITC)-NLS. There are two dominant groups in size for Baa-Lys(FITC)-Lys_8-OH (Fig. 7); one type has a diameter of 40–80 nm with a predominant population at a size of *ca.* 50 nm, and the other population has a diameter less than 20 nm, which may correspond to the size of the fullerene peptides themselves. In contrast, the size distribution of the large aggregates of Baa-Lys(FITC)-NLS is very polydisperse, ranging from 50–150 nm with no obvious dominant population for large aggregates, while smaller aggregates have a size similar to Baa-Lys(FITC)-Lys_8-OH.

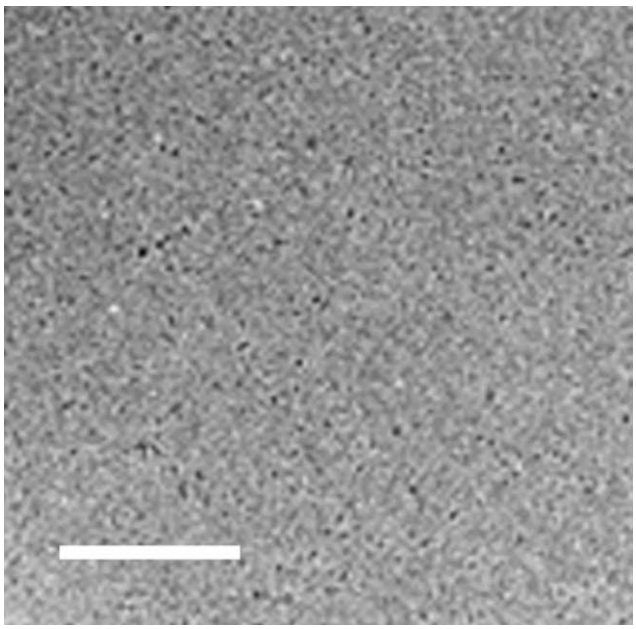

Fig. 7 Vitreous ice cryo-TEM micrograph of the small aggregates formed by Baa-Lys(FITC)-Lys_8-OH in PBS buffer (1 mg mL^{-1} @ pH = 7). Scale bar = 200 nm.

In order to test the stability of the fullerene-peptide linkage, samples of Baa-Lys(FITC)-Lys_8-OH and Baa-Lys(FITC)-NLS were exposed to bovine plasma for up to 3 days and analyzed by HPLC. No degradation of the sample was observed suggesting that both the fullerene amine linkage and the Baa-peptide linkages are stable under conditions relevant to the present study. Furthermore, no alteration of fluorescence frequency or intensity is observed.

Cellular uptake

An immortalized human embryonic kidney epithelial cell line (HEK-293) was used for our initial cellular uptake studies, although similar results were obtained with the human liver cancer cell line (HepG2). After treatment, the cells were washed and observed by a fluorescent microscope equipped with FITC and red filters. The concentration of Baa-derived peptides used in this study is 10^2–10^3, lower than concentrations where cell viability is diminished.[15]

Optical microscope images of HEK-293 cells incubated with Lys(FITC)-Lys_8-OH and Baa-Lys(FITC)-Lys_8-OH shown in

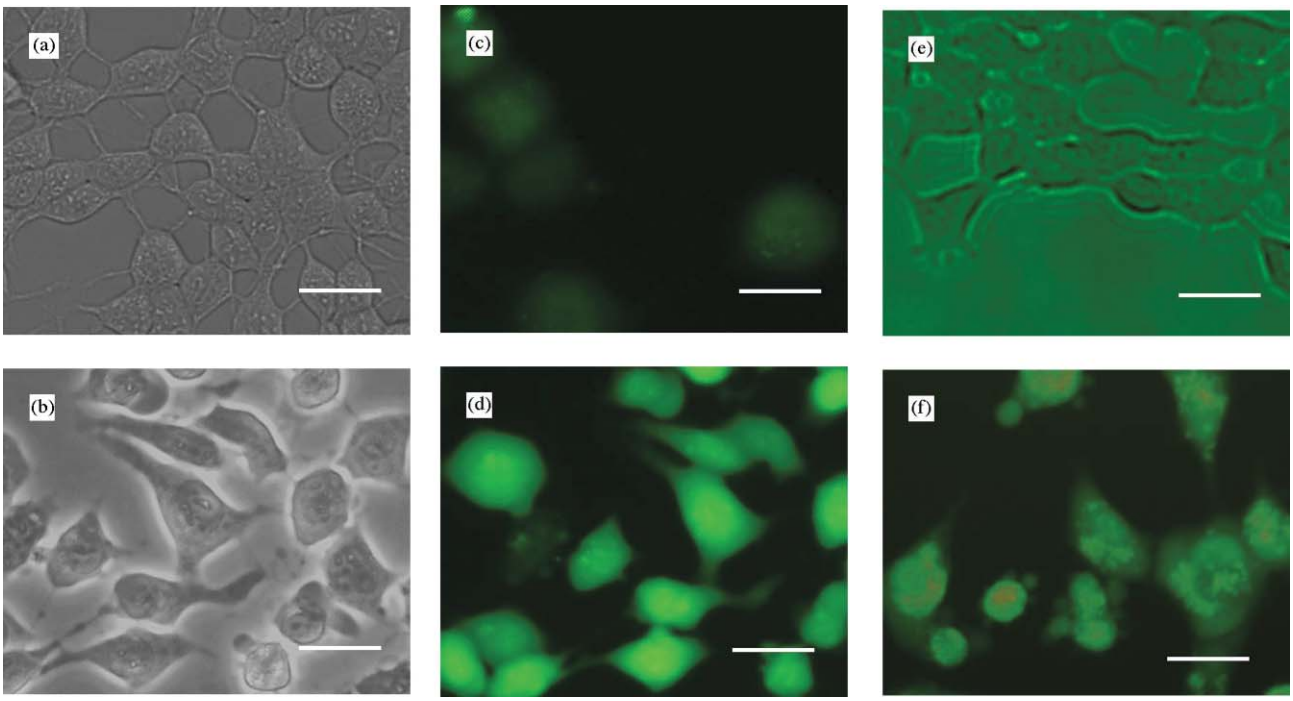

Fig. 8 Optical micrographs of HEK-293 cells incubated with (a) Lys(FITC)-$(Lys)_8$-OH and (b) Baa-Lys(FITC)-$(Lys)_8$-OH, and fluorescence images of HEK-293 cells incubated with (c) Lys(FITC)-$(Lys)_8$-OH, (d) Baa-Lys(FITC)-$(Lys)_8$-OH, (e) Phe-Lys(FITC)-NLS and (f) Baa-Lys(FITC)-NLS. Scale bar = 150 μm.

Fig. 8a and b, respectively, are indistinguishable but the fluorescence images show a dramatic difference. While the cationic peptide Lys(FITC)-Lys_8-OH shows no ability to cross over the cell membrane (by fluorescence, Fig. 8c), the addition of Baa to the sequence facilitates the intracellular localization of the peptide as shown by strong green fluorescence within the cytoplasm (Fig. 8d).

The non-fullerene phenylalanine-NLS derivative (Phe-Lys(FITC)-NLS) shows no uptake into HEK-293 cells (Fig. 8e). In contrast, Baa-Lys(FITC)-NLS shows a localized intense fluorescence in the center of the cells (Fig. 8f). Treatment with DAPI nuclei staining dye showed that while there is a correlation between the location of the Baa-Lys(FITC)-NLS and the nuclei, the peptide is not located exclusively within the nuclei. It would appear therefore that the H-Baa-Lys(FITC)-NLS is located in the nucleus region of the cell, but transport across the nuclear membrane is not extensive under the present conditions.

In order to show the generality of our results we have also studied the uptake of H-Baa-Lys(FITC)-NLS into the neuroblastoma cell line (IMR 32). Neuroblastoma is the most common extracranial solid tumor in children and is responsible for 15% of pediatric cancer deaths. Neuroblastoma cells are known for their difficulty in transfection through the cell membrane. H-Baa-Lys(FITC)-NLS was incubated with IMR 32 cells and after washing with PBS buffer, treated with DAPI nuclei staining dye. Fig. 9 shows intense point fluorescence in the cytoplasm, and homogeneous intense fluorescence around nuclei closely associated with the blue of the DAPI nuclei staining dye. As was observed for the HEK-293 cells, there is a correlation between the localization of H-Baa-Lys(FITC)-NLS and the nucleus.

Cell uptake studies performed at 4 °C showed no cellular uptake activity for either fullerene peptide. Cellular studies

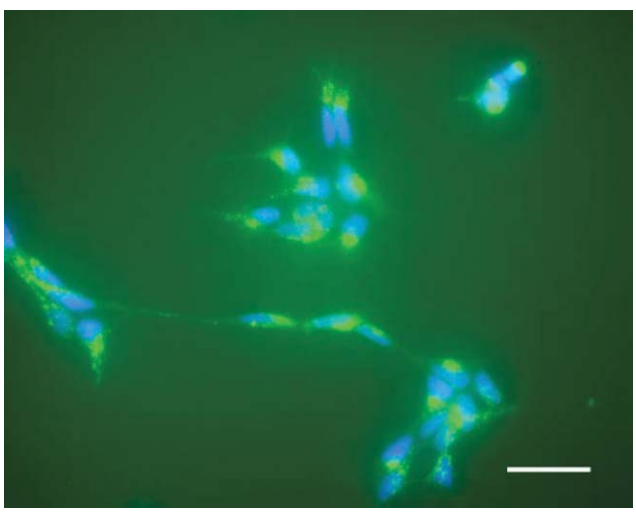

Fig. 9 Fluorescence micrographs of neuroblastoma cells incubated with (green) Baa-Lys(FITC)-NLS and (blue) DAPI. Scale bar = 150 μm.

show that the uptake intensity for the anionic peptide Baa-Lys(FITC)Glu_4Gly_3Ser was greatly reduced in comparison with the cationic fullerene peptides of the same concentration. The cellular uptake is also decreased by the addition of increasing concentrations of γ-cyclodextrin (CD).

Discussion

Several approaches have been previously taken towards the synthesis of fullerene-based amino acids. The simplest approach involved the reaction of an amino acid with C_{60}; however, in

these derivatives only the carboxylic acid functional group remains available for reaction, limiting subsequent incorporation into peptides. Prior to our studies, truly bi-functional fullerene substituted amino acids (those in which both the carboxylic acid and amine functionalities are available for reaction) have been limited to those employing ester or amide linkages.[17,18] Fullerene peptides have shown potential applications in medicinal chemistry,[19] and the use of fullerene amino acids in solution[20] and solid-state[21–23] peptide synthesis has been demonstrated. However, in each case the fullerene amino acids employed contained either the ester or amide linkages giving rise to potential instability. Our use of a hydrolytically stable amine linkage in Baa results in excellent stability under conditions for solid phase peptide synthesis (SPPS).

The incorporation of fullerene peptides using SPPS has previously focused on the use of Fmoc (9-fluorenylmethoxycarbonyl protected) chemistry. We have confirmed that this approach does not lead to high yields[16] and these difficulties in purification led us to develop Boc chemistry for Baa-peptides. The yield of the Baa-terminated peptides appears to be independent of the length of the peptide,[16] but is affected by the charge of the peptide: anionic peptides are made in lower yields possibly due to problems of removal from the resin.

A significant fluorescence quenching in many donor-linked fullerenes can occur; however, in the case of the Baa-Lys(FITC) derivatives, the fluorescence of the FITC label is unaffected by the C_{60} residue. The calculated structure of Baa-Lys(FITC)-NLS shows that the fullerene and FITC tag are about 2.2 nm apart (Fig. 10). It is a result of both this intra-peptide $C_{60} \cdots$ TITC distance and possibly the structure of aggregates formed by the Baa-peptides that results in the retention of the fluorescence of the FITC label. Irrespective of the rational, the retention of fluorescence is a useful indication of the lack of degradation of the peptide.

It has been reported that Lys(FITC)-NLS shows no uptake into cells in the absence of a conjugate.[24] Similarly, our phenylalanine derivative, Phe-Lys(FITC)-NLS, shows no uptake into HEK-293 cells suggesting that the presence of a simple hydrophobic amino acid is insufficient to promote intracellular transport. Furthermore, the cationic peptide Lys(FITC)-Lys_8-OH shows no ability to cross over the cell membrane. However, for both sequences the addition of Baa to the sequence completely alters the transport of peptides into cells. Thus, we report herein that the attachment of a fullerene-derived amino acid enables the transport into cells of peptides that ordinarily cannot enter the cell.

The uptake into the cells for both Baa-Lys(FITC)-NLS and Baa-Lys(FITC)-Lys_8-OH was found to be temperature dependent, suggesting that the cellular uptake activity of the fullerene peptides is an energy dependent process, typically a characteristic of an endocytosis process. It has been previously suggested that the endocytic translocation of the cell penetrating peptides (CPPs) is triggered by the electrostatic interaction of their net positive charge with the negatively charged phospholipid membrane.[25] If a similar process is occurring for our fullerene-functionalized peptides then the use of a negatively charged sequence should reduce or preclude the cellular uptake. Cellular studies show that the uptake intensity for Baa-Lys-(FITC)-Glu_4-Gly_3-Ser-OH was greatly reduced in comparison with the cationic fullerene peptides of the same concentration. Thus, the charge of the peptide does limit the efficacy of the Baa to promote intracellular uptake of a peptide.

Our results strongly suggest that fullerene amino acid actively assists in the cellular uptake. The discovery, combined with our knowledge in the cellular uptake of fullerene amino acid,[15] makes us believe that the hydrophobic fullerene core is playing an important role in assisting the delivery of the fullerene peptide to the interior of the targeted cells. The importance of the hydrophobic nature of the fullerene is demonstrated by the decreased uptake observed by the addition of γ-cyclodextrin (CD). It is well known that CD can change the hydrophobicity of fullerene dramatically through the formation of a complex. If the hydrophobic nature of the fullerene in Baa is an important parameter in providing enhanced transport of the attached peptide, then complexation

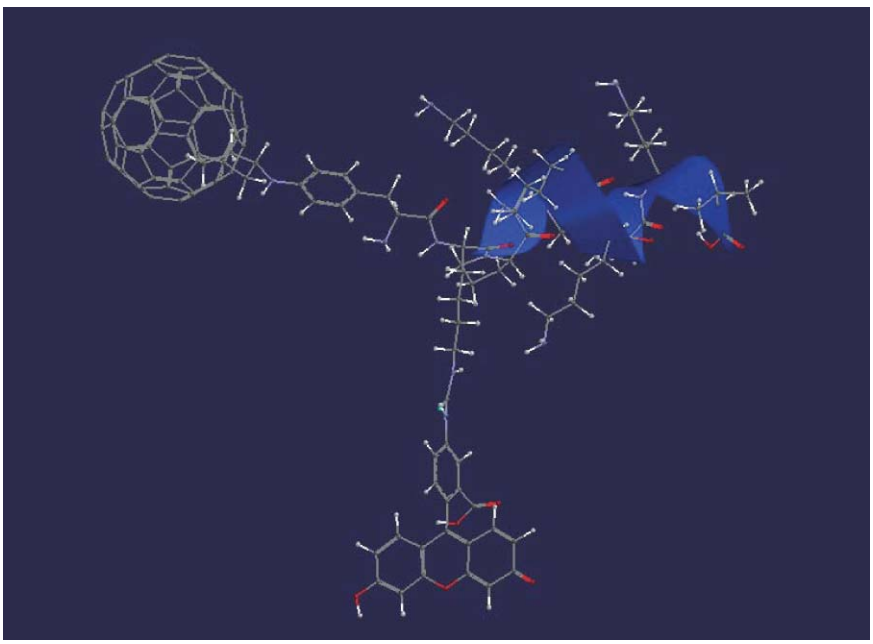

Fig. 10 Calculated structure of Baa-Lys(FITC)-NLS showing the special relationship between the C_{60} and FITC residues.

of the fullerene will have a detrimental effect. This is indeed observed: the higher the CD concentration, the lower the fluorescence intensity is observed inside the cytoplasm. The fullerene-substituted peptides exhibited strong aggregation behavior in aqueous solution, a similar phenomenon demonstrated by other water-soluble fullerene derivatives.[26] CD is also known to break up fullerene aggregates in solution, and thus the lowering of cellular uptake may also be a consequence of the destruction of the Baa-peptide aggregates and it is the aggregate that is the active species in intracellular transport. While further studies are required to define the detailed mechanism of the cellular uptake of fullerene peptides, it is apparent that both the hydrophobic fullerene core and peptide sequences play important roles in assisting the delivery of the fullerene peptide to the interior of the targeted cells.

In summary, we have discovered that the addition of a fullerene-based amino acid (Baa) to cationic peptides facilitates their intercellular translocation into cells where the parent peptides show no such uptake. In this regard, the fullerene provides a passport for the peptide sequence for transport across the cell membrane, while the peptide provides water solubility and reciprocated transport for the fullerene. The hydrophobic fullerene, in combination with the hydrophilic peptide sequence, may form an amphipathic cell penetrating peptide.

Our approach is potentially highly useful because the Baa residue is relatively small, stable under physiological conditions, and readily added to any sequence. Within limitations of peptide charge, it is now possible to prepare an almost inexhaustive range of cell penetrating peptides through the simple and expedient addition of Baa to the sequence. We propose that this concept is a new approach for overcoming the barrier for the effective delivery of membrane impermeable molecules.

Experimental

Boc-Baa was prepared as previously reported.[14] Wang resin was obtained from Novabiochem, USA. Amino acids were purchased from Novabiochem and used as received. The synthesis of amino acid sequences, including a Lys(Mtt) residue, but without Baa was carried out on an automated APEX 396 Multiple Peptide Synthesizer using preloaded Fmoc-Lys(Boc)-Wang resin as the solid phase in a manner similar to that described for the Baa-peptides (see below). MALDI-TOF mass analysis was performed on a linear Protein-TOF Bruker instrument using sinapinic acid as the matrix. UV–Vis spectra were recorded on a Varian Cary 5000 spectrometer. PBS buffer prepared from HPLC grade water was used as solvent and was filtered through a 0.10 mm cup filter (Millipore, Express). TEM measurements were performed on a JEOL 2010 TEM at 200 kV. The images were taken at a concentration of 1.0 mg mL^{-1} for both peptides. Samples for cryo-TEM studies were prepared by dipping a copper grid coated with amorphous carbon-holey film into the sample solution. The TEM images were mainly taken in the hole region of the TEM grid to minimize the artificial effect from the samples or ice.

Synthesis of Baa-containing peptides

The coupling of amino acid sequences, including a Lys(Mtt) residue, but without Baa was carried out on an automated APEX 396 Multiple Peptide Synthesizer using preloaded Fmoc-Lys(Boc)-Wang resin (469 mg, 0.30 mmol) as the solid phase. Fmoc deprotection was performed using 25% piperidine in DMF solution. A portion of the resin was swollen with DMF. A 3-fold excess of Boc-Baa (157 mg, 0.15 mM) dissolved in DMF–DCM (2 : 1, 9 mL) activated with PyBOP–HOBt–DIPEA (1 : 1 : 1 : 3) was mixed with the resin, and shaken for 1 day. After washing thoroughly with DMF and DCM, the resin was treated with 1% TFA and 5% TIPS in DCM, and swelled in DMF; after which the resin was shaken with a solution of FITC (65 mg) in DMF (8 mL) and DIPEA (130 μL). After washing with DMF, DCM and shrunk with MeOH, the resin was dried *in vacuo* overnight. Cleavage of the peptide was achieved with TFA–TIPS–thiolanisole–H_2O (92.5 : 2.5 : 2.5 : 2.5) cocktail. After filtration, the peptide solution was concentrated and precipitated with cold Et_2O. RP-HPLC purification was carried out on a Phenomenex Luna C5 column using an isocratic gradient of 0.1% TFA in water, and 0.1% TFA in isopropanol. Baa-Lys(FITC)-Lys_8-OH: isolated yield, 43.4%; MALDI-MS (*m*/*z*), 2496 [M^+], 2107, [M^+-FITC]. Baa-Lys(FITC)-Pro-Lys-Lys-Lys-Arg-Lys-Val-OH (Baa-Lys(FITC)-NLS): isolated yield, 50.6%; MALDI-MS (*m*/*z*), 2337 [M^+ + H], 1948 [M^+ + H-FITC]. Baa-Lys(FITC)-Glu_4-Gly_3-Ser-OH: isolated yield, 7.4%; MALDI-MS (*m*/*z*), 2270 [M^+ + Na].

Cellular uptake studies

Cells were cultured in RMPI 1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 and at 37 °C in a humidified incubator. The medium was supplemented with penicillin (100 U cm^{-3}), streptomycin (100 μg cm^{-3}), and glutamine (2 mM). For experiments and microscopy, cells were seeded at 1×10^4 cells/well on 6-well plates and grown for two days in RPMI. Cells were incubated with purified fullerene peptides for up to 1 day in a concentration of 10 μM at 37 °C in a humidified incubator (5% CO_2). After treatment, cells were washed once with RPMI supplemented with 10% FBS and three times with PBS (10 mM). Cells were visualized with an Olympus IX70 fluorescence microscope. For nuclear staining, cells were treated with DAPI for 5 minutes, and washed with PBS before imaging.

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