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PAPER

Self-assembling dipeptide-based nontoxic vesicles as carriers for drugs and other biologically important molecules†

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Self-assembling short peptides can offer an opportunity to make useful nano-/microstructures that find potential application in drug delivery. We report here the formation of multivesicular structures from self-assembling water-soluble synthetic amphiphilic dipeptides containing a glutamic acid residue at the C-terminus. These vesicular structures are stable over a wide range of pH (pH 2–12). However, they are sensitive towards calcium ions. This causes the rupturing of these vesicles. Interestingly, these vesicles can not only encapsulate an anticancer drug and a fluorescent dye, but also can release them in the presence of calcium ions. Moreover, these multivesicular structures have the potential to carry biologically important molecules like cyclic adenosine monophosphate (cAMP) within the cells keeping their biological functions intact. A MTT cell-survival assay suggests the almost nontoxic nature of these vesicles. Thus, these peptide vesicles can be used as biocompatible delivery vehicles for carrying drugs and other bioactive molecules.

Introduction

Molecular self-assembly offers a wonderful strategy to make various supramolecular structures including nanotubes,[1] nanorods,[2] nanofibers,[3] nanovesicles[4] and others.[5] Vesicles (enclosed spherical assemblies) seem to be a fascinating structure, because of their structural resemblance with primitive biological cells.[6] Vesicles are of technological interest for various applications including encapsulation and sustained release of drugs,[7] chemical sensing,[8a–b] biosensing,[8c] nanoscale reactors[9] and enzyme encapsulation.[10] Antonietti *et al.* have reported that the vesicle formation is a two-step self-assembly process, an amphiphile first forms a bilayer, which then in a second step closes to form a vesicle.[7c] Previous reports include that vesicles are obtained from various building blocks including block copolymers,[11] oligonucleotides,[12] lipid-based molecules,[4a,4h–i,7c–d] polysaccrides,[13] polypeptides[11b–e,14] and a few synthetic organic molecules like calixarenes,[15] cyclodextrins,[16] and curcubit[6]urils.[17] Peptide assemblies are biocompatible, as is evidenced by their successful applications in biological and medical fields including stem cell differentiation, tissue engineering, *in vivo* nerve regeneration and others.[18] Vesicles obtained by the self-assembly of the polypeptides are termed as "peptosomes" and they have been studied extensively by different research groups.[11b–e,14] Deming and his coworkers made a significant contribution in constructing vesicles from self-assembling charged amphiphilic block copolypeptides and they have used these vesicles as an intracellular delivery system.[11b–e] Formation of vesicles depends on various factors including the proper choice of self-assembling molecular scaffold,[4d,4j,19] pH,[20] concentration[21] and some other factors.[22] However, examples of short peptide-based vesicles are relatively fewer than polypeptide-based vesicles. Raches and Gazit have reported that diphenylglycine, the most generic form of an aromatic dipeptide, diphenylalanine, can form spherical nanometric assemblies and these hollow nanospheres have remarkable stability, like nanotubes formed by diphenylalanine.[23] In a recent report, it is demonstrated that nanovesicles are formed from self-assembling dipeptides with a conformationally constrained α,β-dihydrophenylalanine residue (ΔPhe). These vesicles can encapsulate small drugs and other biologically important molecules including riboflavin, vitamin B_{12}, bioactive peptides and even a protein. They are resistant to the proteolytic enzyme, proteinase K.[24]

There are many reports of self-assembled biomolecule based vesicular structures. However, vesicular assemblies which contain small vesicles within their interior compartments, similar to the discrete organelles within eukaryotic cells, are very rare in the literature[25] and the structural control of such assembly has not yet been achieved properly. There is a need for nano- or microvesicles composed of biological building blocks, which can deliver the encapsulated molecules at the target site in response to a stimulus and these vesicles should exhibit minimum or no toxicity, so that they can be used as good delivery vehicles for carrying drugs and other biologically important molecules. Peptides **1**, **2** and **3** (Fig. 1) have been selected in such a way that each peptide contains a C-terminally located hydrophilic glutamic acid residue and the

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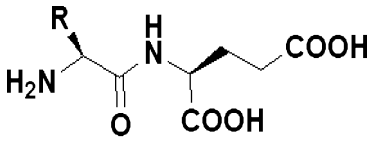

For **peptide 1**, R= $CH(CH_3)_2$

For **peptide 2**, R= $CH_2CH(CH_3)_2$

For **peptide 3**, R= $CH_2CH_2CH_3$

Fig. 1 Chemical structures of peptides **1**, **2** and **3**.

N-terminally positioned hydrophobic residue. The reason behind that is all these amphiphilic peptide molecules (**1**, **2** and **3**) are anionic at slightly acidic to neutral pH as well as in basic pH and they are sensitive to bivalent cations. In this study, we present the formation of multivesicular architectures (vesicles containing small vesicles within their interior compartments) from the self-assembling water-soluble synthetic dipeptides containing glutamic acid as the C-terminal residue. To the best of our knowledge, this is the first report of multivesicular architectures obtained from water-soluble synthetic anionic dipeptides. In this report, it has been demonstrated that these peptide-based vesicles are stable over a wide pH range (pH 2–12), and responsive to Ca^{+2} ions. Moreover, a fluorescent dye (eosin B) and an anticancer drug (doxorubicin) can be encapsulated inside these vesicles and these encapsulated materials can be released in the presence of calcium ions. The nontoxic nature of these dye/drug-loaded vesicles has also been demonstrated using MTT (3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell survival assay. A cellular uptake study using vesicles loaded with biologically important molecules like cyclic adenosine monophosphate (cAMP) demonstrates that these vesicles not only enter into the cells but also can successfully deliver the entrapped molecules within the cells keeping their biological functions intact. These functional peptide-based vesicles are endowed with fascinating properties and this opens up an opportunity to use these vesicles as vehicles for delivering drugs and other biologically important molecules inside the cytosolic counterpart of cells.

Results and discussion

Fig. 1 shows chemical structures of peptides which were used in this study. Each peptide consists of a glutamic acid residue at the C-terminal end and the N-terminal position is occupied by any of the hydrophobic residues Val/Leu/Nva (norValine) for peptides **1**/**2**/**3**. Thus, three hydrophilic functionalities (one amino group and two carboxylic acid groups) are present in each of these peptides. All these peptides are soluble in water and they were visibly clear in the entire duration of the study.

A dynamic light scattering (DLS) experiment has been performed to assess the formation of supramolecular assemblies. It is a rapid screening method to identify supramolecular well-defined structures and no nanostructures can be formed without any discrete peak intensity. It was found from the DLS study (Fig. S10, ESI†) that the average hydrodynamic diameters are 280, 310, 370 nm, respectively, for peptides **1**, **2** and **3**. It is important to note that DLS cannot provide meticulous insight into the size of the regular structures formed. Rather, it can capture the average size. Hence, several microscopic studies were carried in order to examine meticulous morphological information for these self-assembled structures.

A field emission scanning electron microscopic (FE-SEM) study has been performed to investigate the morphological features of these self-assembled peptides. Each of these peptides (0.06 mmol) was dissolved in 1 mL water of pH 6.96 and allowed to age for 24 h. The FE-SEM study clearly demonstrates (Fig. 2) the formation of spherical aggregates (nanospheres) having diameters within the range of 90–100 nm for peptide **1**, 90–100 nm for the peptide **2** and 80–90 nm for the peptide **3**, respectively.

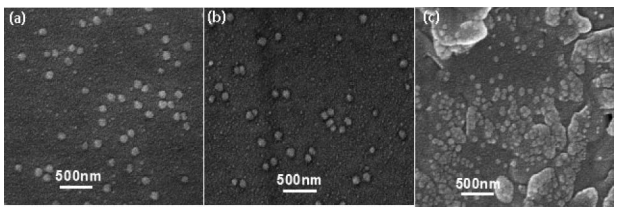

Fig. 2 Field emission scanning electron microscopic (FE-SEM) images of (a) peptide **1**, (b) peptide **2** and (c) peptide **3**.

Transmission electron microscopy (TEM) experiments were carried out to examine the internal structure of these spherical aggregates, which were obtained from self-assembling peptides **1**, **2** and **3**. No clear and credible images with distinct structural features was obtained in the TEM experiments (Fig. S15, ESI†) at 0.06 mmol mL^{-1} peptide concentration (*i.e.* under the conditions of the FE-SEM experiment).

So, all TEM experiments were performed at a lower concentration of peptides (0.02 mmol of each peptide in 1 mL of water). These TEM images vividly illustrate the architectural arrangement of multivesicular assemblies for these self-assembled peptides, in which one or more vesicles have been enclosed within a relatively larger one (Fig. 3 and Fig. S19, ESI†). These peptide vesicles are stable within a wide range of pH values (pH 2 to 12). It is interesting to note that the vesicles within the vesicles exist throughout the experimental conditions of different pH values (Fig. 3, 4, 5). This is probably due to the fact that a change of pH (2–12) cannot change the amphiphilic nature of the molecule and the self-assembly pattern in the formation of the vesicular structure. In some cases, these assemblies are similar to the organization of biological eukaryotic cells and their subcellular organelles (Fig. 3a,b, 4c, 5a,c).[25] The sizes of these vesicles obtained from FE-SEM and TEM experiments are different as the peptide concentrations that were used for these two experiments were not the same. TEM experiments were carried

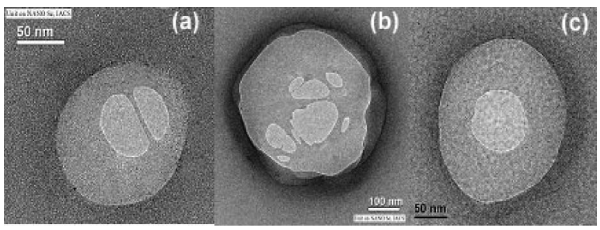

Fig. 3 Transmission electron microscopy (TEM) images of (a) peptide **1**, (b) peptide **2** and (c) peptide **3** at pH 6.96.

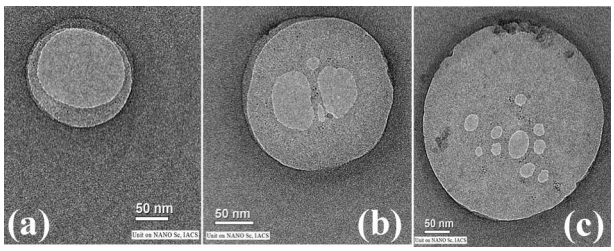

Fig. 4 Stability of vesicles at pH 12. (a), (b) and (c) are the vesicles obtained from peptides **1**, **2** and **3**, respectively, at pH 12.

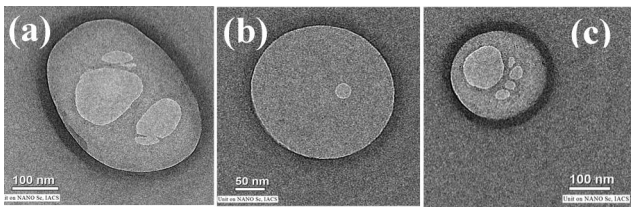

Fig. 5 Stability of vesicles at pH 2. (a), (b) and (c) are the vesicles obtained from peptides **1**, **2** and **3**, respectively, at pH 2.

out using much more dilute solutions of peptides compared to those of the FE-SEM experiments. A time dependent confocal fluorescence microscopic study was also performed to understand the kinetics of the self-assembly process forming vesicles. Different confocal fluorescence microscopic images were taken at different time intervals after preparing the samples (Fig. S20, ESI†). Fig. S20 shows that there is no significant change in the morphology of vesicle formation for the samples of 30 min, and up to 385 min of incubation time.† This clearly indicates that the kinetics of vesicle formation are fast. A confocal fluorescence microscopic study in the solution state was performed using the fluorescent dye eosin B-loaded vesicles, and encapsulated vesicles were imaged by fluorescence microscopy. The image (Fig. S22, ESI†) clearly shows the formation of vesicular architectures in the wet solution state. An AFM study was performed to examine the topography and the height of the vesicles in the *z*-range. The AFM analysis suggests the spherical configuration of these vesicles (Fig. S11, S12, and S13, ESI†) obtained from the self-assembling peptides **1**, **2** and **3**. The heights of these vesicles were found to be 27.71, 9.86 and 12.33 nm, respectively, for vesicles obtained from self-assembling peptides **1**, **2** and **3**.

It is interesting to note that the vesicular morphology of these self-assembling peptides was retained from acidic to basic pH values (pH 2 to 12) (Fig. 4 and 5). Now, we address the question of whether these peptide vesicles are stable in the presence of bivalent cations (Ca^{+2}/Mg^{+2}) or not. The stability of these vesicular structures was also tested in the presence of calcium ions. These peptide-based vesicles were incubated with aqueous solution containing calcium ions for six hours under very mild conditions (at peptide and metal ion concentration 1 : 2). This triggers the rupturing of these vesicular morphologies and the energy depressive X-ray (EDX) analysis confirmed the presence of calcium (Fig. S14, ESI†) within the debris of vesicles.

Vesicles obtained from the self-assembling molecules of biological origin can be considered to be an important delivery vehicle to carry drugs and other bioactive molecules inside cells.[7d–f] Our main interest is to examine the entrapment of various guest molecules within the dipeptide-based vesicles to explore the possibilities of using these vesicles as delivery vehicles.

Vesicles were co-incubated with the fluorescent dye eosin B (EB) for the encapsulation to allow the detection of dye-loaded vesicles using fluorescence microscopy. Confocal laser scanning fluorescence microscopic (CLSM) images (Fig. 6) show the entrapment of dye eosin B within these vesicles. It is important to note that the presence of calcium ions (at peptide/metal ion concentration ratio 1 : 2), can rupture these vesicles and the CLSM images (Fig. 7) also support the rupturing of the dye-loaded vesicles in the presence of calcium ions. From the kinetics of release study (Fig. S21, ESI†) it is evident that after the addition of calcium ions (0.12 mmol mL^{-1}) most of the encapsulated dye has been released within 0.5 h. Furthermore, these peptide-based vesicular systems were tested for the encapsulation of an anticancer drug, doxorubicin. CLSM images clearly demonstrate the encapsulation of doxorubicin (Fig. S16, ESI†) within the vesicles, and the release of encapsulated doxorubicin is also possible by treating them with calcium ions as stated above (Fig. S17, ESI†). Thus, it can be envisaged that these peptide-based vesicles can be used in the future as drug delivery vehicles.

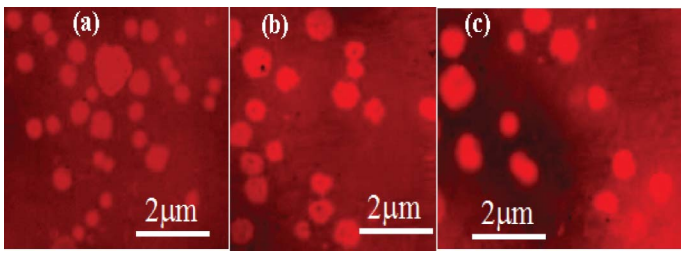

Fig. 6 Encapsulation of eosin B (EB) within the vesicles upon co-incubation of the respective peptides (0.06 mmol) with EB (0.03 mmol) in 1 mL water at pH 6.96 for 48 h (a) for peptide **1**, (b) for peptide **2** and (c) for peptide **3**.

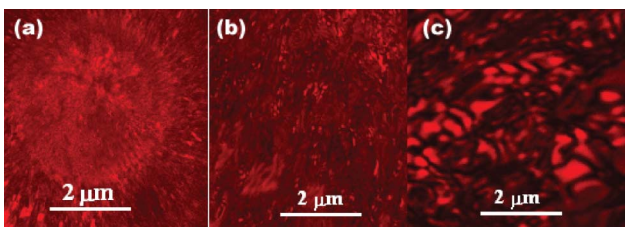

Fig. 7 Entrapped EB release study in the presence of calcium ions (at concentration 0.12 mmol mL^{-1}). Release of EB from vesicles of (a) peptide **1**, (b) peptide **2** and (c) peptide **3**.

We studied the potential of these vesicles to transport biologically important molecules into the cytosolic compartment of cells. This could have far reaching implications in modern molecular therapeutics, because in many cases a drug by itself may not be able to penetrate through the cell membrane.

To investigate this, vesicles were loaded with a fluorescent dye, eosin B (EB) and these dye-loaded vesicles were incubated with C_6 glioma cells for 48 h. The confocal laser scanning microscopic (CLSM) study clearly demonstrates (Fig. 8) that the dye-loaded vesicles are localised into the cytosolic compartment of cells.

A time kinetic entry of dye-loaded vesicles within the C_6 glioma cells was also accomplished. A significant difference in

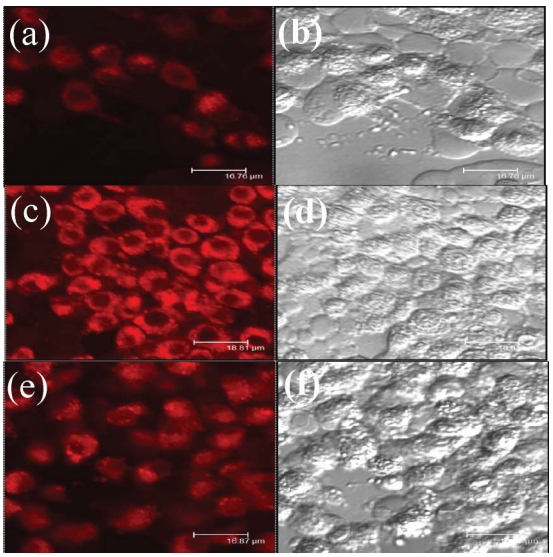

Fig. 8 Internalization of eosin B-loaded vesicles within the cytosolic compartment of cells. Confocal fluorescence microscopic images of EB-loaded vesicles of peptide **1** within the cell (a), peptide **2** (c), and peptide **3** (e). (b), (d) and (f) are the corresponding bright field images.

fluorescence intensity of EB was observed after six hours (Fig. S18, ESI†) in the membrane fraction and in cytosol. This further supports the internalisation of dye-loaded vesicles inside the cells.

Although the EB-loaded vesicles were internalized within the C_6 glioma cancer cells, the potential cytotoxicity needs to be addressed. There are many reports of peptide-based and block copolymer-based vesicular structures formation and their applications in dye/drug encapsulation and release studies.[11b–e,24] However, toxicity measurement of those vesicles containing dye/drug has rarely been addressed.[11b,26] The toxicity of these vesicles has been examined using the MTT cell-survival assay. It was observed that even after incubation of the C_6 glioma cells with the EB-loaded vesicles for 48 h, there was no significant decline in the number of viable cells (Fig. 9). This suggests that these dipeptide-based vesicles are almost nontoxic in nature. Experiments with EB-loaded vesicles established the fact that these vesicles are capable of transporting molecules inside the cell.

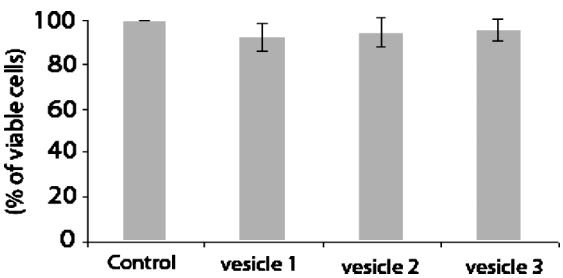

Fig. 9 MTT assay shows that toxicity of the peptide vesicles is insignificant, as almost 90% cells have remained viable after 48 h of treatment.

However, it is important to evaluate whether these peptide-based vesicles can be able to successfully release the molecules keeping the function of the cargo intact after entry into the cell.

For this, we explored the ability of the reported dipeptide-based vesicles in carrying biologically important molecules like cyclic adenosine monophosphate (cAMP) which has an important role in intracellular signal transduction.[27]

However, cAMP by itself is unable to enter into the cells to induce morphological changes in astrocytes upon internalization.[28] C_6 glioma cultures were treated with peptide **1** vesicles loaded with cAMP (final conc. 0.25 mM) for 24 h and the morphologies of these cells were visualized by immunofluorescence staining with Glial fibrillary acidic protein (GFAP) antibody. Unlike untreated controls as well as cells treated with only cAMP (1 mM), vesicles loaded with cAMP initiated differentiation of cells within 24 h, with the formation of process bearing cells (Fig. 10 A, B and C).

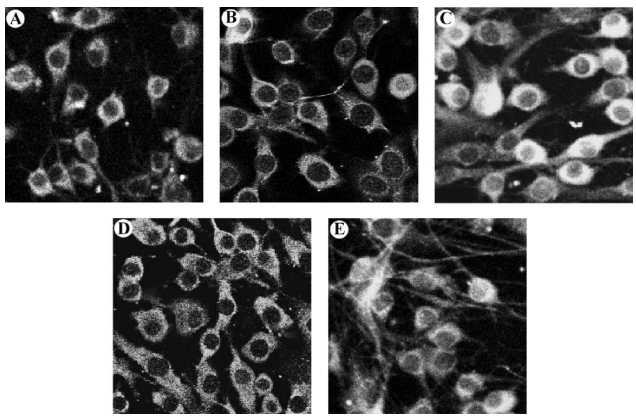

Fig. 10 Effect of vesicles loaded with cyclic AMP on the morphology of C_6 glioma cells. Cells were cultured in DMEM containing 10% serum on coverslips until 50% confluency. Cells were then grown in DMEM containing 0.2% serum for 24 h followed by treatment with the same medium containing vesicles loaded with cAMP for an additional 24 h (C). In (B) the C_6 glioma cells were instead incubated with media containing 1 mM cAMP for 24 h and served as control. Additionally, instead of treatment with vesicles, cells were cultured for a further period of 24 h in DMEM containing 0.2% serum or DMEM containing 10% serum or DMEM containing 0.2% serum and 1 mM dibutyryl cAMP in (A), (D) and (E), respectively. Following treatment, the C_6 glioma cells were immunofluorescent stained for GFAP and examined by confocal microscopy. Experimental details are described in Materials and methods. Photomicrographs show representative fields from three independent experiments. Magnification, ×400.

The morphology of these cells is very similar to cells treated with 1 mM of the cAMP analog, dibutyryl cyclic adenosine monophosphate (dbcAMP) (Fig. 10E). The above mentioned experiment has unequivocally established that these peptide-based vesicles play a critical role in carrying biologically important cargo like cAMP, which otherwise cannot enter into a cell by itself. Moreover, it ensures the release of molecules upon entry, so that they can exert their biological functions.

Conclusions

Water-soluble synthetic amphiphilic dipeptides containing glutamic acid as the C-terminal residue form multivesicular structures upon self-association in aqueous solution. These vesicular structures exhibit pH stability over a pH range of 2 to 12. However, calcium ions trigger the rupturing of these vesicles. Interestingly, these peptide-based vesicles not only can encapsulate an anticancer drug and a fluorescent dye, but also can release them in the

presence of calcium ions. A MTT cell survival assay confirmed that these peptide vesicles are almost nontoxic in nature. Moreover, these nontoxic multivesicular structures have the potential to carry biologically important molecules like cyclic AMP within the cells keeping their biological function intact. Although the structural basis of the formation of these multivesicular assemblies remains to be explored, such functional peptide vesicles may be used as biocompatible delivery vehicles for carrying drugs and other bioactive molecules at the target site and hold future promise to offer potential applications as new functional biomaterials.

Experimental and general method

All amino acids used in the preparation of peptides, coupling reagents dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenztriazole (HOBt) and others that were used in this study, were purchased from Sigma Aldrich.

The reported peptides were synthesized by conventional solution phase methods by using a racemization-free fragment condensation strategy. The Boc group was used for N-terminal protection and the C-terminus was protected with a methyl ester. Deprotection of the ester group was performed using the saponification method, and removal of the Boc group was done with trifluoroacetic acid (TFA). Couplings were mediated by dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (DCC/HOBt). Final compounds were fully characterized by NMR and IR spectroscopy, and mass spectrometry.

NMR studies were carried out on a Brüker DPX 300 MHz spectrometer at 300 K. Compound concentrations were in the range 1–10 mM in $CDCl_3$, $(CD_3)_2SO$ and D_2O.

Mass spectra were recorded on a HEWLETT PACKARD Series 1100MSD and Micromass Qtof Micro YA263 mass spectrometer by positive mode electrospray ionization.

Specific rotations of these compounds reported were measured in Perkin-Elmer instruments, model 341LC Polarimeter.

The DLS study was done in a Nano ZS MALVERN Instrument UK using a solution of 0.06 mmol of each peptide in 1 mL water solution.

Each of the peptide solutions having a concentration of 0.02 mmol mL^{-1} was prepared and it was then aged for 6 h. A drop of this solution was placed on a carbon-coated copper grid (300 mesh) and evaporated. It was dried under vacuum for 10 h. With these grids, TEM studies were carried out using a JEOL JEM 2010 electron microscope. For SEM experiments, a solution (0.06 mmol mL^{-1}) of the reported peptide was taken on glass cover slips and evaporated to dryness for 24 h. A gold coating was applied on the top of the sample to make it conductive for analysis. Then, it was studied on a JEOL JSM 6007 F instrument at 3.0 kV voltage and 20000× magnification

The peptide solution (0.06 mmol of each peptide in 500 μL water) was dried by slow evaporation on a microscopic cover glass and then AFM study was performed using an AUTOPROBE CP BASE UNIT, diCP-II instrument, Model no. AP-0100 instrument.

The confocal fluorescence microscopic study was carried out using a LEICA DM 1000 fluorescence microscope at 100× magnification.

Vescicles of individual peptides were prepared by incubating 0.06 mmol of each of these peptides (**1**, **2** or **3**) in 1 mL water for two days at 37 °C. Eosin B (EB) was then loaded into the vesicles by incubating the solution of vesicles with 0.03 mmol EB in 1 mL water for another two days at 37 °C. Any free EB in the solution was removed by dialysing for 2 h at room temperature in phosphate buffered saline (PBS) using three changes.

For internalisation studies, C_6 glioma cultures, grown on cover slip upto approximately 50% confluency, were incubated with 10 μL of the EB-loaded vesicles for 8 h in a Forma-CO_2 Incubator (5% CO_2/95% air) at 37 °C. Coverslips were washed with PBS buffer twice to remove any free EB-loaded vesicles and the cells were examined under confocal fluorescence microscope at 100× magnification.

For studying the time kinetics the entry of the EB-loaded vesicle inside C6 glioma cells, cells cultured in 24 well plates were treated with 400 μL DMEM (Dulbeco's Modified Eagle's Medium) + sera and 10 μL peptide **1** vesicles for different time intervals. Treatment was terminated by washing the cells with ice cold 50 mM Tris-HCl, pH 7.2. The cells were scraped in 1 mL of the same buffer and cell suspensions were sonicated thrice for 20 s each. Samples were centrifuged at 20 000 rpm for 20 min. The supernatant contained the cytosolic fraction and the pellet contained the membrane fraction. The membrane fraction was dissolved in 0.1 N NaOH. The fluorescence intensity of both fractions was measured in a Perkin Elmer fluorometer at an excitation of 520 nm and emission of 570 nm.

Cyclic adenosine monophosphate (cAMP) was loaded in peptide **1** vesicles using similar concentrations and procedure as those used for loading the dye eosin B (EB). C_6 glioma cells were cultured on coverslips with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) until approximately 50% confluency. The medium was replaced with low serum (0.2%) containing DMEM for a further period of 24 h and then incubated with 1 mM cAMP or 1 mM dibutyryl cAMP or vesicles loaded with cAMP for 24 h. Cells were then fixed in ice cold methanol for 10 min, washed three times with phosphate buffered saline (PBS) and incubated with mouse monoclonal anti GFAP (Sigma, USA) (1 : 10 dilution) followed by FITC conjugated goat anti-mouse IgG (1 : 10 dilution) (Sigma, USA). All incubations were carried out for 30 min at 37 °C. These coverslips were mounted onto glass slides in buffered glycerol (pH 7.8) and examined under confocal microscope.

Cells were grown on 24 well plates, and 1 μL of EB-loaded vesicle were added after 2 days of culture for 8 h as described earlier. After treatment, the medium was removed. 400 μL of fresh medium was added to each well followed by 40 mL of MTT (5 mg mL^{-1}). After 4 h, the MTT solution was carefully removed, the purple crystals were solubilized in 1.4 mL of DMSO, and absorbance was measured at a test wavelength of 550 nm with a reference wavelength of 620 nm. The absorbances obtained from cells with vesicles were expressed as percentages of the absorbances obtained from normal cells.

Acknowledgements

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