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ENCAPSULATION OF ANTENNAPEDIA (PENETRATIN) PEPTIDE IN A POLYMERIC PLATFORM FOR EFFECTIVE TREATMENT OF INTRACELLULAR BACTERIA

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Antimicrobial peptides (AMP) and cell-penetrating peptides (CPP) are two classes of peptides that share some structural and physicochemical similarities. Antennapedia or penetratin (ANT) is one of the most known CPPs, that was proven to have antimicrobial activity against certain strains of planktonic bacteria. ANT can enter the cells but has no activity against intracellular bacteria. This is attributable to the inability of the peptide to reach bacteria reside within cellular components as well as low delivery efficiency, due to loss of activity by proteolysis and poor specificity. The aim of this work is to develop a formulation that can effectively reach and attack intracellular bacteria. To achieve this goal, ANT was encapsulated in PLGA platform as nanoparticles with the size range of 500-1000 nm, which allows for selective uptake by macrophages where bacteria mostly reside. ANT was loaded with high loading efficiency (12.7%) inspite of high water solubility. ANT-nanoparticles (ANT-NP) had no cytotoxicity on J774a.1 macrophages and were readily taken up by macrophages as confirmed by fluorescence microscopy. Antibacterial activity of ANT-NP remains to be tested against different intracellular bacteria.

INTRODUCTION

Antimicrobial peptides (AMPs) refer to cationic peptides that consist of 12 to 50 amino acids, of which up to 50% are hydrophobic ones. This amphipathic secondary structure allows them to partition into the lipid bilayer of bacterial membrane[1].

CPPs are either polycationic which contain plentiful of positively charged amino acids such as (arginine, lysine) or amphipathic containing an interchanging sequence of polar, charged amino acids and non-polar, hydrophobic amino acids[2].

Cell-penetrating peptides (CPPs) have been used to deliver various molecular components (nanoparticles, small chemical molecules or DNA fragments) within the cells[3]. CPPs carry their "cargo" either through covalent or non-covalent interactions[4&5].

CPPs are used to deliver chemotherapeutic drugs[6] or antibiotics to help their entry to cells and access intracellular targets[7]. Their application is however hindered by several problems, such as high toxicity, proteolytic instability and poor specificity[8].

Some of the CPPs already had some antibacterial activity[9]. The antimicrobial activities of CPPs have been extensively investigated due to their potential clinical uses[10&11]. Some CPPs and AMPs have the same characteristics, including basic amino acids composition and cationic charge[12&13]. For example, Antennapedia peptide, also called

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penetratin (Fig. 1), is a 16 amino acid peptide corresponding to the region within the Drosophila Antennapedia DNA binding domain responsible for cell entry[8]. Penetratin can translocate through the plasma membrane to the cytosol of living cells. Its molecular weight is 2246.8 Da and it has an amino acid sequence of RQIKIWFQNRRMKWKK (Arg - Gln - Ile - Lys - Ile - Trp - Phe - Gln - Asn - Arg - Arg - Met - Lys - Trp - Lys - Lys – OH)[14]. It is cationic with a net charge of +7, and 37% of the amino acids are hydrophobic[9]. Antennapedia peptide is considered a CCP, although the mechanism of its cellular entry remains controversial[15].

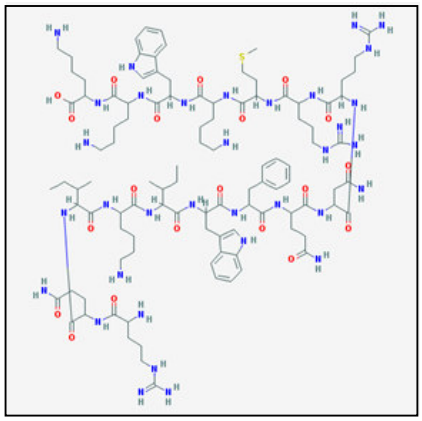

Fig. 1: Structure of Penetratin peptide[16].

Penetratin has been reported to enter cells either via the receptor- and metabolic energy-independent pathway or endocytic pathway, even when conjugated to large hydrophilic molecules[5]. Penetratin was tested against certain isolates of methicillin-susceptible and *methicillin-resistant S. pseudintermedius* (MRSP) isolated from infected dogs and it was found effective with a minimum inhibitory concentration MIC_{50} of 8 mM and it showed no cytotoxic effect to mammalian cells at concentrations up to 256 mM[9].

Although penetratin is effective against planktonic bacteria, it is ineffective against intracellular ones despite its ability to enter cells. This may be attributed to either intracellular degradation or inability to reach bacteria hiding in the cells[4&9]. We hypothesize that encapsulation of penetratin in PLGA nanoparticles will be beneficial to its entry and reaching bacteria residence inside cells without degradation. The nanoparticles are made in 500-1000 nm to help its uptake by macrophages where bacteria mostly reside so that it serves as an effective treatment of intracellular bacteria[17].

MATERIALS AND METHODS

Materials

Antennapedia (Penetratin) peptide was synthesized by GenScript USA (Piscataway, NJ, USA). Poly (Lactic-co-Glycolic) acid (PLGA) (acid end cap, 100-200 kDa, LA:GA= 85:15) and Rhodamine B-conjugated PLGA (10-30 kDa, LA:GA= 50:50) were purchased from Akina Inc. (West Lafayette, IN, USA).Polyvinyl alcohol (PVA) (6000 Da) was purchased from Polysciences, Inc. (Warrington, PA, USA). Dichloromethane (DCM), Fetal bovine serum (FBS), Dulbecco's Modified Eagle's medium (DMEM), Penicillin and Streptomycin were purchased from Thermofisher (Columbus, OH, USA).(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was purchased from Invitrogen (Eugene, OR, USA). Dimethyl sulfoxide (DMSO), Acetonitrile and Trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Preparation of drug loaded PLGA nanoparticles

ANT-loaded PLGA NPs (150 kD, LA:GA= 85:15) was formulated via the single emulsion solvent evaporation method. Drug was dissolved in 0.2 ml DMSO. Drug/DMSO were then added dropwise to PLGA/DCM mixture consisting of 40 mg PLGA and 2 ml dichloromethane (DCM). The organic polymer phase was then emulsified in 20 ml aqueous 1% PVA containing 150 mM sodium chloride (NaCl) using a Silverson homogenizer (East Longmeadow, MA) at 5000 rpm for 3 minutes. Emulsion was dispersed in deionized water (DI) containing 150 mM NaCl, stirred for further 30 minutes to allow evaporation of DCM, and evaporated by a rotary evaporator for another 30 minutes to ensure DCM evaporation. NPs were collected via centrifugation at 8,891 rcf for 15 minutes and were washed twice using DI water to remove any residual DCM and PVA.

When the double emulsion technique was used, the drug was dissolved in 0.1 ml DI water and then was added dropwise to PLGA/DCM mixture and continue as above.

For preparation of fluorescently labeled NPs, 10% of PLGA was replaced with Rhodamine B-conjugated PLGA.

Ant-NP characterizations

The particle size and zeta potential of NPs were determined using Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). For the size measurement, NPs were suspended in DI water at a concentration of 1 mg/ml. For measuring zeta potential of NPs, NPs were dispersed in phosphate buffer (5 mM, pH 7.4).Particle morphology was imaged by FEI Tecnai T20 transmission electron microscopy. For TEM measurement, NPs dispersed in water at 1 mg/mL were mounted on formvar-coated carbon grid (400 mesh) and directly examined.

Peptide loading capacity

To determine ANT loading, amount of 1 mg of NPs was dissolved in 0.5 mL of DMSO and then 0.5 mL DI water was added to induce polymer precipitation. The suspension was centrifuged at 12,126 rcf for 20 minutes to separate a supernatant, which was analyzed by high pressure liquid chromatography (HPLC).

HPLC analysis of antennapedia

HPLC analysis was performed with an Agilent 1100 HPLC system and a Phenomenex Luna 5 µm equipped with a C18(2) column (100 Å, 250 × 4.6 mm) with a column temperature of 40°C. The mobile phase was a mixture of solvent A (0.05% Trifluoroacetic acid (TFA) in acetonitrile) and solvent B (0.05% TFA in water) and run at a flow rate of 0.7 mL/min. Initially, the column was equilibrated with a mobile phase consisting of 10% solvent A and 90% solvent B for 3 minutes. Solvent A was increased in a linear gradient manner from 10% to 100% over 8 minutes. Subsequently, the column was eluted with 100% solvent A for 5 minutes, and the mobile phase was returned to the initial condition and re-equilibrated, ANT was detected at 10.45 minutes out of total 22 min elution time. ANT was detected at 280 nm in the UV detector.

***In-vitro* cytotoxicity study**

Cytotoxicity of ANT-NP was tested with J774a.1 macrophages. Macrophages were seeded in 96-well plate at a density of 20000 cells/well in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37°C in a 5% carbon dioxide (CO_2) atmosphere until 70% confluence. The cells were treated with Blank NP (no drug) and ANT-NP at different concentrations for 24 h. The cells were washed and then the MTT reagent and stop/solubilization solution were sequentially added with a 4 h interval. The absorbance was measured at 562 nm with a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

All statistical analysis was performed with GraphPad Prism 7 (La Jolla, CA). All data were analyzed with two-way ANOVA test to determine the difference of means among groups, followed by the recommended multiple comparisons test such as Sidak's, multiple comparisons tests. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To enhance the stability of the particles at physiological condition, PLGA was used as the main platform to encapsulate penetratin. PLGA is one of the most widely used synthetic approved FDA polymers. PLGA is biocompatible and biodegradable, exhibits a wide range of erosion times and has tunable mechanical properties[18].

The size of ANT-NP was 713.7±62.8 nm, and zeta potential was -1.19±0.593 mV, which are suitable for macrophage uptake and systemic administration[17&19]. TEM images of particles are shown in figure 2.

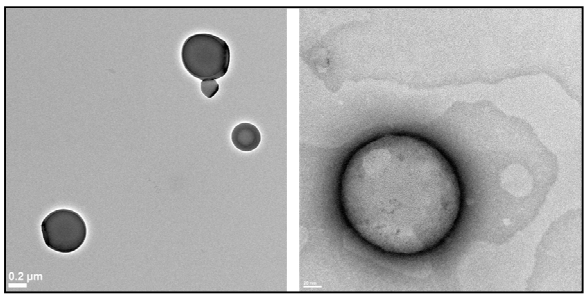

Fig. 2: TEM image of PLGA NP.

The formulation and drug loading of different ANT-NPs are summarized in table 1. The drug loading (%) was defined as the weight of drug in the NPs divided by the weight of NPs. Drug loading was estimated with HPLC at 280 nm in the UV detector (retention time 10.45 min). Standard calibration curve of ANT in the mobile phase is shown in figure 3. A straight line was obtained with a correlation coefficient of 0.9998 (r^2= 0.9998, indicating good linearity over the investigated concentration range (0.0125-0.4 mg/ml).

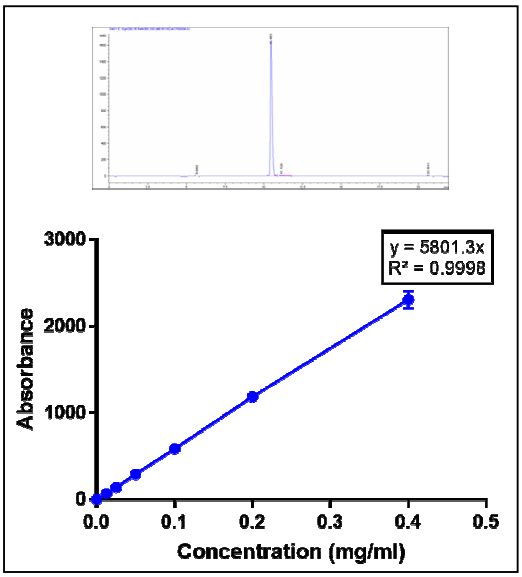

Fig. 3: Standard calibration curve of ANT in the used mobile phase. Figure shows HPLC chromatogram of 0.1 mg/ml ANT at 280 nm.

Loading efficiency depends mainly on the drug solubility in aqueous/ organic phase. Encapsulation of water-soluble drugs in PLGA is challenging and mostly very low due to escaping of drug from the inner aqueous phase to the outer aqueous phase[20].

PLGA degrades when exposed to water and the degree of degradation depends on the LA:GA ratio in the polymer. The rate of degradation increases with increasing the GA ratio[18]. So, considering the grade/type of PLGA is very important. PLGA with LA:GA ratio of 85:15 may result in less degradation and better encapsulation of drug.

First, ANT was encapsulated via double emulsion technique (F1), but loading was 0.42%. This low loading may be due to the high-water solubility of penetratin, where the aqueous continuous phase diffuses into the dispersed phase creating water channels, leading to pore formation within polymer matrix[21].

In F2, polymer amount was increased from 20 to 40 mg. This high polymer concentration is expected to increase the viscosity and delay the diffusion of the drug from the solution. In addition, increasing the polymer amount may prevent drug diffusion across the phase boundary because polymer precipitates faster on the surface of the dispersed phase[21]. Sodium chloride (NaCl) was also added in the external or continuous water phase (W2) to stabilize the encapsulation process. The addition of salts is expected to generate osmotic pressure gradients between the two aqueous phases which will confer stability to the encapsulated drug[22]. These modifications increase the drug loading up to 3.15% in F2.

When ANT was encapsulated in PLGA via the O/W single emulsion method, the drug loading increased to 4.2% (F3). ANT was further increased to reach maximum amount of drug loading (12.7%) (F5). However, further increase of ANT amount rather interfered with NP production.

Table 1: Formulation and peptide loading of different ANP-NPs.

Formula No.	Single or double emulsion	PLGA (mg/2 ml DCM)	ANT feed (mg)	NaCl (150 mM)	Peptide loading (%)	TLE (%)
F1	double	20	2	x	0.42	4.76
F2	double	40	2	√	3.15	4.76
F3	Single	40	2	√	4.19	4.76
F4	Single	40	4	√	7.27	9.09
F5	Single	40	6	√	12.7	13.04
F6	Single	40	8	√	--	16.6
F7	single	40	10	√	--	20

TLE: Theoretical loading efficiency.
(√): NaCl is added, (x): NaCl is not added.

Although penetratin is active against planktonic bacteria and can enter cells[9,12&23]. It is not active against intracellular bacteria,

likely due to proteolytic instability and inability to access the bacteria[8]. Encapsulation of ANT within PLGA-NP and optimization of particles in the size range of 500-1000 nm provide an efficient and safe strategy for the delivery of ANT to macrophages. As macrophages are the target cells where bacteria mostly reside, ANT-NPs toxicity was tested against J774a.1 macrophages. ANT-NPs showed insignificant ($p>0.05$) toxicity to the J774a.1 macrophages compared to blank PLGA NPs as shown in figure 4.

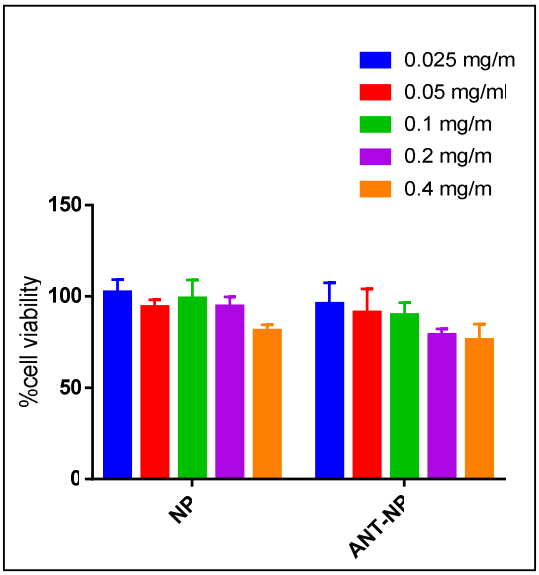

Fig. 4: Cytotoxicity of NPs, ANT-NPs (selected formulation F4) on J774A.1 macrophages. No statistical difference from the control groups (blank PLGA NP) by Sidak's multiple comparisons test.

Cellular uptake of rhodamine-labeled nanoparticles (designated as *NP) by J774a.1 macrophages was observed by fluorescence microscopy (Fig. 5). After 3 h incubation, *NPs appear around the nucleus of J774a.1 macrophage. This indicates that macrophages readily take up *NPs, likely due to the size (713.7±62.8 nm) optimal for macrophage uptake.

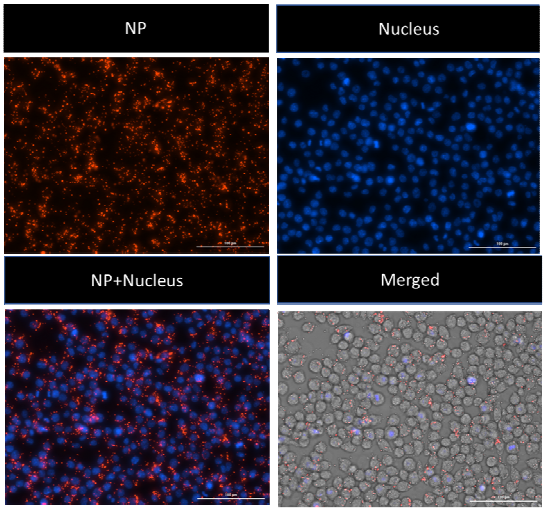

Fig. 5: Cellular uptake of rhodamine labelled nanoparticles *NP (0.025 mg/ml) in *J774a.1 macrophhages using citation 3 flourescence microscope.

Conclusion

Penetratin (ANT) peptide was successfully encapsulated in PLGA nanoparticles with a good loading percentage (12.7%). ANT-NPs had no cytotoxicity on J774a.1 macrophages. On the basis of the size, particles are phagocytosed by macrophages, target cells in the therapy of intracellular infection. This formulated platform may be used for encapsulation of different antibacterial peptides.

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نشرة العلوم الصيدلية
جامعة أسيوط

تغليف الببتيد انتينابيديا (بينتراتين) في قالب بوليمري لعلاج فعال للبكتيريا داخل الخلايا

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الببتيدات المضادة للميكروبات (AMP) والببتيدات المخترقة الخلايا (CPP) هما فئتان من الببتيدات التي تشترك في بعض أوجه التشابه البنيوية والفيزيائية. يعد انتينابيديا (بينتراتين) (ANT) واحدًا من الببتيدات المعروفة ، والذي ثبت أنه له نشاط مضاد للميكروبات ضد سلالات معينة من البكتيريا. يمكن أن يدخل ANT الخلايا ولكن ليس له نشاط ضد البكتيريا داخل الخلايا. هذا يعزى إلى عدم قدرة الببتيد على الوصول إلى البكتيريا الموجودة داخل المكونات الخلوية وكذلك انخفاض كفاءة التوصيل ، بسبب فقدان النشاط عن طريق تحلل البروتين وضعف الخصوصية. الهدف من هذا العمل هو تطوير تركيبة يمكنها الوصول إلى البكتيريا داخل الخلايا ومهاجمتها بشكل فعال. لتحقيق هذا الهدف ، تم تغليف ANT في قالب بوليمري PLGA كجسيمات نانوية في حجم يتراوح بين ٥٠٠–١٠٠٠ نانومتر ، مما يسمح بالدخول الانتقائي بواسطة البلاعم حيث توجد البكتيريا في الغالب. تم تحميل ANT بكفاءة تحميل عالية (١٢,٧٪) على الرغم من درجة الذوبان العالية في الماء. لم يكن للجسيمات النانوية (ANT-NP) أي سمية للخلايا البلعومية J774a.1 وتم تناولها بسهولة عن طريق الخلايا البلعومية كما تأكد بالمجهر الفلوري. ويبقى النشاط المضاد للجراثيم من ANT-NP ليتم اختبارها ضد البكتيريا داخل الخلايا المختلفة.