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ENCAPSULATION OF miRNA IN CHITOSAN NANOPARTICLES AS A CANDIDATE FOR AN ANTI-METASTATIC AGENT IN CANCER THERAPY

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ABSTRACT

MicroRNAs (miRNA) have been utilised as a repressor molecule for metastasis of tumours, as it inhibits fundamental processes related to cellular and physiological pathway of the tumour at the mRNA level. However, therapeutic application of miRNAs is impaired by premature degradation in the extracellular environment by endonucleases. This research describes the optimisation, chemical, and morphological characterisation of nanoparticles for effective encapsulation of miRNA-186 and evaluate its efficiency as anti-metastatic agent in non-small cell lung carcinoma monolayer. Through ionic gelation methods, the miRNA was encapsulated in chitosan nanoparticles (CNPs), a drug carrier with high particle stability, low cellular toxicity, and robust preparation methods. Physiochemical and morphological characterization analysis through light scattering analysis showed miRNA-CNP sizes below 200 nm, with a low polydispersity index and exhibition of irregular spherical shape of the nanoparticles synthesised through FESEM analysis. Additionally, *in vitro* nanoparticle efficacy evaluated through scratch assay suggests a decrease in invasion ability of cancer cells exhibited by miRNA-CNP.

Key words: Chitosan nanoparticles, miRNA-186, anti-metastatic agent, cancer therapy

INTRODUCTION

The global increase in cases and mortality due to cancer has been significantly alarming, with a projected 21.7 million new cancer cases and 13 million cancer-afflicted deaths by 2030 (American Cancer Society, 2015). Locally the age-standardised incidence rates (ASR) for cancer in 2007 was 179.5 per 100 000 populations, and exponentially increased 37.74% to 288.3 in 2012 (Ariffin & Nor Saleha, 2011). Cancer is the uncontrollable growth of abnormal cells and is often deadly in its malignant form from which the tumour spreads from the origin site to other part of body tissues. Termed metastasis, cancer relapse is highly probable even upon successful treatment (National Cancer Institute, 2015). Various treatments are presently available to treat, remove or suppress cancer growth and metastasis, including surgery, chemotherapy, radiation, photodynamic, stem cell transplant, immunotherapy, hormonal therapy, lasers, complimentary and targeted therapies depending on a patients' needs. However, each one of it often comes with multiple side effects and even worse, damaging adjacent healthy tissues.

Appropriately, microRNAs (miRNAs) have been recently established as potential tumour suppressors. This class of nucleic acid encompass a large family of non-coding small RNAs which occur as single-stranded RNAs of approximately 22 nucleotides (nt) in length (range between 19 to 25 nt) (Ruvkun, 2003). MicroRNAs regulate gene expression by the sequence-selective targeting of mRNAs, leading to translational repression or mRNA degradation and so, they have been implicated in the control of many fundamental cellular and physiological processes such as cellular differentiation, proliferation, apoptosis and stem cell maintenance (Rothscchild, 2014). Among others, miRNA-186 is an example of miRNA derived from *Homo sapiens* that has been suggested to serve as a tumour-suppressor in the development and progression of

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non-small cell lung carcinoma (NSCLC). Studies in 2014 found the expression of miRNA-186 in NSCLC cells is abrogated and markedly reduced, resulting in uncontrolled cell cycle progression and consequently increased cell proliferation (Cai *et al*., 2012). Previously, Li *et al* (2013) identified pituitary tumour transforming gene (PTTG1) modulated by miRNA-186 inhibits the invasion and metastasis of NSCLC through several functional assays.

Applications of miRNAs in cancer therapy is an alternative from traditional cancer treatment. However, as a double stranded nucleic acid, miRNA is exposed to nuclease degradation (Alexis *et al.,* 2008), activate innate immune response (Hornung *et al.,* 2005), and may encounter off-target effects since miRNA is also capable of affecting multi-target genes (Conde *et al.,* 2015). On the other hand, encapsulating therapeutic agents such as miRNA in polymer nanomaterials such as chitosan nanoparticles confers two main advantages. The first is miniaturization to sizes below 1000 nm, enabling the nanoparticles to extravagate through the endothelium in inflammatory sites, epithelium, tumours, or microcapillaries (Desai *et al.,* 1997) and the second is its biodegradability properties allows a sustain drug release in days or weeks within the target site (Singh & Lillard, 2009).

Hence, in this study chitosan nanoparticles (CNP) were used to encapsulate the miRNA-186 through ionotropic gelation methods due to the materials' stability, low toxicity, robust preparation methods, and its ability in controlling the release of active agents (Nagpal *et al*., 2010). This study was aimed at optimising the synthesis of chitosan nanoparticles to encapsulate miRNA-186, performing morphological characterization and finally evaluating its efficiency as an anti-metastatic agent in lung cancer tissue.

MATERIALS AND METHODS

Preparation and synthesis of miRNA-186, CNP and miRNA-CNP

Bacterial heat shock treatment was used to transform the precursor miRNA-186 plasmid into DH5á competent cells. On the following day, single colonies were grown in Luria broth supplemented with ampicillin overnight, extracted and analysed for plasmid concentration at 260nm. The precursor plasmid was then stored in 4^{o}C until further use. Chitosan nanoparticles (CNP) were synthesised using sodium tripolyphosphate (TPP) as a cross-linker with chitosan (CS) through ionotropic gelation using modified methods explained previously (Calvo *et al.,* 1997). Sterilised distilled water was used to solubilise TPP (1.0 mg/mL in deionised water) and CS (1.0 mg/mL in acetic acid). Approximately 0.5 mg/mL of CS and 0.7 mg/mL of TPP were prepared and subsequently CNP were prepared at a range of different TPP volumes. For encapsulation of miRNA-186, the precursor plasmid was initially complex with CS, followed by addition of TPP to form miRNA-CNP. The optimised parameters were then selected to undergo further analysis.

Characterisation analysis and cellular treatment

Dynamic light scattering (DLS) was used to determine average size and polydispersity index (pdi) of CNP with all measurements were made in triplicate. Field emission scanning electron microscopy (FESEM) was then used for morphological analysis. Using techniques described by Moutasim *et al* (2011) scratch assay technique which provide straightforward method, have particular suitability to study cell migration by cell-cell interaction *in vitro* and mimics almost similar behaviour on the migration of cells *in vivo* (Liang *et al.,* 2007) was used to access the invasiveness of A549 non-small cell lung carcinoma at different time points by creating a "wound" in monolayer of A549 cells then their ability to migrate and closing the wound was measured and assessed using inverted microscope (Moutasim *et al.,* 2011).

Technically, A549 cell were seeded and achieved 80-90% confluency in 24-well plate prior to treatment. On the treatment day, media was removed, monolayer cell scratched using 200 µl tip and rinsed with 1% of phosphate buffered saline (PBS). miRNA-CNP, CNP and miRNA-186 alone was added to the cell respectively and as control, complete growth media was added in another well. Cells were viewed under microscope at several time points. The distance of the scratch created was measured and three replicate of treatments were made.

Statistics

Each group of data are expressed as mean ± SEM and two-way ANOVA with Bonferroni Test was performed using GraphPad Prism version 5.02 (for Windows, GraphPad Software, San Diego California USA). Statistically significant was considered when $p < 0.05$, $p < 0.01$ or $p < 0.001$.

RESULTS AND DISCUSSION

Morphological characterization of nanoparticles synthesised

CNP and miRNA-CNP samples were successfully synthesised through ionic gelation methods. The average size of samples should be below 1000 nm which indicate nanoparticles size

while low polydispersity index (pdi) representing single size mode and low aggregations. Figure 1A and 1B represents the particle size and pdi of samples following synthesis. The precursor miRNA-186 used prior encapsulation conferred high pdi values, with sizes less than 12 nm and show various irregular shapes of the plasmid through morphological analysis (Figure 2B). A range of TPP volumes were then used to synthesise CNP, with optimised parameter determined at 200 ul TPP as presented in Figure 1A; with a particle size around 100 nm. This parameter also exhibited the lowest pdi value, indicating a low aggregation of synthesised CNP. This parameter was further used to optimise encapsulation of miRNA-186 in CNP.

Intuitively, different volumes of miRNA-186 were used for encapsulation and results were optimised using 100 ml. Trends showed an increase in particle size (Figure 1A) thus suggesting the formation of nanoparticles with the precursor plasmid within the CNP core. Particle size for miRNA-186 encapsulated-CNP correlated with pdi values below 0.5 in Figure 1B and increased particle morphology (Figure 2D), compared to CNP samples (Figure 2C). The spherical shape of both synthesised nanoparticles possess a higher surface area as compared to other possible shapes (Ismail *et al.,* 2015). Explicitly, particle size of optimized CNP was 111.80 nm and increased 97.25% to 220.53 nm upon addition of 100ul miRNA-186; this parameter further utilised in cellular treatments of A549 lung cancer cells. Therefore, it was assumed that miRNA-186 was successfully encapsulated in CNP at 90ng/ml. Theoretically, the formation of both CNP and miRNA-186 encapsulated-CNP occurred through an electrostatic interaction of oppositely charged polymers (Kauper & Forrest, 2012). Protonated amine groups of CS electrostatically bind to anionic phosphate group of TPP and/or miRNA which finally produced smaller and fine nanoparticles (Katas & Alpar, 2006).

Nanoparticle efficacy through cellular treatment

Scratch assay was performed by making scratch wounds on a cell monolayer grown on 24-well plates. The ability of cancer cells to close the wound gap was observed at several time points to stimulate metastasis processes *in vitro*. In Figure 3, A549 cancer cells without any treatments (control) was shown to grow and close the scratched gap rapidly within a 48 hour timeframe with 60% decrease in length. Interestingly, both miRNA-CNP and CNP alone significantly hindered the migration rate of A549 cells when compared with control group through 48 hours observation. Specifically, A549 cells treated with miRNA-186 encapsulated-CNP shown an increase in gap distance at 19% with clusters of cells floating and clumping in media. Meanwhile, treatment using CNP caused a slightly increase of the gap at only 1.1% within 48 hours with CNP exhibits potential inhibition to invasion properties of the cancer cells. Loch-Neckel *et al* (2015) observed a similar effect of chitosan-coated nanoparticles containing curcumin (Cur-CCNP) on melanoma cells assessed using the scratch assay. Treatment of Cur-CCNP had prevented cellular growth in the scratched area while free curcumin hampered cell migration and also induced cellular death (Loch-Neckel *et al.,* 2015). In contrast, directly treated miRNA-186 caused floating and clumping of cells without visible scratched gap since the first hour. Similarly, transducing naked miRNA-186 has been reported to slow down A549 cells as analysed using MTT assay (Cai *et al.,* 2012). Due to this observation, it is worth noting that the mechanisms and elucidation of intake, release and action of this system would govern more insight in future studies.

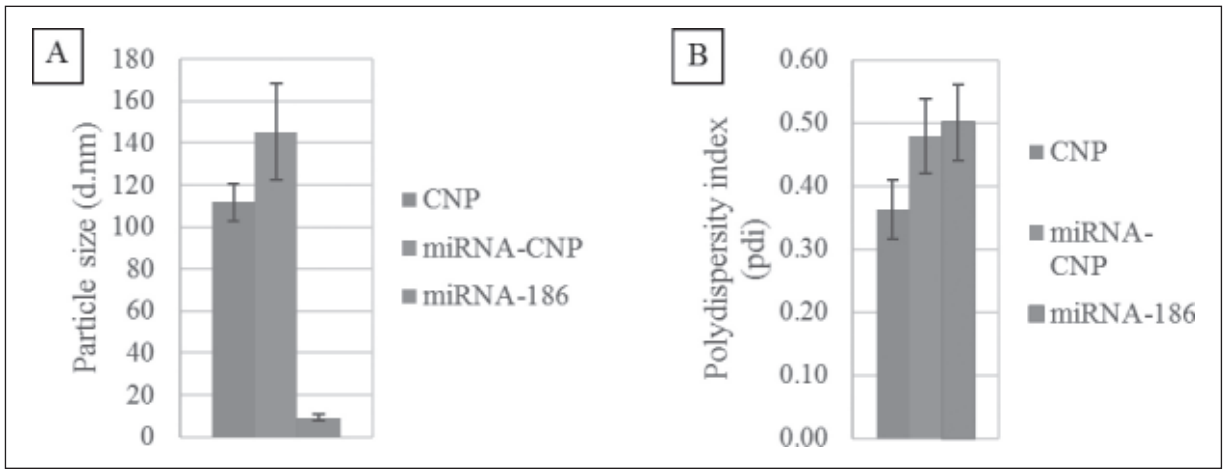

Fig. 1. Light scattering data of (A) particle size & (B) polydispersity index of optimized CNP, miRNA-CNP and miRNA-186. Data were obtained in triplicate and presented as mean ± SEM.

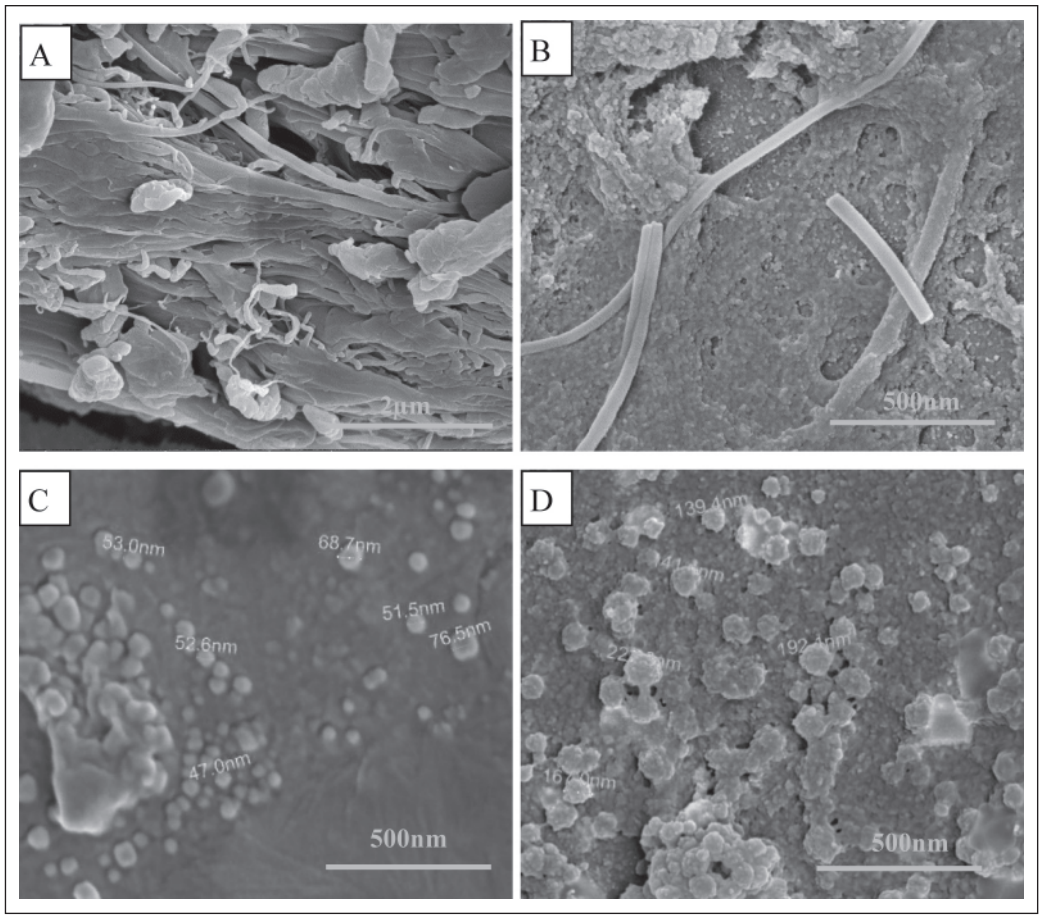

Fig. 2. FESEM analysis on morphology of (A) chitosan, (B) miRNA-186, (C) CNP and (D) miRNA-186 encapsulated-CNP at 100,000x magnification.

CONCLUSION

Through this study, miRNA-186 was successfully encapsulated in CNP based on results obtained through DLS analysis and FESEM images. When introduced to A549 lung cancer cells, miRNA-186 encapsulated in CNP showed an ability to reduce cell invasion, possibly caused by inhibition of cell proliferation. Therefore, miRNA-186 encapsulated-CNP is suggested to possess an anti-metastatic in A549 lung cancer cells, and governs further exploration of the miRNA-186 delivery system into cancer cells.

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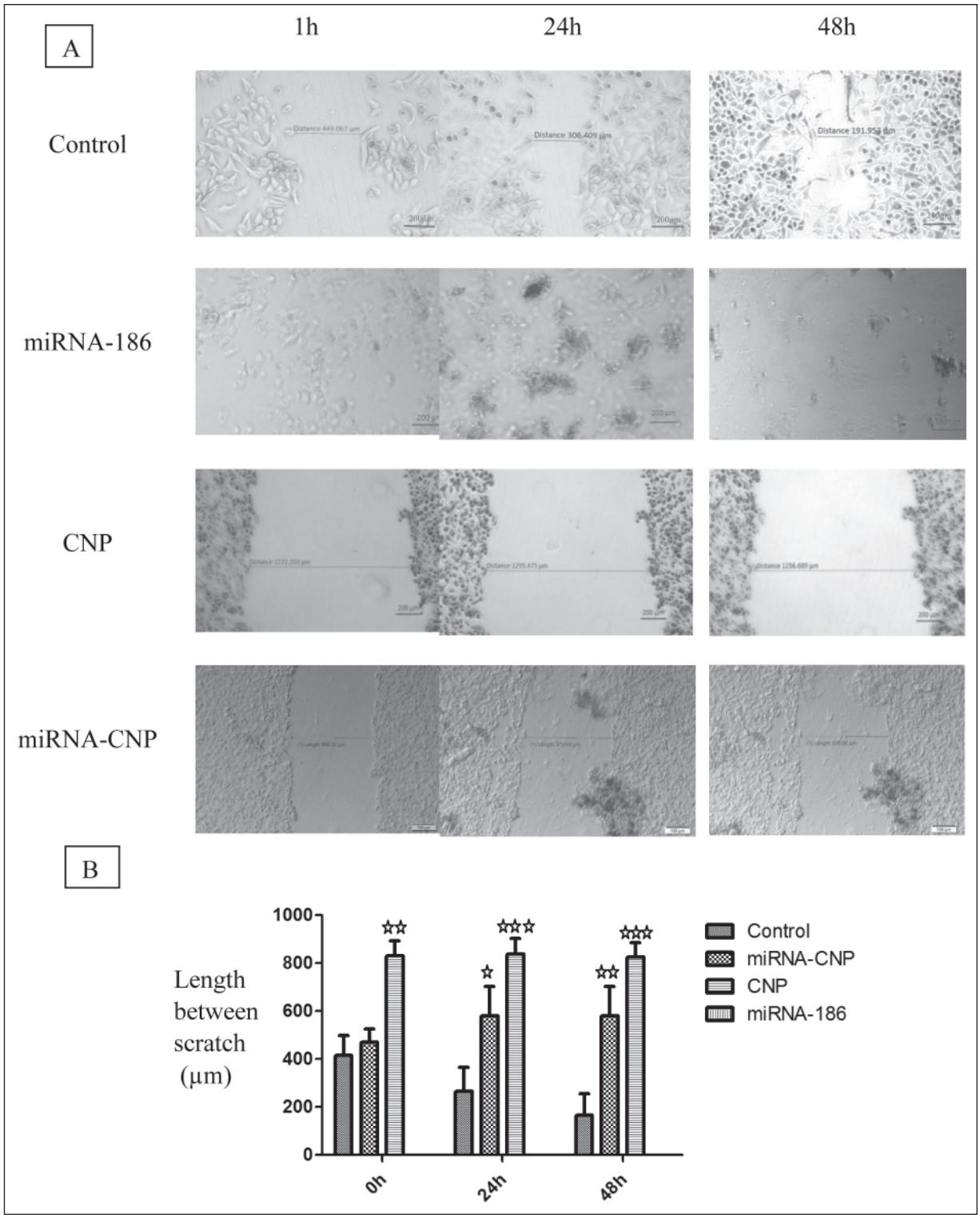

Fig. 3. Effect of miRNA-CNP, CNP and miRNA-186 in the migration of A549 cells using the *in vitro* scratch assay and viewed under 10x magnification of inverted microscope with untreated cells used as control at 0, 24 and 48 hours. (A) Length between scratch represent the migration of the cells by quantifying the total distance between two edges of the scratch. (B) Graph data are presented as mean ± SEM from triplicate experiments (☆☆☆$p < 0.001$) when compared with control group. Two-way ANOVA was performed followed by Bonferroni test.

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