FABRICATION OF p53 PLASMID LOADED POLY LACTIC-CO-GLYCOLIC ACID (PLGA) NANOPARTICLES AS A POTENTIAL TREATMENT FOR MALIGNANT MELANOMA

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Abstract

Malignant melanoma has been listed as one of the poor prognosis diseases in the world due to the ineffective treatments. One of the risk factors for melanoma is the mutated p53 gene which eventually causes cancer formation. Restoring the normal function of p53 has become one of the potential approaches in treating melanoma. In this study, the extracted p53 plasmid was evaluated in terms of pDNA integrity by using agarose gel electrophoresis and encapsulated in double emulsion water in oil in water (W/O/W) of PLGA nanoparticles. The nanoparticles were characterised in terms of size, charge and morphological surface by using Zeta analysis and scanning electron microscope (SEM). p53 loaded PLGA nanoparticles were exposed to A375 Human Malignant Melanoma cell lines at different concentration i.e. 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml for 24 and 48 hours. Cell viability assay (MTT assay) was performed to evaluate the effectiveness of the cells' treatment in terms of nanoparticles concentration and duration of nanoparticles exposure. The evaluation of cell viability assay indicated that p53 loaded PLGA nanoparticles significantly reduced the cell viability of A375 as the time of exposure increased. These findings indicated that p53 plasmid loaded PLGA nanoparticles have the potential to become one of the alternative treatments for malignant melanoma.

Keywords: p53, PLGA, nanoparticles, double emulsion, malignant melanoma, A375

1. Introduction

Malignant melanoma is one of the skin cancers which developed from the melanocytes. It is considered as a rare disease which contributes about 1% of all of the skin cancer cases (American Cancer Society, 2019). However, the incidence is recently on the rise with 5- years survival rate found to be less than 5% (Kalal, Upadhya, & Pai, 2017); and a remarkably high percentage of melanoma cases are found in white and fair skin population (Geggel, 2017). This disease is also characterised by a poor prognosis as it has a high proliferation and metastasise rate, which contributes to 80% death among the skin cancer patients (Kalal et al., 2017).

p53 mutation has become one of the potential factors that lead to skin cancer as its mutation has been recorded approximately in 50% of human skin cancer types (Zhang, Zeng, & Lu, 2014). p53 is a tumour suppressor gene which is responsible for controlling the cell cycle process. The activated p53 will arrest cells which possess DNA damage, activate the DNA repair proteins and induce apoptotic activity if the cells are severely impaired and cannot be repaired (Ozaki & Nakagawara, 2011). On a contrary, the mutated p53 can pose the oncogenic potential by inhibiting the production of wildtype p53 which eventually leads to cancer formation (Ozaki & Nakagawara, 2011). Therefore, gene therapy has been proposed as one of the alternative treatments in combating malignant melanoma by administering the wildtype p53 proteins which aim to restore the normal function of p53.

Despite its powerful, promising feature, there are some drawbacks of gene therapy. For instance, the gene or therapeutic plasmid that is intended to be inserted into the human body is not stable and can be easily degraded by the armies of the immune system which are sensitive to foreign materials (Hobernik & Bros, 2018). These conditions create the need for targeted therapy through nanoparticle-mediated gene therapy.

Nanoparticle is a very tiny particle made up from organic or inorganic compound which has a size range from 1 – 1000 nm (Salatin & Yari Khosroushahi, 2017). It has the potential to be used either as a diagnostic agent to assist in detecting disease as well as a therapeutic agent which carries drugs to the target site. As claimed by Chen et al. (2013), concerning the small size, nanoparticles have the advantage to concentrate the drugs at the tumour sites and they possess a longer biological lifespan as the immune system does not quickly degrade them. It also supported by Bombelli et al. (2014) who concluded that nanoparticles help in enhancing the pharmacokinetics of drugs. The previous study by Chen et al. (2013) has recorded the usage of nanoparticles in combating malignant melanoma diseases such as liposome, dendrimers, carbon-based nanoparticles and human albumin. All of them have a positive result in treating diseases. However, some modifications should be taken to overcome the unwanted side effects.

In this study, poly D, L- lactic- co- glycolic acid (PLGA) will be used as the polymer in fabricating nanoparticles which act as a vector agent to deliver the wildtype p53. Sharma, Parmar, Kori, & Sandhir (2016) described, PLGA as a polymer which has a record of safety administration,

approved by the Food and Drug Administration (FDA) and European Medical Agency (EMA). It also has a remarkable a long-standing track of usage in biomedical applications. The main objective of this study is to fabricate p53 loaded PLGA nanoparticles by using a double emulsion water-oil-water (w/o/w) solvent evaporation method. PLGA nanoparticles were intended to be used as a carrier to allow the extracted p53 plasmid to be targeted to the specific area in treating malignant melanoma. Then cell viability was evaluated by conducting (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) assay.

2. Materials and methods

Extraction of p53 plasmid

p53 transformed *Escherichia coli* was cultured on nutrient agar supplemented with *kanamycin* for 24 hours to yield a single colony. Then the bacteria were further cultured in lysogeny broth supplemented with *kanamycin*. After 8 and 12 hours of bacteria preparation, p53 plasmid was extracted by using PureYield™ Plasmid Maxiprep System. The experiment protocol was adapted from Promega Corporation (2009).

Evaluation of pDNA purity and integrity

The extracted p53 plasmid was evaluated in terms of purity and pDNA integrity by using Nanodrop 1000 Spectrophotometer and Agarose Gel Electrophoresis respectively.

Fabrication of PLGA nanoparticles

PLGA nanoparticles were fabricated by using double emulsion water-oil-water (WOW) solvent evaporation method adapted from Ismail et al., (2016). Accurately 0.1 g of PLGA solid crystal was weighed and dissolved in 2 ml of ethyl acetate to form the oil phase. Then, the first aqueous phase containing 200 µl of distilled water and 6 µl of p53 plasmid DNA were added into the oil phase. The mixture was then homogenised for 2 minutes (30 seconds on, 15 seconds off) and sonicated for 30 seconds (5 seconds on, 3 seconds off) to create the first emulsion. Next, primary emulsion was added to the second aqueous phase containing 5.5 ml of 4% polyvinyl alcohol and 16.5 ml of distilled water. The mixture was homogenised, sonicated with the same parameter as before and stirred for 13 hours to produce a stable structure of the nanoparticle.

Next, the nanoparticle solution was centrifuged with a parameter of 13000 rpm at 20°C for 15 minutes. The pellet was washed for two times with distilled water and finally resuspended with 5 ml of distilled water. Water bath sonicator was used to aid the resuspension process. The same procedure was repeated for the nanoparticle formulation without the addition of p53 plasmid in the first aqueous phase.

Characterisation of nanoparticles

The fabricated nanoparticle was characterized in terms of size, zeta potential and morphological surface by using Zeta Sizer, Zeta Potential and Scanning Electron Microscope (SEM) respectively. Before SEM observation and examination, the sample was freeze-dried and proceed with sputter coating (gold).

Culture and maintenance of A375 cell lines

A375 Human Malignant Melanoma Cell lines were cultured and maintained in 10% Complete Growth Medium. The cells were seeded into 96 well-plate after the cells reached 80% confluency.

Cells treatment

After 24-hours of incubation, the seeding cells were divided into four group which were (i) treated with p53 loaded PLGA nanoparticles, (ii) treated with p53 plasmid extracts (positive control), (iii) treated with blank nanoparticles (negative control), and (iv) untreated cells. For the treatment group, five concentrations were set up consisted of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The treatment was added according to the designed concentrations. The plates were then incubated for 24 and 48 hours at 37 °C supplemented with 5% CO_2.

Cell viability assay

After 24 and 48 hours of treatment, MTT assay was performed by incubating the treated cells with 0.5 mg/ml of MTT reagent for 4 hours at 37 °C incubator supplemented with 5% CO_2. Formazan crystal in purple colour was produced by the viable cells once the MTT reagent absorbed into the mitochondria of the cells. After the incubation period, 100µL of DMSO was added into each well and incubated for 1 hour to dissolve the crystal. Then, the absorbance was measured by using a microplate reader with 570 nm of optical density.

Data analysis

All of the data collected from the experiment were conducted in triplicate. The mean, as well as the standard deviation were measured. The collected data were analysed by using IBM SPSS Statistics 25 with One Way ANOVA test. A p-value of <0.05 was considered as statistically significant.

3. Results

pDNA purity and integrity

The purity of the plasmid has been confirmed through two measurements of 260/280 and 260/230 ratio which are 1.90 and 2.12 respectively. While the stock concentration of extracted p53 plasmid recorded from the Nanodrop spectrophotometer reading was 178.6 ng/µl.

The integrity of the pDNA was analysed by using agarose gel electrophoresis. Four bands have been formed and analysed by using ImageJ software analysis which the intensity of the four bands have been measured and compared. A set of range has been set up from the darkest black colour with the intensity of 0.71 to the whitest white colour with the intensity of 255.00 as a standard comparison. Figure 1 shows the band formation of p53 plasmid viewed under ultraviolet rays. B3 has the highest intensity among the four bands with the value of 234.60 indicating the accumulation of plasmid at that area which denotes the confirmation structure of pDNA. The confirmation structures of pDNA consist of supercoiled, open circular or linear (Li, Bo, Wang, Shao, & Huang, 2011). Supercoiled DNA has the least resistance towards the gel; hence it is the fastest moving DNA, followed by open circular and linear. Based on Figure 1, the extracted DNA has the most open circular DNA structure (B3).

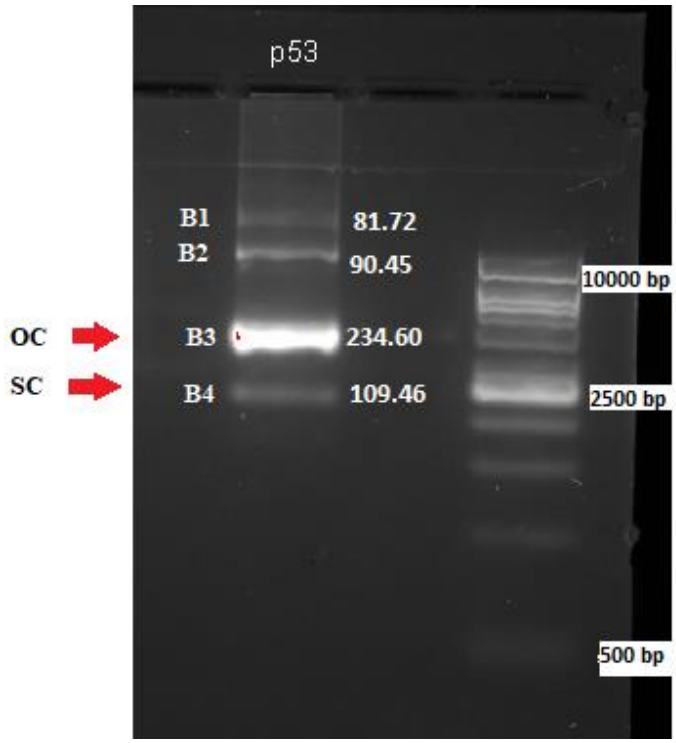

Figure 1 p53 bands formation under Agarose Gel Electrophoresis assay which further analysed by using ImageJ-software to measure and compare the intensity of the four bands (B1-B4). The right-side values (besides the band) are referring to the intensity value measured by ImageJ software. While the red arrows were pointing out the pDNA isoforms which are OC = open circular and SC= super-coiled.

Nanoparticles characterisation

Size and zeta potential

The fabricated blank nanoparticles and p53 loaded nanoparticles have been characterised three times per sample by using Zeta Sizer and Zeta potential machine. From the reading, it was recorded that the size of the nanoparticles increased after the encapsulation process, which reaches up to 266.67 ±

46.97 nm. While the zeta potential has shown slight reduction -29.53 ± 3.07 mV for p53 loaded nanoparticles. The size and zeta potential of the nanoparticles were summarised as in Table 1.

Table 1 Characterization of blank and p53 loaded nanoparticles in terms of size and zeta potential.

	Unloaded/Blank Nanoparticles	**p53 loaded Nanoparticles**
Particle Size (nm)	153.33 ± 63.13	266.67 ± 46.97
Zeta Potential (mV)	-30.1 ± 3.40	-29.53 ± 3.07

Morphological surface

Through the morphology examination in Figure 2, it was found that the nanoparticles have a spherical shape with a smooth external surface. No pores and perforated particle walls were observed. Although the size distribution of the nanoparticles was not equally even, this does not reflect the average size of the nanoparticles fabricated since the image does not represent the overall population of the nanoparticles.

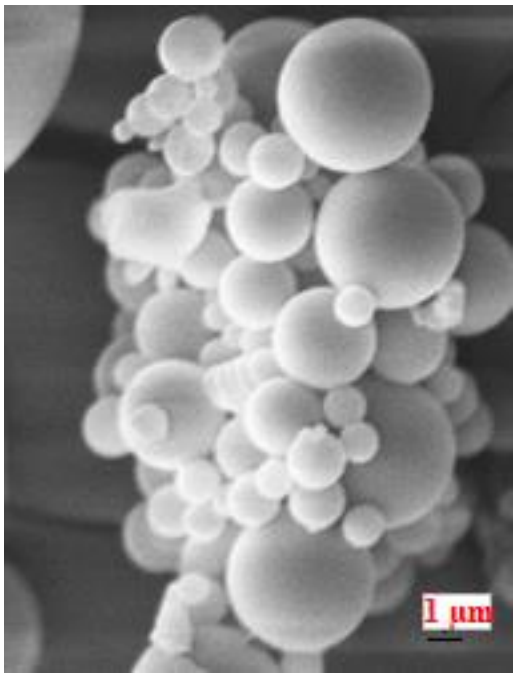

Figure 2 The morphology of p53 loaded nanoparticles under magnification of 2.00Kx of Scanning Electron Microscope.

Cell viability assay

Two sets of data have been set up (i) to compare the mean between the concentration of p53 loaded nanoparticles and cell viability of A375 cell lines and (ii) to compare the mean between duration of nanoparticles exposure to the cells and cell viability.

Concentration of nanoparticles vs cell viability

The cell viability was expected to reduce as the concentration of p53 loaded nanoparticles increased. Although the data was found to be not statistically significant with a p-value of 0.097, however through Figure 3.0, the trend of the graph indicates that as the concentration of p53 loaded nanoparticles increases, the cell viability would decrease.

Duration of nanoparticles exposure vs cell viability

Through One Way ANOVA analysis, it was found that there is a statistically significant difference between the duration of exposure and cell viability with the p-value of 0.035. Figure 3 indicates the longer the duration of exposure the lower the cell viability.

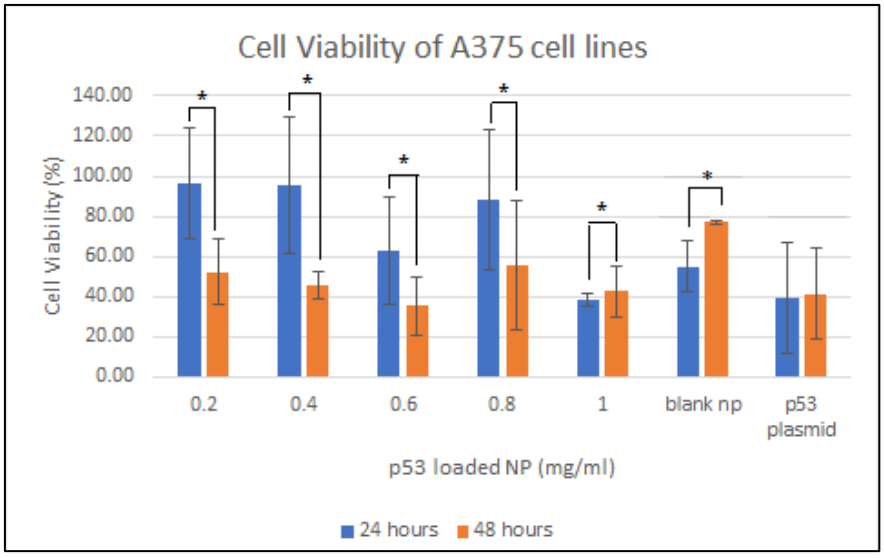

Figure 3 The range of concentration of p53 loaded nanoparticles and their effects on cell viability of A375 cell lines in 24 and 48 hours. Blank nanoparticles and p53 plasmid were used as a control. Statistical test of concentration effect on cell viability was not significant (p value = 0.097). However, the effect of different duration of exposure on cell viability was found to be significant (p value = 0.035). *p<0.05, determined as described in materials and method. The line with * sign indicates that there is a significant different between group of 24 and 48 hours.

4. Discussions

pDNA integrity

In recent years, a study on pDNA stability has been conducted by Doolaanea, Ismail, Mansor, Mohd Nor, & Mohamed, (2015). The study emphasised that supercoiled (SC) isoform of pDNA possess the highest biological activity compared to the other two isoforms. However, in the present study, agarose gel electrophoresis analysis has revealed that the most isoform of p53 extracted plasmid is in the open circular (OC) form. These finding, indicating that the biological activity of the extracted p53 plasmid was fewer than initially expected. The conversion of pDNA isoform from SC to OC, considered as degradation which might happen during the plasmid extraction or nanoparticle's fabrication process. Doolaanea et al., (2015) have highlighted the importance and effects of different surfactants on pDNA stability. From the study, it was found that Tween blend was the best surfactant which can preserve the pDNA stability. In contrast, the present study only used 1% PVA as the surfactant which is not enough to preserve the plasmid. In the future study, a combination of PVA and Tween blend may result in higher SC of pDNA confirmation isoform.

Nanoparticles formulation and stability

Among the crucial characteristics of nanoparticles in delivering drug are size, shape, and surface charge of the nanoparticles. In the present study, blank/ unloaded nanoparticles were smaller than p53 loaded. The increment of size in p53 loaded nanoparticles up to 266.67 nm may be due to the additional large size of p53 plasmid encapsulated in the nanoparticles. According to Biswas, Islam, Choudhury, Mostafa, & Kadir (2014), the preferential size of nanoparticles for drug delivery is less than 200 nm. However Murugan et al., (2015) has stated that nanoparticles which possess the size of less than 500 nm are still able to produce high cellular uptake. Therefore, the size of the fabricated p53 loaded nanoparticles is still acceptable to be the carrier agent of a plasmid.

Next, the surface charge measured in zeta potential has shown a decreasing charge in p53 loaded nanoparticles. This observation may be due to the charge of the plasmid which affects the charge of the nanoparticle as a whole.

Meanwhile from the morphological examination, it was illustrated that the fabricated nanoparticles were spherical with a smooth external surface and had no pores. Some previous studies, including Behzadi et al., (2017) and Sousa et al., (2017) have claimed that spherical shape of nanoparticles enhanced the cellular uptake through endocytosis process. In contrast, Salatin & Yari Khosroushahi (2017) found different evidences suggesting that rod-shaped nanoparticles are more efficient in terms of cellular uptake compared to the spherical ones. It is because the rod shape nanoparticles have higher surface area that enables them to interact more with the cell membrane. However, Behzadi et al., (2017) justified their stand by suggesting that rod-nanoparticles need a longer time for membrane wrapping of the endocytosis process compared to the spherical nanoparticles. Hence, the researchers were prone to suggest that the spherical nanoparticles are more

effective in drug-delivering mechanism. This debate is still on- going and most of the researchers are firm on their stand with finding evidence.

Cell Viability

As illustrated in Figure 3, p53 plasmid extract as the positive control has shown a reduction in cell viability at both 24 and 48 hours. This finding is parallel with previous researches which highlighted the ability of p53 gene in inducing apoptotic activity of irreversible damage cells (Ozaki & Nakagawara, 2011 and Box, Vukmer, & Terzian, 2014).

However, the blank nanoparticles group which act as the negative control showed an unexpected result. As stated in many studies including (Danhier et al., 2012; Samani & Taghipour, 2014; Sharma et al., 2016; Cappellano, Comi, Chiocchetti, & Dianzani, 2019), PLGA is a biodegradable and biocompatible polymer which has been approved by FDA and EMA. Hence, by exposing the cells to non-capsulated PLGA nanoparticles, it was expected that there would be no cell viability reduction. Sousa et al. (2017) also have indicated in their study that the unloaded PLGA nanoparticles were not causing toxicity to the exposed cells. In contrast, (NanoComposix, 2012) has mentioned the possibility of endotoxin contamination in nanoparticles that may happen due to the material used or during the fabrication process. The endotoxin contamination can cause toxicity effect to the cells; as endotoxin is the significant component of the cell wall of gram-negative bacteria. Referred to as lipopolysaccharides (LPS), a high amount of this substance can induce cytokines production and immune response (Smulders et al., 2012). Therefore, the low percentage of cell viability in 24 hours of cells exposed to the blank nanoparticles might be due to the endotoxin contamination which causes the toxicity effect to the cells. Different batches of blank nanoparticle samples were exposed to the cells in 24 hours and 48 hours suggesting different results of cell viability at both hours. Both batches have been fabricated by using the same formulation and materials, however different containers used to load and store the nanoparticles as well as the handling technique may cause the contamination to occur. The endotoxin contamination may happen during the storage phase of the nanoparticles as it is one of the ambient contaminants in the environment and it possesses a thermoresistant characteristic. This quality enables the endotoxin to persist in the environment even without the presence of gram-negative bacteria. Future work should be done on endotoxin contamination evaluation by using a few methods as in the study conducted by Smulders et al (2012).

p53 loaded nanoparticles have shown a decrease in cell viability as the concentration of nanoparticles increased. However, the results were found not to be statistically significant. Besides, the cell viability at a concentration of 0.8 mg/ml was higher than the previous concentration in both 24 and 48 hours. This unexpected result might happen due to a few reasons. The first factor is the stability of the nanoparticles. As the size of the nanoparticles in the present study is less than 300 nm (considered as small size), the stability of the nanoparticles become a point of concern. Mohanraj & Chen (2006) addressed the issue where the aggregation between the particles is much easier to take

place when the size of nanoparticles is small. The authors also pointed out about the poor storage stability of the nanoparticles which contributes to the aggregation process. Hence, the aggregation of nanoparticles might be the possible reason causing high cell viability in cells exposed to 0.8 mg/ml p53 loaded nanoparticles. The aggregation reaction makes the nanoparticles failed to deliver the plasmid into the cells. Another factor that may contribute to the non-statistically significant result might be due to the negative charge of the nanoparticles. A study by Salatin & Yari Khosroushahi (2017) found that positive charge nanoparticles enhanced cellular uptake. It is due to the electrostatic interaction between the positive charge nanoparticles and negative charge membrane of the exposed cells. Besides, Danhier et al., (2012) have mentioned about the disadvantage of double emulsion W/O/W formulation of nanoparticles. In this formulation, the high mechanical force needs to be applied to the compound in order to produce the nano-size particles. However, the shear force applied can cause nucleic acid degradation. Therefore, the efficacy of the plasmid itself will be reduced.

Apart from the above-mentioned possible reasons, there are several factors which may influence the result of the absorbance reading of cell viability assays such as the MTT concentration and the incubation period, the number of viable cells and their metabolism activities and the medium of the cell culture during the cell viability assay procedure. According to Riss et al., (2013) longer incubation period will enhance the sensitivity of the cells toward MTT. However, MTT itself is cytotoxic which the overextend period of incubation will affect the actual reading of the result. In the present study, the MTT reagent was exposed to the cells for four hours which is still in the range of optimum incubation period (Barnabe, 2017). Nevertheless, in a future study, the cells may be incubated for 3 hours or less to reduce the toxicity risk. While, poor culture medium such as overgrowth of the cells will reduce the ability of the viable cells to reduce MTT to formazan crystals due to the low metabolism of the cells. This condition will lead to linearity loss between the absorbance and the cell number, which also contribute to the inconsistency of the cell viability results (Riss et al., 2013).

Even though the concentration range of nanoparticles was not statistically significant; the second set of the experiment which focused on the duration of nanoparticles exposure to the cell viability was turn otherwise. 48 hours of treatment exposure to A375 cell lines showed a lesser percentage of cell viability compared to 24 hours. The finding suggests that the exposure of nanoparticles should be lengthened in order to allow more nanoparticles to be absorbed into the cells.

5. Conclusion

The general purpose of the current study was to assess the effectiveness of p53 plasmid in restoring the function of wildtype p53 and increasing the apoptosis rate in A375 melanoma cell lines. The extracted p53 plasmid was pure from any contaminants; however, they exert most of the open circular isoform compared to the super-coiled which has the highest biological activity. Other than that, this study also aimed to evaluate the ability of the fabricated PLGA double emulsion W/O/W

nanoparticles in delivering the plasmid into the cells. The fabricated nanoparticles have been characterised as small size, spherical, and smooth surface with no porosity which enhances the cellular uptake of the nanoparticles. However, the negative surface zeta potential became the limitation for the study. Meanwhile, the cell viability of A375 cell lines was found to be reduced after the exposure of p53 loaded PLGA nanoparticles in 24 and 48 hours. Even though, there is no significant difference in the cell viability as the concentration of p53 loaded PLGA nanoparticles increased, the difference in the time exposure was found to be significant.

Findings from this study demonstrate that p53 plasmid can be one of the opportunistic gene therapy approaches that can be developed as one of the alternative treatments for melanoma. However, the formulation of nanoparticles should be improved in the future in terms of stability, the positivity of the nanoparticles' charge and proper storage to prevent the aggregation process which can reduce the efficacy of the nanoparticles as the plasmid delivering agent. Besides, this study has shown that more prolonged nanoparticles exposure is more significant in inducing higher apoptotic rate to the cancer cells as more nanoparticles are being endocytosed into the cells. In the future study, improvements can be done by increasing the volume of the plasmid being encapsulated in the nanoparticles and increasing the concentration of the plasmid encapsulated nanoparticles that exposed to the cells; as well.

Acknowledgement

The authors are grateful to the Advanced Drug Delivery Lab, Pharmaceutical Department of Kulliyah of Pharmacy IIUM Research Team for their support; and IIUM Research Acculturation Grant Scheme (IRAGS) with grant number of IRAGS18-026-0027.

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