Novel Nano-biomaterials for Inhibition of Respiratory Syncytial Virus

Sameer Joshi[1], Daniel Kirby[2], Yvonne Perrie[3] Shree R. Singh[1]

[1]Center for NanoBiotechnology Research, Alabama State University, Montgomery, Alabama, USA.
[2]Aston Pharmacy School, Aston University, Birmingham, UK.
[3]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK.
E-mail: s_ndds@hotmail.com

ABSTRACT

Respiratory syncytial virus (RSV) infection is a major concern for the population across all age groups and it is the single serotype with two major antigenic subgroups circulating together but one leads [1]. Considering published outcomes related to treatment, as well as the impact of RSV on global healthcare, a promising treatment or vaccine development remains a priority. The potential of liposomes as a carrier of active pharmacological agents was described decades ago [2-4], whilst liposomes have attracted much attention for their ability to carry antigens as well as immunomodulators [5]. Therefore, the work in this paper was aimed to prepare liposomes < 100 nm using thin film hydration followed by sonication, to effectively conjugate the anti-RSV peptide RF-482 and investigate the efficiency the liposomal formulation in presence and absence peptide RF-482 to inhibit RSV spread.

Keywords: RSV, liposomes, peptide.

1 INTRODUCTION

It was reported that gold nanoparticles (GNPs) can be functionalized with nucleic acid, antibodies, drugs as well as with peptides and these functionalized GNPs then can be applied in diagnosis of treatment of RSV infection [6]. Use of GNPs functionalized with anti RSV peptide RF-482 to inhibit RSV is a patented system [7]. Based on these findings and considering the nature of peptide RF-482, the liposomes were considered as alternative delivery system to deliver peptide RF-482. The peptide was expected to be ecapsulated or adsorbed on the surface of liposomes and bring same or better RSV inhibition compared to GNPs.

The genome of RSV codes immunogenic proteins, which creates scope for DNA vaccines, subunit vaccines and nano vaccines [8]. These vaccines possibly designed as carrier based vaccine using nanoparticulate systems such as liposomes, that expresses adjuvant action by enhanced antigen delivery or inducing innate immune responses [9]. The liposomes were prepared using the conventional thin film hydration method with and without peptide RF-482. The system was tested for peptide loading capacity and RSV inhibition.

2 MATERIALS AND METHODS

2.1 Materials

1,2-disteroylphosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from Sigma-Aldrich Company Ltd. (Poole, UK) was purchased from Sigma-Aldrich Co (St Louis, MO, USA). The peptide RF-482 was purchased from Bachem Americas Inc., Santa Clara, CA, USA. Human epithelial type-2 (HEP-2) cells purcahsed from American Type Culture Collection (ATCC®, Manassas, VA, USA), were used to study the viral infection and inhibition.

2.2 Functionlization of liposomes

The fusion peptide RF-482 is a small as well as heat sensitive. Therefore, a slight modification was done in the conventional thin film hydration method (Figure 2.2), where the protein was added post sonication when small unilamellar vesicles (SUV) were obtained and not added to the hydration buffer in spite of the hydrophilic nature of the protein. Briefly, a predetermined concentration of protein was added to the SUV suspension and mounted on a shaker for 30 minutes. This was done to facilitate the fusion of protein on to the liposomal surface.

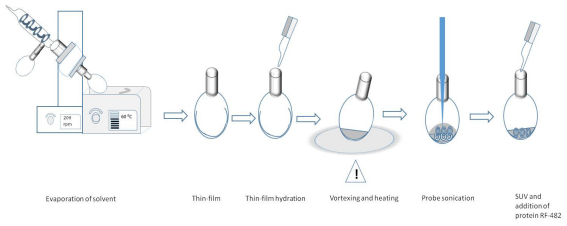

Figure 1 Illustration of thin film hydration based SUV production and loading of fusion protein RF-482.

3 RESULTS AND DISCUSSION

3.1 Confirmation of functionalization

Characterization of size of SUV was performed using dynamic light scattering (Malvern Zetasizer Nano-ZS) and the surface charges were studied using laser doppler velocimetry (Malvern Zetasizer Nano-ZS). RF-482 is a very small peptide and, therefore, a slight increase in the size of

liposomes can be expected to confirm the conjugation. There is a slight change in the size of liposomes (91.78 ± 0.3 nm (PDI 0.2 ± 0.01) and 96.91 ± 0.6 nm (PDI 0.19 ± 0.03) before and after conjugation, respectively). Because DSPC is a neutral lipid, there was no observed change in the zeta potential due to conjugation of RF-482 (-9.9 ± 1 mv and -12.2 ± 1.3 mv before and after conjugation, respectively) (Figure 2).

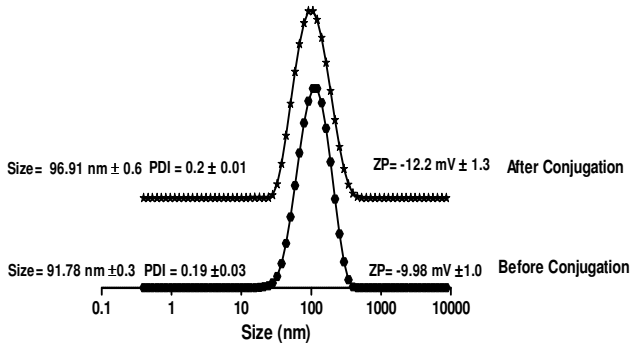

Figure 2 DLS measurement of liposomes before and after RF-482conjugation. (n=3 ±SD).

The first and most supportive evidence of association of peptide with liposome was observed when FITC labelled peptide RF-482 was used for the conjugation. After the separation of non-conjugated peptide, the liposomal suspension was dried, covered with phosphotungstic acid for better resolution and observed under the fluorescence microscope. Imaging at low and high maginification shows the presence of peptide suggests the association of peptide RF-482 with liposomes (Figure 3).

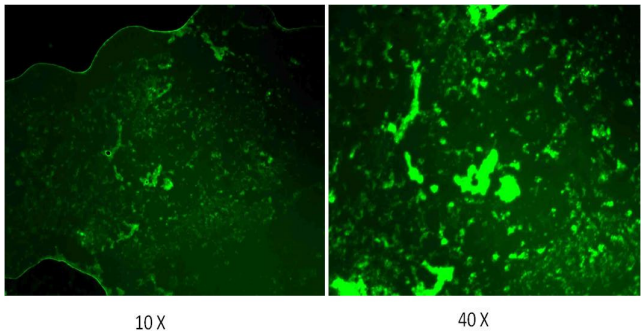

Figure 3 Fluorescence microscopy analysis liposomes conjugated with FITC labelled peptide RF-482 (Green). 10X and 40X magnification.

Subsequently after the fluorescence imaging, the empty liposomes and the peptide conjugated liposomes were imaged under TEM. Association of protein was again indicated by a cloudy environment observed around the liposomes and a change in morphology (Figure 4b), that was not observed in the case of empty liposomes (Figure 4a).

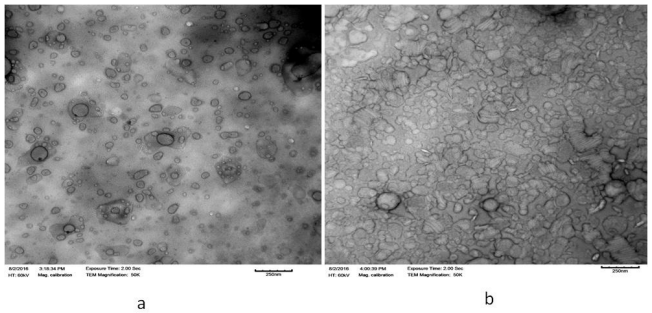

Figure 4 Transmission electron microscopy analysis. Comparison between (a) empty liposomes and (b) RF-482 peptide associated liposomes Images taken at 50X magnification and 60kv HT.

3.2 Cytotoxicity analysis

Human epidermoid type-2 (HEP-2) cell were used for the assay. These HEP-2 cell were proliferated using minimum essential medium (MEM) having added 10 % fatal bovine serum (FBS) mM L-glutamine, 75 U/ml penicillin, 100 mg/ml kanamycin and 75 mg/ml streptomycin. Empty and functionalized liposomes as well as peptide RF-482 was tested for the cell toxicity by using dye MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide). The reduction assay was performed using CellTiter 96® Non-Radioactive cell proliferation assay kit (Promega, Madison, WI, USA). 25,000 cells per well were seeded using MEM-10 (10% FBS). Two different concentrations of peptide RF-482, empty liposomes and functionalized liposomes were tested for the toxicity. Cell toxicity was assessed 72 hours post incubation. MTT assay was performed as per protocol provided (Promega, Madison, WI, USA). The absorbance was measured at 570 nm using the plate reader (TECAN^{TM}, Morrisville, NC, USA). There was no toxicity observed with both the concentrations of all samples. For all the chosen concentration of all the samples, more than 80 % (n=3 ± SD) cell viability was observed after 72 hours of incubation (Figure 5).

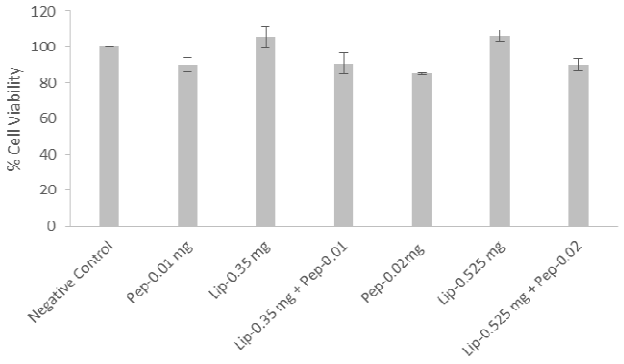

Figure 5 Screening of Peptide, Liposomes and peptide encapsulated against RSV. Plaques were counted and the mean plaque was count of for each sample was expressed as a percentage of the mean count of the control. (n=3 ±SD)

3.3 Qualitative analysis RSV inhibition

Initially, the immunofluorescence imaging was used as a qualitative tool to study the RSV inhibition by liposomal system. the HEP-2 cells were incubated for 48 h with peptide RF-482, empty liposomes and functionalized liposomes, followed by fixing in paraformaldehyde-glutaraldehyde and washing with buffer (1X PBS). An appropriate chamber from the 8 chambered slide was observed under the fluorescence microscope for the RSV activity.

The obtained results confirm the effectiveness of the system to inhibit the RSV fusion to the HEP-2 cells (Figure 6). Moreover, recently it was reported that the phosphatidylinositol (PI) inhibits the RSV as the PI binds the RSV with high affinity, inhibiting its fusion to the epithelial cells [10, 11]. Both PI and phosphocholine (PC) are deivatives of phospholipids , therefore the inhibition of RSV by DSPC liposomes alone can be expected.

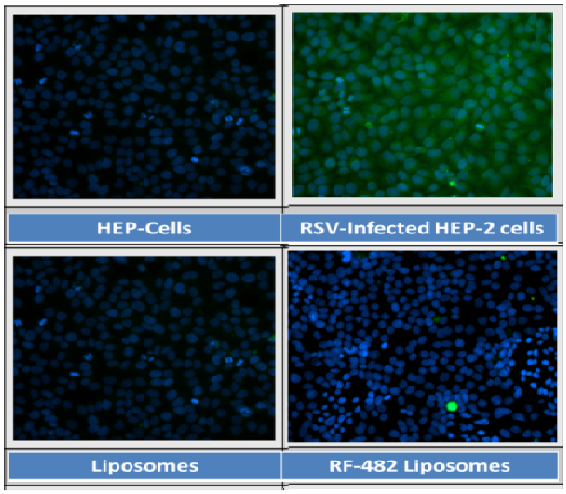

Figure 6 Fluorescence microscopy analysis. FITC (Green): RSV and DAPI (Blue): HEP-2 Cell nucleus. In liposomes and RF-482 liposomes the blue colour represents survived cells and green colour represents presence of RSV.

3.4 Quantitavie analysis of RSV inhibition

The plaque assay is one of the most common and reliable methods of determination of viral/antiviral activity by counting plaques in the cell culture. Also, the plaque reduction assay is known as the optimum standard for antiviral activity analysis [12].

Liposomes with and without their conjugation product with the RSV fusion protein were tested against RSV infection to the HEP-2 cells. When these plaques were counted, it was observed that, in the presence of peptide RF-482/liposomes/peptide conjugated liposomes, the total number of plaques are significantly ($p<0.005$ ANOVA, post hoc-Dunnett's multiple comparison test) less than in the absence of the same (Figure 7).

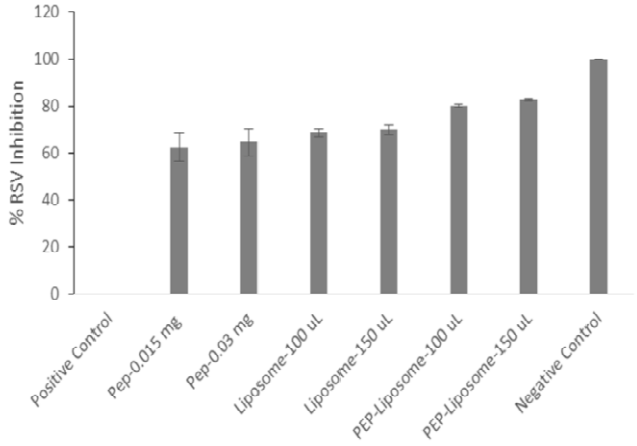

Figure 7 Plaques were counted and the mean plaque was count of for each sample was expressed as a percentage of the mean count of the control. (n=3 ±SD).

Having confirmation of viral inhibition from the microscopic as well as from the plaque assay results, it was necessary to have another establised technique to validate the results obtained. Polymerase chain reaction (PCR), is one of the establised techniques available in the current biological research, employed for the quantification of the target sequence using real time, by detecting the number of amplicons generated at the end of every amplification cycle [13].

qRT-PCR provides rapid and quantitative data for analysis of viral presence. However, before the amplification of cDNA via RT-PCR, the RNA was extracted from the samples and cDNA was synthesis edusing the superscript. After amplification, the obtained cycle threshold 'Ct' values were used as a tool for the comparison between the samples.

Significant difference ($p<0.005$, ANOVA, post hoc-Dunnett's multiple comparison test) were observed between the Ct value of virus samples and samples having peptide/liposomes/conjugated liposomes (Figure 7). Whereas, there is no significant difference ($p>0.05$, t-test) observed between peptide/liposome alone as well as the conjugated liposomes. Although, the conjugated liposome samples displayed a slight trend for higher Ct values,further suggesting more inhibition when used in combination (Figure 8).

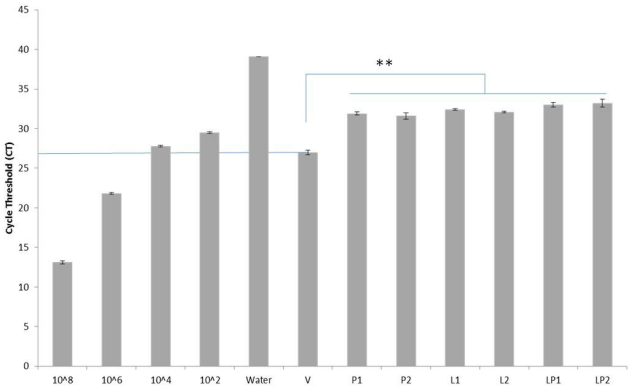

Figure 8 Screening of RSV-F gene amplicon dilution with water as negative control. Water represents blank or sample with no gene amplicon. Blue line: indicating the threshold of virus samplehas higher gene amplicon than the gene amplicon of standard 10^4. Comparison of viral gene amplicon (V) and Peptide (P1 & P2), Liposomes (L1 & L2), functionalized liposomes (LP1 & LP2). (n=3 ±SD) (* and ** in the figure represents $p<0.05$ and $p<0.01$ respectively).

4 CONCLUSION

With the quest of finding an alternative carrier for delivering the anti-RSV peptide RF-482, a liposome formulation has been prepared and tested for the inhibition of RSV infection. The functionalization of liposomes was confirmed by various techniques including dynamic light scattering (Figure 2), the surface charge, fluorescence imaging (Figure 3) and transmission electron microscopy (Figure 4). The cytotoxicity of various concentrations of these liposomes was tested by MTT assay and it was found that liposomes of two different concentrations of lipids with and without protein conjugation did not render any cytotoxic effect and molecular effect on the host HEP-2 cells (Figure 5). The anti-RSV effect was then confirmed in plaque assay (Figure 7), immunofluorescence imaging (Figure 6) as well as in qRT-PCR (Figure 8). In plauqe assay significant difference was observed in number plaques observed for liposomes with and without protein (Figure 7). Similarly, in qRT-PCR, the cycle threshold (Ct) for liposomes of two different concentrations of lipids with and without protein conjugation was observed significantly higher ($P <0.005$, t-test) than the virus cycle threshold (Ct) (Figure 8). Overall, the results indicate that the liposome are a new potential candidate for delivery of anti-RSV peptide RF-482. However, exact mechanism of action of liposomes in inhibiting RSV need explorations foreseeing possibility of lipsomal vaccine for RSV infection.

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