Chapter 4

Results and Discussion

4.1 Procurement and selection of minicells producing strains

Proposed work focused on targeted delivery of shRNA specific for VEGF A mRNA through folic acid conjugated bacterial minicells. In order to develop minicells as delivery systems, large numbers of purified minicells were required. Hence to achieve these, strain that was capable of producing large number of minicells was selected from the available minicell producing strains. Minicell producing capacity of each strain was observed qualitatively by gram’s staining and best strain was selected. The available viz. E.coli K-12 X$^{984}$, E.coli P678-54 and E.coli PB114 has been revived from the procured stocks and enriched in the growth medium.

4.1.1 Enrichment of E.coli K-12 X$^{984}$ and E.coli P678-54

E.coli K-12 X$^{984}$ and E.coli P678-54 were provided as a dry powder form adsorbed on the filter disc. Strains were required to be revived from dormant state to active state for the further experimentation. As mentioned in the previous section, filter disc was placed in the centre of LA plates and sterile broth was added drop wise in order to spread culture evenly in the plate. Subsequently, plates were incubated at 37°C for overnight. After overnight incubation, lawn growth was observed in case of both the strains (Figure 4.1). Figure 4.1 represents a photograph of LA plate which shows loan growth of E.coli P678-54. Similar result was obtained in case of E.coli K-12 X$^{984}$. These results indicate the successful revival of E.coli K-12 X$^{984}$ and E.coli P678-54. Both the strains were inoculated in LB medium and used for the grams staining.
Figure 4.1: LA Plate of *E.coli* P678-54. LA plate showing lawn growth of *E.coli* P678-54. Arrow indicate filter disc.

4.1.2 Enrichment of *E.coli* PB 114 minicell producing strain

*E.coli* PB114 was provided as stab culture stock. As described in the previous section, a colony was inoculated in LB broth containing kanamycin. The flask was incubated for overnight incubation at 37°C and 180 rpm. After overnight incubation in shaking incubator, growth was observed. LB broth became turbid after the overnight incubation which suggested the successful revival of *E.coli* PB114. Subsequently, it was used for gram’s staining in order to observe it’s morphology and minicells producing capacity.

4.1.3 Gram’s staining of *E.coli* K-12 X^{984}, *E.coli* p678-54 and *E.coli* PB 114

All the strains were stained with gram’s staining procedure for the preliminary screening of minicell production and to compare their minicell producing capacity. Figure 4.2 (a-c) shows photographs of light microscopy after staining, at 1000X magnification. It can be observed from the figure 4.2 that all the three minicells producing strains of *E.coli* turned pink upon staining thus confirming the presence of gram negative organism. It also showed the production of minicells which are indicated with arrows. However, all minicells producing capacity were different for all the three strains. It can be visualized that numbers of minicells production by *E. coli* PB114 were very high as compared to *E.coli* K-12 x^{984} and *E.coli* p678-
54 (Figure 4.2). Hence, this strain (E.coli PB114) was selected for further experimentations. In the next step, detailed morphology and minicells production was observed by Transmission Electron Microscopy (TEM).

**Figure 4.2(a):** Gram’s staining of *E.coli* K-12 $x^{984}$. Light microscopy image of *E.coli* K-12 $x^{984}$ after gram’s staining. (1000X magnification)

**Figure 4.2(b):** Gram’s staining of *E.coli* P678-54. Light microscopy image of *E.coli* P678-54 after gram’s staining (at 1000X magnification).
Figure 4.2(c): Gram’s staining of *E.coli* PB114. *Light microscopy image of E.coli PB114 after gram’s staining (at 1000 X magnification).*

### 4.2 Transmission Electron Microscopy of *E.coli* PB 114.

To observe detailed morphology, minicell production and characterization of *E.coli* PB 114, Transmission Electron Microscopy (TEM) was performed using Tecnai 20 microscope. **Figure 4.3 (a-c)** shows TEM images of *E.coli* PB 114. Figure 4.3 depicts sequential event of minicell production from parent cell which is indicated by arrows. Figure 4.3(a) shows bacterium in which minicell production was just initiated from the pole. Figure 4.3(b) shows minicell in the stage of its division from parent bacterium. Figure 4.3(c) shows minicell divided from the parent bacterium. Hence, from TEM images ultrastructure of *E.coli* PB114 has been deduced and minicell production from this strain has been characterized. In the next step, shRNA expression vectors were procured in order to transform in *E.coli* PB114 and encapsulate into minicells.
Figure 4.3: Transmission Electron Microscopy image of *E.coli* PB114. (20000X magnification). Figure 4.3 (a), (b) and (c) shows sequential minicell production from the parent *E.coli* PB114 which is indicated by arrows.

### 4.3 Procurement and maintenance of expression vectors

Figure 4.4 shows expression maps of various vectors used in the study. Figure 4.4 (a) shows an expression map of pEZ43G-D plasmid. pEZ43G-D has ampicillin resistant gene as a selectable trait and GFP as a reporter gene express in the bacteria. pEZ43G-D was used to transformed *E.coli* PB114 in order to study segregation of protein and plasmid DNA into the minicells. Moreover, pEZ43G-D transformed *E.coli* PB114 was also used to visualize minicells purification more clearly under the fluorescence microscope.

Figure 4.4 (b) shows an expression map of pSUPERneo plasmid. pSUPERneo was used as a backbone expression vector which was used to clone shRNA sequences against VEGF A gene and scramble shRNA (control). pSUPERneo has geneticin resistant gene as a selectable
marker. It was also used as a negative control in all the experiment pertaining to gene silencing with active shRNA. **Figure 4.4 (c and d)** shows an expression map of psNIPERDH1A1 and psNIPERDU6A2, respectively. psNIPERDH1A1 and psNIPERDU6A2 are expression vectors having shRNA 1 and shRNA 2 sequences under H1 and U6 promoter, respectively. Sequences of both the siRNA were given in the previous section. Both the expression vectors were tested for their gene silencing *in vitro* and best shRNA was selected for *in vitro* and *in vivo* delivery. **Figure 4.4(e)** shows an expression map of pSUPERneoScramble. pSUPERneoScramble is an expression vector having scramble shRNA sequence. **Figure 4.4(f)** shows an expression map of pRNAT plasmid. pRNAT was used as a backbone vector to clone shRNA sequence against BCL-2 gene. pRNAT has geneticin resistant gene as a selectable marker and GFP as a reporter gene under the U6 promoter which express in the mammalian cells. pRNAT was used to study the expression of transgene after the *in vitro* delivery through folic acid conjugated minicells.

**Figure 4.4(a) Expression map of pEZ43G-D plasmid vector.** *pEZ43G-D has ampicillin resistant gene as a selectable trait and GFP as a reporter gene express in the bacteria.*
Figure 4.4(b) Expression map of pSUPER.neo plasmid vector. pSUPER.neo has geneticin resistant gene as a selectable marker under H1 promoter.

Figure 4.4 Expression map of (c) psNIPERDH1A1, (d) psNIPERDU6A2 plasmid vector. psNIPERDH1A1 and psNIPERDU6A2 are expression vectors having shRNA 1 and shRNA 2 sequences under H1 and U6 promoter, respectively.
Figure 4.4 (e) Expression map of pSUPERneoScramble plasmid vector. pSUPERneoScramble has geneticin resistant gene as a selectable marker.

Figure 4.4(f): Expression map of pRNAT plasmid vector. pRNAT has geneticin resistant gene as a selectable marker and GFP as a reporter gene under the U6 promoter.
4.3.1 Restriction enzyme digestion of shRNA vectors specific for VEGF A

shRNA expression vectors, psNIPERDH1A1 and psNIPERDU6A2 were digested with restriction enzyme Hind III and Eco R1 to detect the presence of cloned shRNA. Size of psNIPERDU6A2 and psNIPERDH1A1 is 4858 and 4642 bp, respectively. Restriction digestion with Hind III and Eco R1 should yield two fragments for both the vectors. In case of psNIPERDU6A2, double digestion should yield 386 bp and 4472 bp fragments whereas double digestion of psNIPERDH1A1 should yield 170 bp and 4472 bp fragments.

![Figure 4.5: RE digestion](image)

**Figure 4.5: RE digestion.** *Gel electrophoresis of RE digestion of psNIPERDH1A1 and psNIPERDU6A2 a) psNIPERDU6A2 1) digested with hind 3 2) digested with Eco R1 3) double digest 4) control 5) gene ruler 50 bp 6) λ Hind III ladder, b) psNIPERDH1A1 1) gene ruler 50 bp 2) λ Hind III ladder 3) control 4) double digest 5) digested with Hind III 6) digested with Eco R1.*
Figure 4.5 shows agarose gel electrophoresis of digested shRNA vectors. Figure 4.5(a) shows restriction enzyme digestion of psNIPERDU6A2. It shows that double digestion with Hind III and Eco R1 yielded the fragments of predicted size viz. 4472 and 386. Similarly, figure 4.5(b) shows restriction enzyme digestion of psNIPERDH1A1. It shows that double digestion with Hind III and Eco R1 yielded the fragments of predicted size viz. 4472 and 170. Size of bands was compared with λ Hind III ladder and 50 bp gene ruler for confirming their identity. Based on the findings, it is confirmed that shRNA vectors were psNIPERDH1A1 and psNIPERDU6A2 and they posses respective shRNA. Both the vectors can be used for the further experiments.

4.4 Transformation of E.coli PB 114 with pEZ43G-D plasmid

E.coli PB 114 was transformed with pEZ 43G-D plasmid to observe the segregation of it into the minicells. Transformation of E.coli PB 114 was performed by treating them with calcium chloride. Detailed method is described in section 3.5. Figure 4.6 shows LA plates from different groups like negative control, positive control, Test 1, Test-2 and Test-3 after the overnight incubation. It can be visualized from the figure 4.6 that no growth was observed in negative control plate because ampicillin was added but no plasmid DNA was added which posses ampicillin resistant gene. Hence E.coli PB114 failed to grow in the presence of ampicillin. On the other hand lawn growth was observed in positive control plate where neither ampicillin nor DNA was added. Isolated colonies were observed in all the test plates which indicate the transformed E.coli PB114. In all the test plates, ampicillin was added at a concentration of 100 µg/ml. So only those cells can grow which have acquired pEZ43G-D. It was observed that the number of transformed colonies increased with the increasing concentration of plasmid DNA used for transformation. From these results, it can be inferred that transformation of E.coli PB114 with pEZ 43G-D plasmid has been successfully achieved. pEZ43G-D plasmid posses GFP as a reporter gene. So transformation can be further confirmed by expression of GFP in the cells.
Figure 4.6: Transformation of *E.coli* PB114 with pEZ43G-D plasmid. LA plates showing (a) negative control, (b) positive control, (c)Test-1, (d)Test-2 and (e) Test-3 plates. Test1-3 shows *E.coli* PB114 transformed with pEZ 43G-D plasmid.

### 4.4.1 Expression of GFP in transformed *E.coli* PB114

Figure 4.7 shows fluorescence microscopy image of *E.coli* PB114 expressing GFP. Fluorescence microscopy observation of pEZ43G-D transformed *E.coli* PB114 showed bright
green fluorescence protein expression in parent cells as well as minicells (Figure 4.7). It can be observed from the figure that there are large numbers of GFP expressing minicells present in the field. Minicells are indicated with arrows. This result confirms the segregation of plasmid DNA as well as GFP protein into the minicells. However, segregation of plasmid DNA needed to be confirmed further by plasmid isolation from purified minicells. So in the next step minicells were purified from the parent cells and other cell debris in order to check presence of plasmid DNA.

Figure 4.7: Fluorescence microscopy image of *E.coli* PB114 expressing GFP. *(1000X magnification)*. Minicells are indicated by arrows.

4.5 Minicell purification by penicillin lysis method

Initially minicells were purified by penicillin lysis method as reported by S.B Levy, 1970 (Levy, 1970). **Figure 4.8** shows picture of grams stained minicells after the purification. It shows the presence of large number of purified minicells which are denoted by arrows. Along with minicells few parent cells (indicated by circles) and cell debris also appeared as contaminant. These contaminanats need to be removed prior to the use of minicells as a targeted delivery system. Hence, these parent cells and cell debris have to be removed to develop minicells as a drug delivery system. This method was thus modified where ceftriaxone was replaced with penicillin, to obtain purified minicells.
Figure 4.8: Gram’s staining of purified minicells. Light microscopy image of purified minicells after gram’s staining. Minicells were purified by penicillin lysis method. Minicells are indicated by arrows. Circles denotes the parent cells. (1000X magnification).

4.5.1 Purification of minicells by ceftriaxone lysis and filtration

Minicells were purified from GFP transformed *E.coli* PB114 in order to visualize each step of purification by fluorescence microscopy. Figure 4.9 (a-d) shows different stages of minicells purification by ceftriaxone lysis method. Figure 4.9 shows pictures of fluorescence microscopy and corresponding growth of minicell culture after each step of purification. Figure 4.9(a) shows fluorescence microscopy of starting culture of GFP transformed *E.coli* PB114 and its growth on LA plate. It can be observed from the figure that fluorescence microscopy shows large number of parent cells with a GFP expression along with several minicells. Similarly, lawn growth was observed on LA plate when this culture was plated on LA plate. Figure 4.9(b) shows pictures of minicells after differential centrifugation. It can be observed from the figure 4.9(b) that initial differential centrifugation reduced majority of parent cells. It is also evident from the reduced growth in the LA plate. Figure 4.9(c) shows fluorescence microscopy of minicell after antibiotic treatment and its growth on LA plate. Treatment with ceftriaxone killed remaining parent cells which can be observed from the microscopy image and is implied from the corresponding LA plate showing no growth. Here, ceftriaxone was so potent that it killed almost all the parent cells. Residual parent cells which were left become inactive because they failed to grow on LA plate. Eventually, these residual parent cells and cell debris were removed by successive filtration with 0.45µ and 0.22µ filter.
Figure 4.9: Purification of Minicells. Different stages of minicell purification by ceftriaxone lysis method: A) Fluorescence microscopy image and B) Growth of E.coli PB114 on LA plate; a) Starting culture of GFP transformed E.coli PB114, b) Culture of E.coli PB114 after differential centrifugation, c) Minicells after antibiotic treatment, d) Minicells after successive filtration with 0.45µ and 0.22µ filter.
Figure 4.9 (d) shows fluorescence microscopy of minicells after successive filtration and its growth on LA plate. Microscopy image shows highly purified minicells. Moreover, growth was not observed when these minicells were plated on LA plate. Hence, after every step of minicells purification, parent cells decreased which was confirmed by the fluorescence microscopy as well as corresponding growth of minicell culture on LA plate. Moreover, fluid thioglycolate medium did not showed any sign of growth after the 14 day incubation which suggested absence of any slow growing bacterial cells into the final fraction of purified minicells. Thus, ultra pure minicells have been obtained by combining two methods; ceftriaxone lysis and filtration. The size of cells at every step of purification was determined by using dynamic light scattering method (Zetasizer ZS 90, Malvern) for further confirmation about the purity of minicells.

4.5.2 Size measurement of purified minicells

Purity of purified minicells was further confirmed by the size of cells at every step of purification using dynamic light scattering method. Average size and polydispersity index of purified minicells at different stages of purification has been given in Table 4.1.

Table 4.1: Average size and polydispersity index of different fractions of minicell purification.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction</th>
<th>Size (nm)</th>
<th>PDI (polydispersity index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>Starting culture of GFP transformed <em>E.coli</em> PB114</td>
<td>929.4</td>
<td>1.0</td>
</tr>
<tr>
<td>b.</td>
<td>Culture of <em>E.coli</em> PB114 after differential centrifugation</td>
<td>639.9</td>
<td>0.156</td>
</tr>
<tr>
<td>c.</td>
<td>Cells after antibiotic treatment</td>
<td>584.9</td>
<td>0.081</td>
</tr>
<tr>
<td>d.</td>
<td>Cells after successive filtration with 0.45µ and 0.22µ filter</td>
<td>522.3</td>
<td>0.042</td>
</tr>
</tbody>
</table>
Moreover, the graphs of average size at different stages of purification have been shown in figure 4.10. It can be seen from the figure 4.10 and table 4.1 that average size and polydispersity index (PDI) decreased gradually after each step of purification. This decrease in average size and PDI suggested that no parent strain were present in the purified minicells after final step of purification, and obtained were in ultrapure form. Average size of purified minicells was found to be 522.3 nm. This method is fast, cost effective and facilitates high yield of purified minicells, with no parent strain contamination. Subsequently, morphology of purified minicells was observed by TEM and total number of purified minicells was calculated.

Figure 4.10: Average size of Minicells. Graphs showing average size of cells at different stages of minicells purification. a) Starting culture, b) after differential centrifugation, c) after antibiotic treatment, d) Cells after successive filtration with 0.45µ and 0.22µ filter. Graphs shows that average size decrease with each step of purification.
4.5.3 Transmission Electron Microscopy of purified minicells

Integrity of purified minicells was further confirmed TEM. Figure 4.11 shows TEM image of purified minicells at 20000X magnification. Minicells were found to be normal and intact which suggested that antibiotic ceftriaxone had no effect on minicells. Hence, this method is reliable and purifies intact minicells. In the next step, minicells number was calculated to find out the exact yield.

Figure 4.11: Transmission Electron Microscopy of purified minicells (20000X magnification).

4.5.4 Determination of minicell number by spectrophotometric method

OD $A_{600}$ of purified minicells was found to be 0.762

Hence, putting this value in the formula,

\[
\text{OD at } 600 \times 5.0 \times 10^{10} / \text{ ml} \\
0.762 \times 5.0 \times 10^{10} \\
= 3.81 \times 10^{10} \text{ minicells/ml}
\]

Total number of minicells after final step of purification was found to be $3.81 \times 10^{10}$ minicells from the 200 ml of starting culture. Obtained minicell yield was remarkably high in comparison to the other reported methods. For instance, the yield of minicells using the
method reported by S.B. Levy was found to be approximately $1.8 \times 10^8$ minicells from same starting culture (Levy, 1970). A new method for bacterial minicell purification was developed by combining antibiotic (ceftriaxone) lysis and filtration which yielded higher amount of ultrapure minicells with no parent cell contamination (Jivrajani et. al., 2013). Subsequently, plasmid DNA was isolated from purified minicells in order to confirm the segregation of plasmid DNA into minicells.

### 4.6 Isolation of plasmid DNA from purified minicells

Plasmid DNA was isolated by alkaline lysis method. Figure 4.12 shows agarose gel electrophoresis of plasmid DNA isolated from purified minicells. Lane 3 shows isolated plasmid DNA which is indicated by arrow. The plasmid band in Lane 3 corresponds to purified plasmid DNA in lane 2.

![Figure 4.12: Plasmid isolation from purified minicells. Gel electrophoresis of isolated plasmid DNA from minicells. 1) λ Hind-III ladder, 2) Standard pEZ43G-D plasmid, 3) Plasmid isolated from minicells. Bands of isolated plasmid DNA are indicated by arrows.](image)

This suggested that isolated plasmid DNA is same as purified plasmid DNA, i.e. pEZ43G-D and confirmed the presence of plasmid DNA in the minicells. This finding was in agreement
with the theory that when minicells produce from the parent cells, plasmid DNA segregates into the minicells. Similarly, shRNA expression vector can also be packaged in the minicells. Next, plasmid copy number present per minicells was determined by spectrophotometry method.

### 4.6.1 Determination of plasmid copy number

Plasmid copy number present per minicells was determined by spectrophotometric method. Method has been explained in the previous chapter (Section: 3.6.1). Mean plasmid copy number of pEZ43G-D was found to be $198 \pm 9$ per minicell. Copy number obtained is quite high and it is in agreement with previous report (MacDiarmid et. al. 2009). The minicells obtained from *E.coli* PB 114 can be efficiently packaged with large number of plasmid expression vectors. In the next step, cancer cell lines were selected and procured in order for *in vitro* experiments.

### 4.7 Selection, procurement and maintenance of cancer cell lines

As discussed in the previous section, all the human cancer cell lines (A549, LNCaP, HeLa and KB) have been selected on the basis of folate receptor overexpression. LNCaP, HeLa and KB have been selected as positive control whereas A549 was selected negative control, which has very least amount of folate receptor expression.

A549 and LNCaP cell lines were successfully maintained in RPMI 1640 medium whereas HeLa and KB cell lines were successfully maintained in DMEM medium. All the cell lines were grown in 10% fetal bovine serum supplemented with respective medium. **Figure 4.13 (a-d)** shows light microscopy image of all the selected cell lines. From the microscopic observation, it can be inferred that all the cells were healthy and can be used for the further cell based assay. In the next step, shRNA expression vectors, psNIPERDU6A2 and psNIPERDH1A1 were transfected in all the selected cell lines in order to validate down regulation of VEGF A mRNA by shRNA.
Figure 4.13 (a): Light microscopy image of A549 cells. A) 40-50% confluent B) ~100% confluent cells (100X magnification).

Figure 4.13 (b): Light microscopy image of LNCaP cells. A) 30-40% confluent B) ~100% confluent cells (100X magnification).
4.8 Transfection of cancer cell lines

A549, LNCaP, HeLa and Kb cell lines have been successfully transfected with shRNA specific for VEGF A gene i.e. psNIPERDH1A1 and psNIPERDU6A2 which was confirmed by the geneticin selection. **Figure 4.14 (a-d)** shows gradual selection of transfected cells.
(A549, LNCaP, HeLa and KB, respectively) with geneticin. In positive control group, neither plasmid DNA nor geneticin was added. Thus, positive control group cells became confluent very soon. In negative control group, geneticin was added but not plasmid DNA which resulted in cell death with the increase in geneticin concentration.

**Figure 4.14(a): Selection of transfected A549 cells.** (100X magnification) PSN (pSUPERneoScramble vector), H1A1 (psNIPERDH1A1 vector) and U6A2 (psNIPERDU6A2 vector). All the three expression vector possess geneticin resistant marker. Transfected cells were gradually selected by increasing geneticin concentration.

In case of PSN, H1A1 and U6A2 cells were transfected with pSUPERneoScramble, psNIPERDH1A1 and psNIPERDU6A2, respectively. These expression vector posses’ geneticin resistant genes. Cells which have acquired these plasmid, acquired resistant against geneticin. In these experiments, starting concentration of geneticin used was 100 µg/ml. Initially, cells which are not transfected, died because of geneticin treatment. However, cells that have acquired the plasmid were gradually selected by increase in geneticin concentration. Cells have been selected at a concentration of 400 µg/ml geneticin for HeLa and LNCaP cell
lines whereas cells have been selected at a concentration of 450 µg/ml geneticin for A549 and KB cells. Subsequently, these transfected cells have analysed for down regulation of VEGF A gene by RT-PCR.

Figure 4.14(b): Selection of transfected LNCaP cells. (100X magnification) PSN (pSUPERneoScramble vector), H1A1 (psNIPERDH1A1 vector) and U6A2 (psNIPERDU6A2 vector). All the three expression vector posses geneticin resistant marker. Transfected cells were gradually selected by increasing geneticin concentration.
Figure 4.14(c): Selection of transfected HeLa cells. (100X magnification) PSN (pSUPERneoScramble vector), H1A1 (psNIPERDH1A1 vector) and U6A2 (psNIPERDU6A2 vector). All the three expression vector posses geneticin resistant marker. Transfected cells were gradually selected by increasing geneticin concentration.
Figure 4.14(d): Selection of transfected KB cells. (100X magnification) PSN (pSUPERneoScramble vector), H1A1 (psNIPERDH1A1 vector) and U6A2 (psNIPERDU6A2 vector). All the three expression vector posses geneticin resistant marker. Transfected cells were gradually selected by increasing geneticin concentration.

4.9 Gene expression analysis from shRNA transfected cells

Gene silencing of VEGF A in transfected cells was confirmed by RT-PCR. Figure 4.15(a) shows cropped gel picture of amplified PCR product of VEGF A, GAPDH and 18s gene from normal, pSUPERneoScramble, psNIPERDH1A1 and psNIPERDU6A2 transfected A549 cells. It also shows graph of relative gene expression of VEGF A normalize with GAPDH and 18s. It can be visualized from figure 4.15(a) that gene expression of VEGF A has not changed in pSUPERneoScramble transfected cells. Gene expression of VEGF A has been reduced significantly in case of shRNA vectors, psNIPERH1A1 and psNIPERDU6A2 in A549 cells. Additonally, silencing of VEGF A mRNA was more in case of psNIPERDU6A2 transfected A549 cells as compared to psNIPERDH1A1. These results suggested that shRNA 2 was more
potent than shRNA 1 in VEGF A downregulation. Hence, psNIPERDU6A2 was selected for the subsequent *in vitro* and *in vivo* delivery.

**Figure 4.15(a): RT-PCR and gene expression analysis of VEGF A from transfected A459 cells.** A) Cropped gel picture of amplified PCR products from A549 cells 1) Normal 2) cells transfected with pSUPERneoScramble vector 3) cells transfected with psNIPERDH1A1 4) cells transfected with psNIPERDU6A2, B) Graph showing relative gene expression of VEGF A, normalize with GAPDH, C) Graph showing relative gene expression of VEGF A, normalize with 18s (*p<0.05).

Similarly, **figure 4.15(b)** shows cropped gel picture of amplified PCR product of VEGF A, GAPDH and 18s gene from normal, pSUPERneoScramble, and psNIPERDU6A2 transfected LNCaP cells. It can be observed from the gel picture that psNIPERDU6A2 has significantly reduced the gene expression of VEGF A as compared to normal expression in LNCaP cells. In contrast, there is no change in the expression of VEGF A in case of pSUPERneoScramble transfected cells. These results confirmed that active shRNA is required to silence the VEGF A gene.
Similar results were obtained for other two cell lines, HeLa and KB. Figure 4.15 (c) shows cropped gel picture of amplified PCR product of VEGF A, GAPDH and 18s gene from normal, pSUPERneoScramble, and psNIPERDU6A2 transfected HeLa cells. It can be visualized from the gel picture and corresponding graph of gene expression of VEGF A that psNIPERDU6A2 has completely silenced the gene expression of VEGF A as compared to normal and pSUPERneoScramble transfected HeLa cells. These results confirmed the successful transfection as well as validation of shRNA in HeLa cells.

Figure 4.15 (d) shows cropped gel picture of amplified PCR product of VEGF A, GAPDH and 18s gene from normal, pSUPERneoScramble, and psNIPERDU6A2 transfected KB cells. It can be observed from the gel picture that psNIPERDU6A2 has significantly reduced the gene expression of VEGF A as compared to normal pSUPERneoScramble transfected in KB cells. These results confirmed the successful transfection of psNIPERDU6A2 as well as validation of shRNA in KB cells.

Hence, all the cells have been successfully transfected with psNIPERDU6A2 which have shRNA 2 sequences against VEGF A gene. Moreover, pSUPERneoScramble, which is a backbone vector with control shRNA, could not silence the VEGF A gene in all the four cell lines. These results confirmed that active shRNA is required to silence the targeted gene and scramble shRNA has no effect on the VEGF A expression. So, psNIPERDH1A1 and psNIPERDU6A2 have been validated to cause downregulation of VEGF A gene. However, as discussed above psNIPERDU6A2 was found to be more efficient than psNIPERDH1A1, psNIPERDU6A2 was selected for in vitro and in vivo delivery. Subsequently, chemosensitivity of transfected cells was analysed by MTT assay in all the selected cell lines.
Figure 4.15(b): RT-PCR and gene expression analysis of VEGF A from transfected LNCaP cells. A) Cropped gel picture of amplified PCR products from LNCaP cells 1) Normal 2) cells transfected with pSUPERneoScramble vector 3) cells transfected with psNIPERDU6A2, B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s (*p<0.05).
Figure 4.15(c): RT-PCR and gene expression analysis of VEGF A from transfected HeLa cells. A) Cropped gel picture of amplified PCR products from HeLa cells 1) Normal cells 2) cells transfected with pSUPERneoScramble vector 3) cells transfected with psNIPERDU6A2, B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s.
Figure 4.15(d): RT-PCR and gene expression analysis of VEGF A from transfected KB cells. A) Cropped gel picture of amplified PCR products from KB cells 1) Normal cells 2) cells transfected with pSUPERneoScramble vector 3) cells transfected with psNIPERDU6A2, B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s (*p<0.05).

4.10 Chemosensitivity assay

MTT assay was used to investigate chemosensitivity and cell proliferation in normal and psNIPERDU6A2 transfected cells after treatment with doxorubicin. Figure 4.16 (a-d) shows graphs of mean percentage viability against log concentration of doxorubicin for A549, LNCaP, HeLa and KB cells, respectively. It was observed that mean percentage viability decreased significantly in all the cell lines transfected with psNIPERDU6A2 as compared to normal cells. It also reduced the IC\textsubscript{50} values for all the cell lines as compared to normal cells. IC\textsubscript{50} value reduced from 360.99 nM ± 8.76 to 261.925 nM ± 10.21 in A549 cells. IC\textsubscript{50} value reduced from 260.5 nM ± 3.53 to 183.895 nM ± 8.59 in LNCaP cells. Similarly, in Hela cells.
IC₅₀ value decreased from 257.03 nM ± 0 to 198.145 nM ± 15.75. In KB cells, IC₅₀ value decreased from 326.89 nM ± 5.99 to 184.975 nM ± 10.11. These results indicates that in vitro downregulation of VEGF A gene by psNIPERDU6A2 increased the chemosensitivity in all four selected cell lines. It also enhanced the doxorubicin induced cytotoxicity. These results suggested that downregulation of VEGF A gene increased chemosensitivity to doxorubicin, thus reducing the dose of doxorubicin required to kill the cells. In the next step, psNIPERDU6A2 was packaged in the minicells for its in vitro delivery in selected cell lines.

Figure 4.16 (a): Chemosensitivity assay in A549 cells. Graph of mean %viability against log concentration of doxorubicin for (i) normal and (ii) psNIPERDU6A2 transfected A549 cells. (iii) Mean IC₅₀ value (*p<0.05).
Figure 4.16 (b): Chemosensitivity assay in LNCaP cells. Graph of mean %viability against log concentration of doxorubicin for (i) normal and (ii) psNIPERDU6A2 transfected LNCaP cells. (iii) Mean $IC_{50}$ value (*p<0.05).

Figure 4.16 (c): Chemosensitivity assay in HeLa cells. Graph of mean %viability against log concentration of doxorubicin for (i) normal and (ii) psNIPERDU6A2 transfected HeLa cells. (iii) Mean $IC_{50}$ value (*p<0.05).
Figure 4.16 (d): Chemosensitivity assay in KB cells. Graph of mean %viability against log concentration of doxorubicin for (i) normal and (ii) psNIPERDU6A2 transfected KB cells. (iii) Mean IC\textsubscript{50} value (*p<0.05).

4.11 Transformation of E.coli PB114 with shRNA vectors

pSUPERneoScramble and psNIPERDU6A2 were transformed in E.coli PB114 parent cells in order to incorporate these expression vectors into the minicells. Figure 4.17 shows results of E.coli PB114 transformation with pSUPERneoScramble and psNIPERDU6A2. It shows LA plates namely A) negative control, B) positive control, C) E.coli PB114 transformed with pSUPERneoScramble, D) E.coli PB114 transformed with psNIPERDU6A2. As discussed earlier, both the vector possesses ampicillin resistant gene. So, only transformed cells grow in the presence of ampicillin. Figure 4.17 (C, D) shows E.coli PB114 cells transformed with pSUPERneoScramble and psNIPERDU6A2, respectively. E.coli PB114 was successfully transformed with pSUPERneoScramble and psNIPERDU6A2. In the next step, pSUPERneoScramble and psNIPERDU6A2 incorporated minicells were purified from the parent cells by ceftriaxone lysis method.
Figure 4.17: Transformation of *E.coli* PB114 with shRNA vectors. *Transformation of E.coli PB114 with pSUPERneoScramble and psNIPERDU6A2 plasmid. LA plates showing the a) negative control, b) positive control, c) E.coli PB114 transformed cells with pSUPERneoScramble, d) E.coli PB114 transformed cells with psNIPERDU6A2.*

4.12 Purification of minicells from shRNA transformed *E.coli* PB114

Minicells have been successfully purified from pSUPERneoScramble and psNIPERDU6A2 transformed *E.coli* PB-114 by a combination of ceftriaxone lysis and filtration. Purity of these minicells has been confirmed by light microscopy as well as by plating on LA plates. The purity was also checked by incubating purified minicells in fluid thioglycolate medium for 14 days. Growth was not observed in any of the medium thus confirming the purity of these minicells. Subsequently plasmid DNA was isolated from these minicells to confirm the presence of pSUPERneoScramble and psNIPERDU6A2 in minicells.
4.13 Isolation of shRNA vectors from purified minicells

Figure 4.18 shows the gel electrophoresis of isolated plasmid DNA along with purified plasmid DNA. Both the isolated plasmid shows corresponding bands with purified standard pSUPERneoScramble and psNIPERDU6A2 plasmid DNA. It is confirmed that both the plasmid DNA have been segregated into the minicells and hence both the plasmid DNA were present in the minicells. These minicells have been conjugated with folic acid for active targeting to cancer cells.

Figure 4.18: Isolation of shRNA vectors from purified minicells. Gel electrophoresis of isolated psNIPERDU6A2 and pSUPERneoScramble from minicells. Gel showing a) 1) Isolated psNIPERDU6A2 from minicells 2) Purified psNIPERDU6A2, b) 1) Isolated pSUPERneoScramble from minicells 2) Purified pSUPERneoScramble. Bands of isolated plasmid corresponds to standard purified plasmids which confirmed the presence of psNIPERDU6A2 and pSUPERneoScramble plasmid into the minicells.
4.14 Quantitation of shRNA vector copy number

Mean plasmid copy number of pSUPERneoScramble was found to be 145±6 per minicell whereas mean plasmid copy number of psNIPERDU6A2 was found to be 113±8. Copy number obtained for the both plasmid were quite high and were in agreement with the previous report (MacDiarmid et. al., 2009).

4.15 Folic acid conjugation

Folic acid was conjugated with bacterial minicells’ surface protein for active targeting to folate receptor present on cancer cells. Folic acid conjugation was performed using EDC and sulfo-NHS coupling agents. EDC and sulfo-NHS are commonly used water soluble coupling agents which activate carboxyl groups to react with primary amine and form covalent amide bond.

![Figure 4.19: Folic acid conjugation. Schematic representation of folic acid conjugation with psNIPERDU6A2 packaged minicells.](image)
Figure 4.19 shows schematic representation of folic acid conjugation on minicells. EDC react with γ carboxyl group of folic acid to form O-acylisourea intermediate which reacts with free amine present on the minicell surface to form amide bond. However, O-acylisourea intermediate is highly unstable and quickly hydrolyse to form isourea. But presence of sulfo-NHS in the reaction reacts with γ carboxyl group of folic acid to produce amine reactive sulfo-NHS ester. This in turn reacts with free amine present on the minicells surface proteins to form amide bond and produce folic acid conjugated minicells. Addition of DMAP in the reaction increase the yield of folic acid conjugated minicells.

Successful conjugation of folic acid can be qualitatively confirmed by the permanent change in color of minicell pellet. Folic acid imparts its yellow color on minicells after the successful conjugation. Figure 4.20, showed pictures of minicell pellets, before and after folic acid conjugation reaction. It can be observed from the picture that minicell pellets turned yellow in color after the folic acid conjugation. Moreover, this yellow color did not fade out even after repeated washing with PBS. This confirms the folic acid conjugation on the minicells.

Figure 4.20: Minicell pellet. a) Before folic acid conjugation reaction and b) After folic acid conjugation reaction. Yellow colour of minicell pellet indicates successful folic acid conjugation. (Minicell pellet indicated with arrow).
Subsequently, minicells were visualized under microscope to observe the morphology. Microscopic observation revealed that minicells were intact after the reaction. After that, minicells number was calculated by spectrophotometric method as described earlier (section: 3.5). There was a marginal change in optical density (A600) after folic acid conjugation which suggests that conjugation reaction has no detrimental effect on minicells. Eventually, TEM was performed to observe detailed morphology of folic acid conjugated minicells. TEM also showed that minicells were morphologically similar with unconjugated minicells with well characterized cell wall and cell membrane (figure 4.21). Hence, folic acid conjugation was successfully accomplished. Moreover, it has no adverse effect on minicells. These minicells were used for the in vitro and in vivo targeted delivery of psNIPERDU6A2.

![TEM images before and after folic acid conjugation](image)

**Figure 4.21: TEM images before and after folic acid conjugation. TEM images A) before folic acid conjugation B) after folic acid conjugation (20000X magnification). Minicells are indicated with arrows.**

**4.15.1 Quantification of folic acid conjugation**

Amount of folic acid conjugated was calculated by quantifying folic acid at the start and end of the reaction. Amount of folic acid was calculated from the calibration curve shown in the figure 4.22. Amount of folic acid conjugated was found to be 1972.733µg ±112 ~ 2.0 mg/10^10 minicells.
4.15.2 Measurement of size and zeta potential of folic acid conjugated minicells

Average size of folic acid conjugated minicells was found to be 543.2 nm (figure 4.23) whereas zeta potential was found to be -21 mV. The average size obtained for folic acid conjugated minicells is ideal for the tumor targeting drug delivery system.

The size is quite large to cross the blood brain barrier. Moreover, large size also prevents minicells to be filtered through the glomerulus in the kidney (Elnakat & Ratnam 2004). Hence, it avoids non specific targeting of shRNA. Moreover, zeta potential obtained is also in range (-30 to +30 mV) for the ideal nano particle based drug delivery system. In the next step, folic acid conjugated minicells were delivered *in vitro* in the selected cancer cell lines to observe it uptake.
4.16 In vitro delivery of folic acid conjugated minicells

In vitro delivery of folic acid conjugated minicells was studied in order to confirm its uptake in folic receptor positive cell lines. All the selected cell lines have been treated with FITC loaded $10^{10}$ to $10^6$ folic acid conjugated minicells for 1 hour and uptake was visualized in fluorescence microscope. Figure 4.24 shows (A) fluorescence image and (B) phase contrast image of all selected cell lines after in vitro delivery. It can be observed from the figure 4.24 that, there was a very minute amount of minicells uptake in case of A549 cells. A549 cells express very less amount of folate receptor hence there was very less amount of minicells uptake in these cells. In contrast, KB cells have the highest amount of folate receptor expression and showed large amount of minicells uptake. Hela cells have moderate amount of folate receptor expression and corresponding minicells uptake. LNCaP cells also express moderate amount of folate receptor. The large amount of minicells uptake was due to PSMA (prostate specific membrane antigen) which uniquely overexpress by LNCaP cells. Uptake of folate conjugated nanoparticles in LNCaP cells can be attributed to PSMA receptors (Xiang et. al., 2013). Thus, large amount of minicells uptake in LNCaP cells can be attributed to folate receptor as well as expression of PSMA. Additionally, in all the folate receptor positive cell lines uptake of minicells was concentration dependent. The cells treated with FITC at a concentration of $10^{10}$ showed highest number of minicells uptake as compared to the
concentration of $10^6$. The uptake of minicells occurred by receptor mediated endocytosis. However, uptake mechanism needs to be studied at low temperature (4°C) and in the presence of free folic acid in order to confirm this hypothesis.

Figure 4.24: *In vitro* delivery of folic acid conjugated minicells. *Microscopy image of (a) A549 and (b) LNCaP cells after delivery of folic acid conjugated minicells, A) Fluorescence image B) Phase contrast image (400X magnification).*
Figure 4.24: *In vitro* delivery of folic acid conjugated minicells. Microscopy image of (c) HeLa and (b) KB cells after delivery of folic acid conjugated minicells, A) Fluorescence image B) Phase contrast image (400X magnification).
4.16.1 Mechanism of minicell uptake

Uptake mechanism of folic acid conjugated minicells was studied by delivering minicells with and without folic acid conjugation. In the third group folic acid conjugated minicells have been delivered at 4°C. Eventually in the last group folic acid conjugated minicells have been delivered in presence of free folic acid to observe the delivery in presence of free folic acid. It can be visualized from the Figure 4.26 that in all the folate receptor positive cell lines such as LNCaP, HeLa and KB there was a large amount of minicells uptake when incubated at 37°C. In contrast, there was no uptake of minicells in any of the cell lines when incubated at 4°C. Similarly, when minicells were delivered without targeting moiety, i.e folic acid, there was no uptake in any of the cell line. However, delivery of folic acid conjugated minicells in presence of free folic resulted in significant decrease of the uptake of folic acid conjugated minicells in LNCaP, HeLa and KB cell lines.
Receptor mediated endocytosis occurs at 37°C and stops at 4°C. Furthermore, receptor mediated endocytosis do not occurs without the specific ligand of that receptor. Additionally, in the presence of free ligand, competition arise between ligand conjugated nanoparticle and free ligand for the uptake. In this study, folic acid conjugated minicells were not uptaken by cells at 4°C. Similar result was observed when minicells were delivered without targeting moiety. Uptake of folic acid conjugated minicells reduced in presence of free folic acid. These results suggest that minicells taken up by folate receptor overexpressing cells by receptor mediated endocytosis. However, quantification of minicells uptake would give clear indication about the amount of minicells uptaken.

**Figure 4.26: Mechanism of minicells uptake.** *Microscopy image of (a) A549 and (b) LNCaP, cells after delivery FITC loaded minicells 1) \( \text{FA} \) minicells\(_{\text{pNIPERDU6A2}} \) at 37°C 2) minicells\(_{\text{pNIPERDU6A2}} \) at 37°C, 3) \( \text{FA} \) minicells\(_{\text{pNIPERDU6A2}} \) at 4°C 4) \( \text{FA} \) minicells\(_{\text{pNIPERDU6A2}} \) with excess of free folic acid at 37°C A) Fluorescence image B) Phase contrast image (400X magnification).*
Figure 4.26: Mechanism of minicells uptake. Microscopic image of (c) HeLa and (d) KB, cells after delivery FITC loaded minicells 1) $^{FA}$minicells$_{psNIPERDU6A2}$ at 37°C 2) minicells$_{psNIPERDU6A2}$ at 37°C, 3) $^{FA}$minicells$_{psNIPERDU6A2}$ at 4°C 4) $^{FA}$minicells$_{psNIPERDU6A2}$ with excess of free folic acid at 37°C A) Fluorescence image B) Phase contrast image (400X magnification).

4.16.2 Quantification minicells uptake by fluorimetry

Minicells uptake was quantified in KB and A549 cells after the *in vitro* delivery of FITC loaded $^{FA}$minicells$_{psNIPERDU6A2}$ in presence and absence of free folic acid. Minicell uptake was quantified in different groups by fluorimetry and represented per milligram of protein. Primarily, minicell uptake was quantified in two cell lines i.e KB (folate receptor positive) and A549 (folate receptor negative). It can be seen from Figure 4.27 that at 37°C uptake of $^{FA}$minicells$_{psNIPERDU6A2}$ was highest (1290 mV/mg protein) in KB cells. But when free folic acid was added, uptake reduced significantly (432 mV/mg protein). In other two groups viz. cell incubated with minicells$_{psNIPERDU6A2}$ at 37°C and with $^{FA}$minicells$_{psNIPERDU6A2}$ at 4°C, no fluorescence was detected. Whereas in A549 cells, there was very minute uptake of
minicells\textsubscript{psNIPERDU6A2} at 37°C (149 mV/mg protein). Similarly, in other groups fluorescence was undetectable. These results were in agreement with the \textit{in vitro} minicells uptake study and confirmed that uptake of minicells was through receptor mediated endocytosis.

Figure 4.27: Quantification of minicells uptake by fluorimetry. \textit{Graph showing mean fluorescence/mg of protein in different groups (\textit{*}, \textit{**p}<0.05).}

4.17 \textit{In vitro} delivery of \textsuperscript{FA}minicells\textsubscript{pRNA}\textsubscript{T}

In the previous studies, we studied and confirmed the receptor mediated endocytosis of folic acid conjugated minicells. The objective of this experiment was to study gene expression after the delivery of folic acid conjugated minicells. To study this pRNA\textsubscript{T} expression vector was packaged in minicells. Folic acid was conjugated on these minicells and subsequently delivered to all selected cell lines. pRNA\textsubscript{T} has GFP as a reporter gene. So cells having this vector express GFP. It can be observed from the \textbf{figure 4.28} that A549 cells have hardly one or two GFP positive cells per field after delivery of \textsuperscript{FA}minicells\textsubscript{pRNA}\textsubscript{T} which are indicated by arrows. In contrast, other cell lines such as LNCaP, HeLa and KB large number of GFP positive cells were present per field. Among these cell lines, GFP expression was found to be
highest in KB cells. A549 cells showed very low level of folate receptor expression; hence folic acid conjugated minicells could not deliver pRNAT into these cells, efficiently. On the contrary, LNCaP, HeLa and KB expressed high level of folate receptor and showed large number of GFP positive cells per field. These results were also in agreement with other *in vitro* minicell delivery studies. Hence, from this experiment it can be inferred that folic acid conjugated minicells can efficiently deliver packaged expression vector, which later on expressed the transgene.

**Figure 4.28 (a):** *In vitro* delivery of $^{FA}_{\text{minicells}}p_{\text{RNAT}}$ in A549 cells. *In vitro* gene expression in A549 cell lines after $^{FA}_{\text{minicells}}p_{\text{RNAT}}$ delivery. Microscopy image of A549 cells after $^{FA}_{\text{minicells}}p_{\text{RNAT}}$ delivery. A) Fluorescence image B) Phase contrast image (100X magnification) Cells expressing GFP are indicated with arrows.
Figure 4.28 (b): *In vitro delivery of* $\text{FA}_{\text{minicells}}{p}_{\text{pRNA}}$ *in LNCaP cells.* *In vitro gene expression in LNCaP cell lines after* $\text{FA}_{\text{minicells}}{p}_{\text{pRNA}}$ *delivery. Microscopy image of LNCaP cells after* $\text{FA}_{\text{minicells}}{p}_{\text{pRNA}}$ *delivery. A) Fluorescence image B) Phase contrast image (100X magnification).

Figure 4.28 (c): *In vitro delivery of* $\text{FA}_{\text{minicells}}{p}_{\text{pRNA}}$ *in HeLa cells.* *In vitro gene expression in HeLa cell lines after* $\text{FA}_{\text{minicells}}{p}_{\text{pRNA}}$ *delivery. Microscopy image of HeLa cells after* $\text{FA}_{\text{minicells}}{p}_{\text{pRNA}}$ *delivery. A) Fluorescence image B) Phase contrast image (100X magnification).
Figure 4.28 (d): *In vitro* delivery of $^{FA}$minicells$_{pRNAT}$ in HeLa cells. *In vitro* gene expression in KB cell lines after $^{FA}$minicells$_{pRNAT}$ delivery. Microscopy image of KB cells after $^{FA}$minicells$_{pRNAT}$ delivery. A) Fluorescence image B) Phase contrast image (100X magnification).

4.18 *In vitro* delivery of $^{FA}$minicells$_{psNIPERDU6A2}$ and gene expression

In this final *in vitro* experiment, $^{FA}$minicells$_{psNIPERDU6A2}$ were delivered in all four selected cell lines and expression of VEGF A was analysed. Figure 4.29(a-d) shows results of gene expression analysis by RT-PCR after *in vitro* delivery of 1) $^{FA}$minicells$_{Scramble}$, 2) minicells$_{psNIPERDU6A2}$, 3) $^{FA}$minicells$_{psNIPERDU6A2}$. It can be visualized from figure 4.29(a) that expression of VEGF A did not change after the delivery in any of the group which is in harmony with minicell uptake study in A549 cell line. On the other hand, expression of VEGF A decreases significantly in $^{FA}$minicells$_{psNIPERDU6A2}$ when compared with other two groups in remaining cell lines (figure 4.29 (b-d)).
Figure 4.29(a): *In vitro* delivery of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ in A549 cells. RT-PCR and gene expression analysis of VEGF A from A549 cells after in vitro delivery of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ A) Cropped gel picture of amplified PCR products from A549 cells 1) $^{FA}_{\text{minicellsScramble}}$, 2) $^{FA}_{\text{minicellspsNIPERDU6A2}}$, 3) $^{FA}_{\text{minicellspsNIPERDU6A2}}$ B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s.
Figure 4.29(b): \textit{In vitro} delivery of $^{\text{FA}}_{\text{minicellsp}_{\text{psNIPERDU6A2}}}$ in LNCaP cells. RT-PCR and gene expression analysis of VEGF A from LNCaP cells after in vitro delivery of $^{\text{FA}}_{\text{minicellsp}_{\text{psNIPERDU6A2}}}$ A) Cropped gel picture of amplified PCR products from LNCaP cells 1) $^{\text{FA}}_{\text{minicellss}_{\text{Scramble}}}$, 2)minicellsp_{psNIPERDU6A2} 3)$^{\text{FA}}_{\text{minicellsp}_{\text{psNIPERDU6A2}}}$ B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s (*p<0.05).
Figure 4.29(c): In vitro delivery of $^{FA}\text{minicell}_{p\text{NIPERDU6A2}}$ in HeLa cells. RT-PCR and gene expression analysis of VEGF A from HeLa cells after in vitro delivery of $^{FA}\text{minicell}_{p\text{NIPERDU6A2}}$ A) Cropped gel picture of amplified PCR products from HeLa cells 1) $^{FA}\text{minicell}_{\text{Scramble}},$ 2) $^{FA}\text{minicell}_{p\text{NIPERDU6A2}},$ 3) $^{FA}\text{minicell}_{p\text{NIPERDU6A2}}$ B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s (*p<0.05).
Figure 4.29(d): In vitro delivery of $^{FA_{\text{minicell}}}_{ps_{\text{NIPERDU6A2}}}$ in KB cells. RT-PCR and gene expression analysis of VEGF A from KB cells after in vitro delivery of $^{FA_{\text{minicell}}}_{ps_{\text{NIPERDU6A2}}}$ A) Cropped gel picture of amplified PCR products from KB cells 1) $^{FA_{\text{minicell}}}_{Scramble}$, 2) $^{FA_{\text{minicell}}}_{ps_{\text{NIPERDU6A2}}}$, 3) $^{FA_{\text{minicell}}}_{ps_{\text{NIPERDU6A2}}}$ B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s ($^{*}p<0.05$).
These results suggest that uptake of shRNA packaged minicells was dependent on folate receptor which overexpress in LNCaP, HeLa and KB cells. Whereas in A549 cells (negative control), \(^{14}\text{FA}\)minicells\(_{\text{psNIPERDU6A2}}\) could not deliver psNIPERDU6A2 due to the low level of folate receptor expression. The delivery of pSUPERneoScramble did not change the expression of VEGF A in any of the selected cell lines. This indicated that active shRNA must be delivered to silence the targeted gene. In this way, folic acid conjugated minicells have effectively delivered psNIPERDU6A2 in folate receptor positive cell lines through receptor mediated endocytosis. Moreover, psNIPERDU6A2 has successfully silenced the VEGF A gene. After successful \textit{in vitro} delivery, \textit{in vivo} delivery of \(^{14}\text{FA}\)minicells\(_{\text{psNIPERDU6A2}}\) was checked in tumor xenograft model.

### 4.19 Development of immunocompromised mice

In \textit{vivo} experiments were initiated with the development of immunocompromised mice to develop tumor xenograft model. Nude mice are widely employed to develop tumor xenograft and subsequent \textit{in vivo} study of any anticancer therapeutics. In spite of sole advantage of easy and efficient xenograft development, nude mice have several disadvantages \textit{viz.} difficulty in transportation and maintenance and high mortality rate. Due to such reasons, we have focused on immunosuppression protocol to develop tumor xenograft in C57 BL6 mice. Cyclosporine and ketoconazole were used to induce immunosuppresion. Detailed protocol is mentioned in the previous chapter (section: 3.19). \textbf{Figure 4.30} shows graph of mean WBC and lymphocyte count in different group of mice at the end of treatment. From the graph, it can be seen that efficient immunosuppression was found in the animals of group 6, which was administered with cyclosporine (30 mg/kg) and ketoconazole (10 mg/kg) when compared to control animals. Total WBC and lymphocyte counts were significantly decreased in animals of group 6 when compared to animals of control animals. Hence, animals from group 6 were used for the tumor xenograft development.
Figure 4.30: Mean WBC and lymphocyte count. Graph showing total WBC and lymphocyte count of different groups of mice at the end of immunosuppressive drug treatment (*p<0.05).

4.19.1 Development of tumor xenograft in immunocompromised mice

Cyclophosphamide was administered to the immunocompromised mice in order to eradicate neutrophyls and residual lymphocytes. Cancer cells were injected subcutaneously into the shoulder blade of these animals and tumor growth was observed. After one week of injection, palpable tumor mass was found in every animals. As shown in the Figure 4.31, tumor was developed at the site of injection. Hence, tumor xenograft model was successfully developed. Moreover, xenograft was maintained for more that 60 days. Presence of tumor was confirmed by histopathology. Tumors were excised, sectioned and stained with standard haematoxylin and eosin. Figure 4.32 shows haematoxylin and eosin stained section of KB and LNCaP xenograft. Both the section shows cells with large nucleus with scanty cytoplasm which are characteristic of malignant cells. Hence, it was confirmed that developed xenograft was not a simple hyperplasia but was malignant and invasive tumor. This xenograft model was used to study \textit{in vivo} delivery of FA\textit{minicell}_{PSNIPERDU6A2}. 
Figure 4.31: Tumor xenograft. Immunocompromised C57 BL6 mice bearing tumor xenograft as indicated by circles.

Figure 4.32: Histopathology of tumor xenograft. Photograph of histopathological plates of (a) KB and, (b) LNCaP tumor xenograft. Tumor cells having large nucleus and scanty cytoplasm, are indicated by arrows (At 400X magnification).

4.20 *In vivo* delivery of $^\text{FA}_{\text{minicell}}^\text{PSNIPERDU6A2}$

Figure 4.33, 4.34 and 4.35 shows photographs of mice bearing tumor xenograft from each group at start and end of the treatment from A549, LNCaP and KB xenograft, respectively. Figure 4.36 shows graphs of mean tumor volume at every week during the treatment. Additionally, mean tumor volume for A549, LNCaP and KB xenograft is also given in Table...
4.2, 4.3 and 4.4, respectively. It can be observed from the Figure 4.32 that in case of A549 xenograft there was a gradual increase in the tumor volume till the end of treatment in all four groups. Whereas in case of LNCaP and KB xenograft, there was a significant decrease in tumor volume in \( \text{FA minicells}_{\text{psNIPERDU6A2}} \) treated group as compared to other groups (Figure 4.33 and 4.34). Moreover, this difference in tumor volume was statistically significant.

These results were further confirmed when tumors were excised from each group of animals to observe the angiogenesis (Figure 4.37-4.39). Angiogenesis (formation of blood vessels) is denoted by arrow at the site of tumor xenograft. It can be visualized from the Figure 4.36 that in A549 xenograft, angiogenesis is clearly visible in all the groups. In contrast, in LNCaP and KB xenograft \( \text{FA minicells}_{\text{psNIPERDU6A2}} \) treated group showed reduced angiogenesis as compared to other three groups (Figure 4.37 and 4.38). Figure 4.40 shows excised tumor from each group. From figure 4.39, it can be observed that tumors were significantly reduced in \( \text{FA minicells}_{\text{psNIPERDU6A2}} \) treated LNCaP and KB xenograft bearing mice as compared to other groups.

In brief, significant tumor regression was observed only in \( \text{FA minicells}_{\text{psNIPERDU6A2}} \) LNCaP and KB xenograft. It also showed reduced angiogenesis as compared to other group. Tumor regression was not observed in any xenograft treated with \( \text{FA minicells}_{\text{Scramble}} \) or \( \text{minicells}_{\text{psNIPERDU6A2}} \). These results suggest that tumor regression can be attributed to inhibition of angiogenesis by psNIPERDU6A2 when actively targeted via folate receptors. However, expression of VEGF A is needed to be analysed. Hence, in the next step, expression of VEGF A was analysed by RT-PCR.
Figure 4.33: Mice bearing A549 tumor xenograft. A) at starting and B) end of the treatment. a) treated with saline, b) treated with $^{FA}_{\text{minicell}}_{\text{Scramble}}$, c) treated with $^{\text{minicell}}_{\text{psNIPERDU6A2}}$, d) treated with $^{FA}_{\text{minicell}}_{\text{PSNIPERDU6A2}}$. Location of tumor is indicated with arrows.
Figure 4.34: Mice bearing LNCaP tumor xenograft A) at starting and B) end of the treatment. a) treated with saline, b) treated with $^F_A$ minicellsScramble c) treated with minicells$_{pSNIPERDUS6A2}$, d) treated with $^F_A$ minicells$_{pSNIPERDUS6A2}$. Location of tumor is indicated with arrows.
Figure 4.35: Mice bearing KB tumor xenograft. A) at starting and B) end of the treatment. a) treated with saline, b) treated with $^{FA}_{ minicells_{Scramble}}$, c) treated with minicells$_{ psNIPERDU6A2}$, d) treated with $^{FA}_{ minicells_{ psNIPERDU6A2}}$. Location of tumor is indicated with arrows.
Figure 4.36: Mean tumor volume. Graphs showing mean tumor volume of a) A549, b) LNCaP, c) KB xenograft from different groups at starting and end of the treatment (n=6).
Table 4.2: Mean tumor volume in different groups of A549 xenograft.

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<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
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<tr>
<td>Saline</td>
<td>100.76</td>
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<tr>
<td>FA&lt;sup&gt;+&lt;/sup&gt;minicells</td>
<td>106.71</td>
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<tr>
<td>Scramble</td>
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<td>psNIPERDU6A2</td>
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Table 4.3: Mean tumor volume in different groups of LNCaP xenograft (*p<0.05).

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<td>Scramble</td>
<td>94.37</td>
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<tr>
<td>psNIPERDU6A2</td>
<td>95.38</td>
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Table 4.4: Mean tumor volume in different groups of KB xenograft (*p<0.05).

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<tr>
<td>Saline</td>
<td>99.95</td>
</tr>
<tr>
<td>FA&lt;sup&gt;+&lt;/sup&gt;minicells</td>
<td>97.29</td>
</tr>
<tr>
<td>Scramble</td>
<td>101</td>
</tr>
<tr>
<td>psNIPERDU6A2</td>
<td>104.56</td>
</tr>
</tbody>
</table>
Figure 4.37: Relative angiogenesis in A549 xenograft. Excised tumor showing qualitative angiogenesis in different group of mice from A549 xenograft. a) treated with saline, b) treated with $^{FA}_{\text{minicells}}$ Scramble, c) treated with $^{FA}_{\text{minicells}_{\text{PSNIPERDU6A2}}}$, d) treated with $^{FA}_{\text{minicells}_{\text{PSNIPERDU6A2}}}$. Angiogenesis is indicated with arrows.
Figure 4.38: Relative angiogenesis in LNCaP xenograft. Excised tumor showing qualitative angiogenesis in different group of mice from LNCaP xenograft. a) treated with saline, b) treated with FAminicellsScramble c) treated with minicellspsNIPERDU6A2 d) treated with FAminicellspsNIPERDU6A2. Angiogenesis is indicated with arrows whereas circle indicates reduced angiogenesis.
Figure 4.39: Relative angiogenesis in KB xenograft. Excised tumor showing qualitative angiogenesis in different group of mice from KB xenograft. a) treated with saline, b) treated with $FA_{\text{minicellsScramble}}$, c) treated with $\text{minicells}_{pSNIPERDU6A2}$, d) treated with $FA_{\text{minicellsPSNIPERDU6A2}}$. Angiogenesis is indicated with arrows whereas circle indicates reduced angiogenesis.
Figure 4.40: Excised tumors. Tumor excised from mice of each group of mice from a) A549, b) LNCaP and c) KB xenograft.
4.21 RNA isolation, RT-PCR and *In vivo* gene expression analysis

*Figure 4.41 (a-c)* shows results of gene expression analysis by RT-PCR after *in vivo* delivery of 1) saline, 2) FAminicellsScramble, 3) minicellspsNIPERDU6A2, 4) FAminicellspsNIPERDU6A2. It can be visualized from *figure 4.41 (a)* that expression of VEGF A does not change after the delivery of FAminicellspsNIPERDU6A2 in A549 xenograft which is in harmony with *in vitro* delivery. On the other hand, expression of VEGF A decreases significantly in FAminicellspsNIPERDU6A2 when compared with other groups in LNCaP and KB xenograft (Figure 4.36 b, c). Moreover, these results are in synchronization with tumor regression and reduced angiogenesis in FAminicellspsNIPERDU6A2 treated LNCaP and KB xenograft. In FAminicellspsSUPERneoScramble and minicellspsNIPERDU6A2 treated xenograft expression of VEGF A was comparable with saline treated group. These results confirmed that delivery of shRNA packaged minicells was dependent on active targeting of folate receptor which overexpress in LNCaP and KB cells. Expression of folate receptor is negligible in A549 cells (folate receptor negative cell line). Consequently, FAminicellspsNIPERDU6A2 could not deliver active shRNA subsequent gene silencing.

In can be concluded from the above results that FAminicellspsNIPERDU6A2 were successfully delivered in *in vivo* tumor xenograft which was evendent by significant regression in tumor volume in FAminicellspsNIPERDU6A2 treated LNCaP and KB xenograft. Moreover, this group also showed decrease in angiogenesis as compared to other groups. Eventually, downregulation of VEGF A gene in tumor xenograft confirmed the *in vivo* delivery of FAminicellspsNIPERDU6A2. In the final experiment, *in vivo* biodistribution of FAminicellspsNIPERDU6A2 was studied to observed distribution in other vital organ.
Figure 4.41(a): *In vivo* delivery of $^{FA}\text{minicells}_{psNIPERDU6A2}$ in A549 xenograft. RT-PCR and gene expression analysis of VEGF A from A549 xenograft after in vivo delivery of $^{FA}\text{minicells}_{psNIPERDU6A2}$ A) Cropped gel picture of amplified PCR products from A549 tumor tissue 1) Saline 2) $^{FA}\text{minicells}_{Scramble}$ 3) $\text{minicells}_{psNIPERDU6A2}$ 4) $^{FA}\text{minicells}_{psNIPERDU6A2}$ B) Relative gene expression of VEGF A, normalize with GAPDH, C) Relative gene expression of VEGF A, normalize with 18s. Here, G-1 to G-4 indicates treatment with saline, $^{FA}\text{minicells}_{Scramble}$, $\text{minicells}_{psNIPERDU6A2}$ and $^{FA}\text{minicells}_{psNIPERDU6A2}$, respectively.
Figure 4.41(b): *In vivo* delivery of $^{FA}_{minicells}psNIPERDU_{6A2}$ in LNCaP xenograft. RT-PCR and gene expression analysis of VEGF A from LNCaP xenograft after in vivo delivery of $^{FA}_{minicells}psNIPERDU_{6A2}$ A) Cropped gel picture of amplified PCR products from LNCaP tumor tissue 1) Saline 2) $^{FA}_{minicells}_{Scramble}$ 3) $^{FA}_{minicells}psNIPERDU_{6A2}$. 4) $^{FA}_{minicells}psNIPERDU_{6A2}$ B) Relative gene expression of VEGF A, normalize with GAPDH. C) Relative gene expression of VEGF A, normalize with 18s ($^{*}p<0.05$). Here, G-1 to G-4 indicates treatment with saline, $^{FA}_{minicells}_{Scramble}$, $^{FA}_{minicells}psNIPERDU_{6A2}$, and $^{FA}_{minicells}psNIPERDU_{6A2}$, respectively.
Figure 4.41(c): *In vivo* delivery of $^{FA}\text{minicells}_{psNIPERDU6A2}$ in KB xenograft. RT-PCR and gene expression analysis of VEGF A from KB xenograft after in vivo delivery of $^{FA}\text{minicells}_{psNIPERDU6A2}$

A) Cropped gel picture of amplified PCR products from KB tumor tissue 1) Saline 2) $^{FA}\text{minicells}_{Scramble}$ 3) $\text{minicells}_{psNIPERDU6A2}$ 4) $^{FA}\text{minicells}_{psNIPERDU6A2}$

B) Relative gene expression of VEGF A, normalize with GAPDH

C) Relative gene expression of VEGF A, normalize with 18s ($^*p<0.05$). Here, G-1 to G-4 indicates treatment with saline, $^{FA}\text{minicells}_{Scramble}$, $\text{minicells}_{psNIPERDU6A2}$, and $^{FA}\text{minicells}_{psNIPERDU6A2}$ respectively.
4.22 *In vivo* biodistribution study $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$

**Figure 4.42** shows fluorescence and light microscopy images of tumor and all vital organs after the intravenous delivery of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$. It can be examined from the fluorescence image that majority of fluorescence localized in the tumor followed by liver and heart. Cryosection of spleen also show little fluorescence. On the contrary, cryosection of kidney, brain, lung and skin do not show any fluorescence.

These results suggest that majority of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ were distributed in tumor tissue which is due to both passive and active targeting of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$. Tumor formed more poor and leaky blood vessels through which majority of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ diffused into the tumor tissue by passive targeting. Secondly, $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ actively targeted to tumor cell surface folate receptor by folic acid, followed by receptor engagement and endocytosis. Fluorescence observed in other organ like liver, heart and spleen was found to be in blood vessels.

The results obtained here were in agreement with in vivo delivery of $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ and gene expression analysis. Upon intravenous administration, $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ were passively targeted to tumor microenvironment through leaky blood vessels. There minicells were actively targeted to tumor cell surface by folic acid. Binding of folic acid with the folate receptors overexpress on cancer cell surface resulted in minicells endocytosis, intracellular degradation and released of packaged shRNA and FITC. Expression of folate receptor is absent in most normal tissue so $^{FA}_{\text{minicells}}_{\text{psNIPERDU6A2}}$ would not retained in normal tissue and quickly washed out. Moreover, size of folic acid conjugated minicells is large enough which prevented crossing of blood brain barrier. Furthermore, large size also prevents minicells to be filtered through the glomerulus in the kidney (Elnakat & Ratnam 2004). Hence, it avoids non specific targeting of shRNA. However, further study is required to confirm this hypothesis.
Figure 4.42: *In vivo* biodistribution study $^{\text{FA}}$minicells$_{p\text{NIPERDU6A2}}$. Microscopy image, A) fluorescence image B) light microscopy of HE stained cryosection of a) tumor, b) liver, c) lung and d) kidney after in vivo delivery of FITC loaded tumor $^{\text{FA}}$minicells$_{p\text{NIPERDU6A2}}$. (100X Magnification).
Figure 4.42: In vivo biodistribution study $^{\text{FA}}\text{minicells}_{\text{psNIPERDUGA2}}$. Microscopy image, A) fluorescence image B) light microscopy of HE stained cryosection of e) spleen, f) heart, g) heart and h) skin after in vivo delivery of FITC loaded tumor $^{\text{FA}}\text{minicells}_{\text{psNIPERDUGA2}}$ (100X Magnification).
In brief, folic acid conjugated minicells, i.e. $^{\text{FA}}$ minicells$_{\text{PstNIPERDU6A2}}$ were effectively taken up by folate receptor positive cell via receptor mediated endocytosis. *In vitro* and *in vivo* studies confirmed that, it has efficiently delivered packaged shRNA to targeted cancer cells. Moreover, after delivery, active shRNA has successfully silenced the VEGF A gene, both in cell lines as well as in animal model. These resulted in increased chemosensitivity, decreased angiogenesis and reduced tumor volume. Furthermore, these folic acid conjugated minicells were well tolerated. None of the injected mice showed any adverse reaction during the treatment. However, detailed safety study is required to confirm this claim.