Nanoparticles as a Promising Delivery System to Improve Hepatitis C Treatment
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ABSTRACT
Fluorescently labelled biodegradable polymeric nanoparticles, Rhodamine B isothiocyanate Poly(Glycerol Adipate) nanoparticles [RBITC PGA NPs] were used to investigate nanoparticles (NPs) use as a delivery system for ribavirin (RV) to improve hepatitis C treatment and overcome haemolytic anaemia due to RV uptake by red blood cells (RBCs).

RBITC PGA NPs uptake by RBCs was very low. Huh7.5 cells (as a model of liver cells) infected with hepatitis C virus (HCV) take up RBITC PGA NPs to a higher extent than non-infected cells. Therefore, RV NPs could target RV into infected liver cells and minimize its accumulation into RBCs.

INTRODUCTION
The standard therapy for chronic HCV infection is a combination of RV and PEGylated interferon. Although the future aim for therapy is direct acting antiviral agents without interferon, clinical trials have shown the benefit of RV to achieve a sustained viral response (absence of virus RNA 6 months after stopping treatment) [1]. However, RV is associated with haemolytic anaemia due to RV accumulation into RBCs [2].

If RV could be targeted into virally infected liver cells and avoid uptake into RBCs, hepatitis C treatment could be greatly improved. NPs can target liver cells [3], and RBCs are reported to have no endocytic mechanisms [4]. Although a recent publication showed some uptake of particles by RBCs [5], further investigation is essential to quantitate uptake of NPs by RBCs to evaluate the benefit of their use to improve hepatitis C treatment.

A novel bio-degradable polymer, poly(glycerol-adipate) [PGA] was used to prepare RBITC PGA NPs. These were incubated with RBCs and Huh7.5 cells (virally infected or non-infected) to investigate the uptake of NPs.

EXPERIMENTAL METHODS
RBITC-PGA NPs were prepared by the nano-precipitation method involving addition of the organic solution of polymer into an aqueous solution (HEPES buffer, 10mM, pH7.4) containing the fluorescent dye. The purified NPs (100µl, 100µg) were incubated with human plasma (100µl) for 24h to stabilise the particles and reduce aggregation in phosphate buffered saline (PBS). The NPs/plasma mixture was added to human RBCs (200µl) that were previously washed by PBS and the volume adjusted to 1ml by PBS. Samples were incubated for 1h at 37°C (RBCs + NPs, 1h) under gentle shaking and compared to zero incubation time (RBCs + NPs, zero h) and RBCs that were incubated for 1h at 37°C in absence of NPs (RBCs blank, 1h). Cells were spun down and RBCs were washed 4 times with PBS. RBCs were imaged by fluorescence microscopy.

For quantitative study, washed RBCs (20µl) were diluted by PBS to 1ml and fluorescence was measured by flow cytometry.

RBITC PGA NPs uptake by Huh7.5 cells was evaluated as follows;

Huh7.5 cells were electroporated in either the absence or presence of J6/JFH1 HCV RNA (10µg) at 220V/22ms. Cells were then incubated in Dulbecco’s Modified Eagle Medium (DMEM) [10ml, 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100µg/ml), 1% non-essential amino acids (NEAA)] for 24h at 37°C and 5% carbon dioxide. The cells (100,000/well) were transferred into 6-well plates and incubated for another 24h. Cells were washed with PBS and incubated with RBITC PGA NPs/FBS mixture (400µl each, were previously incubated together for 24h) and DMEM (3.2ml) for different time intervals (0, 0.5, 1, 2, and 4h) at 37°C and 5% carbon dioxide. After incubation, cells were washed 3 times by PBS (1ml). Cells were detached and fixed by a mixture of 4% paraformaldehyde and trypsin (1:1) followed by transfer of cells into flow cytometry tubes. Cells were labelled for virus core protein by primary antibody (mAb C7-50) and secondary antibodies (Alexa488-labelled goat anti-mouse antibody). The labelled cells were examined by confocal microscopy and quantified by flow cytometry. Cells

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were also incubated under the previous condition with an amount of free dye equivalent to the amount that might have leaked from NPs in culture medium and fluorescence was measured by flow cytometry.

Statistical analysis (Two Way ANOVA and Independent T-test) was done by SPSS version 21 at confidence interval (99.9%).

RESULTS AND DISCUSSION

RBCs were associated with some NPs uptake as shown by fluorescence microscopy (Figure 1).

![Fluorescence microscopy image of RBCs incubated with RBITC PGA NPs for 1h at 37°C; RBCs showed unexpected NPs uptake.](image1)

Figure 1: Fluorescence microscopy image of RBCs incubated with RBITC PGA NPs for 1h at 37°C; RBCs showed unexpected NPs uptake.

We next asked whether viral infection modulated NPs endocytosis by Huh7.5 cells. Uptake into virally infected and non-infected Huh7.5 cells was initially investigated by confocal microscopy (Figure 2). This showed that endocytosis of NPs occurred into both infected and non-infected cells as revealed by red fluorescence of RBITC PGA NPs taken up by cells.

![Confocal microscopy images of virally non-infected (A) and infected (B) Huh-7.5 cells incubated with RBITC PGA NPs for 4h at 37°C and 5% CO₂. Virus infection does not interfere with NPs uptake.](image2)

Figure 2: Confocal microscopy images of virally non-infected (A) and infected (B) Huh-7.5 cells incubated with RBITC PGA NPs for 4h at 37°C and 5% CO₂. Virus infection does not interfere with NPs uptake.

A quantitative uptake study using both Huh-7.5 cells and RBCs was then carried out using flow cytometry (Figure 3). NPs uptake by virally infected Huh7.5 cells (MFI=1346±75) was significantly higher (P<0.001, > 2times) than non-infected cells (MFI=645±14) at 4h. Free dye uptake was negligible after 4h and this demonstrated that fluorescence measured was due to actual NPs uptake. After 1h incubation at 37°C, although, NPs uptake by RBCs (13.7±0.3) was significantly higher (p<0.001) than RBCs + NPs (Zero h) (10.97±0.17) and background MFI (RBCs blank, 1h) (8.2±0.05), it is still negligible as compared to Huh7.5 cells.

![Flow cytometry analysis of virally infected/non-infected Huh7.5 cells and RBCs that were incubated with RBITC PGA NPs, results are average of 3 samples.](image3)

Figure 3: Flow cytometry analysis of virally infected/non-infected Huh7.5 cells and RBCs that were incubated with RBITC PGA NPs, results are average of 3 samples.

CONCLUSION

Polymeric NPs have been shown to be taken up preferentially by virally infected Huh7.5 cells. Although there is unexpectedly some uptake of NPs by RBCs, this is at a very low level. These data suggest that NPs could be considered as a useful delivery system for RV to improve hepatitis C treatment as it could minimize drug uptake by RBCs and target the drug into infected liver cells.

REFERENCES


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