A synopsis on Ph.D. work entitled

“Design and Development of PEI Coated Iron Oxide Based Theranostic Nanoparticles for Targeting Brain Tumors”

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A. Introduction

Gliomas, the intrinsic tumours of the brain, are oncological diseases for which there is currently no cure. Although recent progress has been achieved in the identification of aberrant genetic events and signaling pathways, the rate of gliomas still increases remarkably and they continue to be the cause of a disproportionate level of morbidity and mortality across individuals worldwide. Gliomas account for approximately 50% of all primary brain tumours and 80% of all malignant brain tumours in adults. Conventional surgical methods cannot completely remove the tumour cells, and an inevitable relapse always follows. Currently, cytoreductive surgery followed by combination of chemotherapy and radiotherapy is regarded as the most effective gliomas treatment. However, the surgeon encounters challenges including how to completely remove the neoplastic tissue while maximally preserve the normal neurological tissues. Consequently, a crucial challenge is to deliver therapeutic agents effectively to the tumour core and migratory cells in the infiltration zone. However, existing chemotherapy drugs fail to elicit the desired benefit and are associated with serious adverse effects, largely due to their inability to cross the BBB and untargeted accumulation in healthy tissues[1].

Concept of theranostics.

Theranostic nanomedicine is emerging as a promising therapeutic paradigm. It takes advantage of the high capacity of nanoplatforms to ferry cargo and loads onto them for both imaging and therapeutic functions. Theranostic nanoparticles that simultaneously deliver both imaging and therapeutic agents have gained significant attention for disease management in recent years. Disease management not only includes the highly specific diagnosis and treatment of the diseased cells, but also the monitoring of the drug delivery process and therapeutic efficacy. Conventional nanoparticle systems have been previously used to achieve each aspect of disease management separately. However, multiple administrations may be required to fulfil all the necessary functions, which bring concerns of patient compliance and safety. To overcome these limitations, theranostic nanoparticle systems that can perform all the aspects of disease management in a single setting have been developed over the last decade[2].

Magnetic theranostic nanoparticles.

MNPs (Magnetic nanoparticles) are composed of ferromagnetic elements such as iron, cobalt, nickel, or their oxides and alloys. MNPs made of iron oxide (magnetite Fe_3O_4 or magnemite Fe_2O_3) have been widely used as contrast agents in MRI for biological applications due to their ability to dissociate into iron and oxygen inside the body, which can safely be eliminated and utilized in metabolic and oxygen transport systems.When fabricated into nanoparticles of approximately 10 nm in diameter, iron oxide nanoparticles begin to exhibit a superparamagnetic behavior (superparamagnetic iron oxide nanoparticles, SPIONs) leading to improved dispersive properties in the absence of a magnetic field, and later guided to accumulate to the site of interest in the presence of a magnetic field, which is of great importance in targeted drug delivery applications. Gadolinium is a lanthanide element which is also used as MRI contrast agent due to its paramagnetic behaviour because of seven unpaired electrons. MNPs of gadolinium oxide and hybrid nanoparticles prepared by doping gadolinium on iron oxide have also been reported to show MRI contrast (T1 and T2) and hyperthermic behaviour in presence of magnetic field which is useful in cancer therapy[3-14].

Role of mitochondria in cancerous cells.

Prominent features of cancer cells include metabolic imbalances and enhanced resistance to mitochondrial apoptosis. The fact is that tumours rely heavily on glycolysis to meet their metabolic demands. Cancer cells often up regulate the rate-limiting processes and enzymes of glycolysis, including glucose transporters, for instance as a result of the constitutive signalling through the Akt pathway or as a result of the expression of oncogenes. Cancer-specific mitochondrial alterations and bioenergetics may be taken advantage for the development of two novel classes of antineoplastic agents. A first approach would target glycolysis and/or revert the Warburg phenomenon, whereas a second approach would aim at inducing apoptosis by targeting mitochondrial proteins and membranes. Many ligands like PEI, peptides, chemical agents tripolyphosphate which have lipophilic nature have been used to target mitochondria.[15-16]

Lenalidomide

Lenalidomide (Mol. wt. 259.261 g/mol) is a BCS class III thalidomide analogue with immunomodulatory and anti-angiogenic properties that include altering cytokine production, activating T cells, and augmenting natural killer cell function. The oral dose of drug is 2.5 -25 mg/day and is rapidly absorbed after oral administration has short half-life of 3 hrs. requiring frequent dosing as the Cmax reduces upto 36% when drug is co-administered with food. The adverse effects of drug include cytopenia, abdominal pain, nausea, vomiting, diarrhoea, rash, infections, atrial fibrillation, congestive heart failure, myocardial infarction, chest pain, weakness, hypotension, hypercalcemia, hyperglycaemia, pain, dizziness, mental status changes, syncope, renal failure, dyspnoea, pleural effusion, and thrombotic events. Lenalidomide is approved by the U.S. Food and Drug Administration (FDA) for single-agent treatment of myelodysplastic syndromes associated with a 5q deletion and as a combination therapy with dexamethasone for the treatment of multiple myeloma. The drug is under Phase II clinical trials for treatment of glioblastoma multiforme.[17-18]

B. Aims, Objectives, Rationale and Hypothesis

Aim: The aim of present investigation is to develop lanthanide doped polyethyleneimine coated iron oxide based theranostic nanoparticles which will serve as MRI contrasting agent(diagnostic agent) using modified PEI(mitochondrial targeting agent) with therapeutic agents for treatment of brain tumour.

Objectives:

1. Synthesis of magnetic metallic core with Gd doping.
2. Coating of magnetic metallic core with drug.
3. Modification of PEI.
4. Secondary coating of modified PEI on drug loaded core.
5. Evaluation of final formulation by *in vitro, ex vivo* and *in vivo* studies.

Rationale

The formulated theranostic nanoparticles will have the following advantages:

- ✓ The metallic core will be magnetic in nature due to iron oxide which will be useful in targeting by external magnetic fields.
- ✓ The lanthanide doping will provide diagnostic capabilities by T1 and T2 contrast.
- ✓ The metal core shows hyperthermia in presence of magnetic field which is useful for treatment of cancer.
- ✓ The drug coating on the metal core will provide therapeutic benefit.
- ✓ Modified PEI coating will assist in enhanced cellular uptake and mitochondrial targeting.

Hypothesis

It can be hypothesized that the formulated PEI coated drug loaded iron oxide nanoparticles will provide diagnostic capabilities in MRI imaging and also give added therapeutic benefit from drug loading on the nanoparticles with mitochondrial targeting. The formulated nanoparticles can also be targeted to the tumour site with magnetic field thereby reducing the side effects to non-target organs.

C. Highlights of research work completed

1. Synthesis of lanthanide doped metal core.

The following methods were explored for preparation of lanthanide doped metal core:

a. Co-precipitation method.
b. Borohydride reduction.
c. Tannin synthesis.
d. Solvothermal method.
e. Polyol method.

a. Co-precipitation method.

In this method the chloride salts of iron and gadolinium were dissolved in aqueous medium and precipitated using alkaline medium. The precursor medium was injected slowly into the alkaline medium using a syringe while the alkaline medium was stirred using a magnetic stirrer. After precipitation the nanoparticles were separated by magnet.[19-20] The lowest particle size achieved was 144 nm by this method but the control over particle size was poor.

b. Borohydride reduction method.

The method involves use of sodium borohydride which is a reducing agent. The iron and gadolinium precursors were dissolved in aqueous phase and injected into solution containing sodium borohydride while stirring.[21] The nanoparticles were obtained by magnetic separation of the medium. The method was spontaneous and there was no control over particle size. The lowest particle size achieved by this method was 121.1nm.

c. Tannin synthesis.

In this method the iron and gadolinium precursors were dissolved in aqueous phase followed by addition of tannic acid solution 1mg/ml.[22] Upon addition of tannic acid solution the nanoparticles were formed with very large particle size >300nm which were non-magnetic.

d. Solvothermal method.

It involved use of high boiling solvents like 1-octadecene, phenyl ether and diphenyl ether having boiling point >250^{o}C.The iron and gadolinium acetylacetonate precursors were dissolved in high boiling solvents and heated at temperatures above 250^{o}C for decomposition of precursors in inert environment with stirring to yield magnetic nanoparticles.[23, 24] The problem with this method was phase transfer to aqueous medium which reduced yield and was tedious procedure. The lowest particle size achieved was 199.8nm.

e. Polyol method.

In this method the acetylacetonate precursors were heated with glycols which are reducing agents and stabilizers.[25] As washing of magnetic nanoparticles was single step procedure and control over the reaction was sufficient, this method was selected and optimization was done for reaction temperature and reaction time. The lowest particle size achieved was 87.86 nm.

2. Optimization of Gd: Fe ratio/MRI Imaging.

The Gd: Fe precursor ration was optimised by the contrast imaging using 1.5T MRI.The magnetic nanoparticles were prepared by various ratios and were injected into MRI phantoms. The optimised ratio of precursor was selected for further work.[26, 27]

3. Stabilization of magnetic core.

The purified magnetic core was functionalised using citric acid for improved stability and aqueous dispersibility.[28]

4. Coating of drug on magnetic metal core.

Direct conjugation on the drug core was attempted but poor conjugation of drug was observed. The drug lenalidomide was coated on the magnetic metallic core using nanoprecipitation technique. The drug lenalidomide and polymer were dissolved in organic phase and magnetic nanoparticles were dispersed in aqueous phase along with the stabiliser. The organic phase was injected into aqueous phase and allowed to evaporate under stirring.[29]

5. Optimization of drug coating.

The drug coating was enhanced by optimization of process parameters like polymer: payload ratio, type of surfactant, concentration of surfactant.

6. Development and validation of analytical methods for estimation of drug

The UV spectroscopic method was developed and validated as per ICH guidelines Q2 (R1) for estimation of drug in the formulation and *in vitro* drug release using organic solvent and phosphate buffer pH 7.4 and 5.5. [30]

7. Synthesis of modified PEI.

Three analogues of PEI were synthesized using folic acid, triphenylphosphonium and its combination using carboodiimide chemistry. The analogues were purified by dialysis and lyophilised to get solid powder for prolonged further use.[31-33]

8. Characterization of modified PEI.

The synthesized analogues were characterised by physical parameters and the degree of modification was determined by florescamine assay. It was found that the florescence intensity was lower in modified PEI analogues as compared to unmodified PEI (control) which indicates that modification was successful.[34]

9. Coating of modified PEI on drug coated metallic core.

The secondary coating of modified PEI was done on the drug coated core by electrostatic interaction. The zeta potential was measured before and after the coating. The change from negative zeta potential value to the positive zeta potential value indicated the coating of modified PEI.

10. Transmission electron microscopy (TEM).

Transmission electron microscopy of final formulation and uncoated magnetic core was done to see the size and morphology of nanoparticles. It was found that the uncoated magnetic core had size <10 nm while the coated core had size >50nm with semi spherical shape.[35]

11. Energy dispersive X- ray spectroscopy (EDAX)

EDAX was done to know the composition of metals in the metallic core it was found that iron was abundant as compared to gadolinium which was doped on iron oxide.[35]

12. *In vitro* drug release.

In vitro drug release was performed in phosphate buffer 5.5 and 7.4 in receptor compartment while 1 mg equivalent free drug and formulation were filled in dialysis bag which acted as donor compartment. It was found that nanoparticles showed sustained release as compared to free drug.[36]

13. Procurement and sub culturing of cell line.

The U87MG glioblastoma cell line was procured from NCCS, Pune and sub cultured in culture flasks using recommended growth medium.

14. MTT assay.

Various concentrations of free drug, plain core and drug coated nanoparticles were prepared in cell culture media under aseptic conditions. The cells were cultured in 96 well plates followed by treatment by various aliquots for 24 hrs. which was followed by addition of MTT dye to check cell viability.[37]

15. ATP levels.

The ATP levels were determined using ATP determination kit. The U87MG cells were cultured in 6 well plates and treated with aliquots of formulation. After treatment for 24 hrs. Cells were removed, lysed and treated with master mix. The florescence intensity was measured by multiplate reader. It was found that with increase in concentration of the drug the ATP levels were decreasing after initial increase due to stress as compared to control.[38]

16. Mitochondrial membrane potential.

The mitochondrial membrane potential was determined by using Tetramethylrhodamine, Methyl Ester, and Perchlorate (TMRM) kit. The U87MG cells were treated with aliquots of formulation after treatment period the cells were stained with TMRM and quantification of florescence was done at 510/570-600 nm by florimeter.Alteration of membrane potential was observed in treated cells as compared to control.[38]

17. Scratch assay.

The U87MG cells were cultured in 6 well plates and a scratch was made in the centre of the plate using a micro tip. The 5 wells were treated with various concentrations of formulation while one well was kept as control. Imaging of all wells were done for 48 hours after treatment. It was found that higher concentrations of drug was found to inhibit cell proliferation as compared to control which indicates the anticancer activity of the formulation.[39, 40]

18. Confocal microscopy.

The cellular uptake and mitochondrial targeting capability of final formulation was examined by confocal microscopy using HEK 293 cell lines. The formulation was loaded with FITC dye and the mitochondria were stained using lipofectamine transfection. The cell line was treated with formulation and fixed on glass slides and viewed under confocal microscope with suitable filter. Green florescence of FITC was observed in red stained mitochondria indicating localization of nanoparticles in the vicinity of mitochondria.[41-43]

19. Radiolabelling of theranostic nanoparticles.

Radiolabelling of nanoparticles was done by using chloramine T oxidation method. The magnetic core was treated with chloramine T and I-131(gamma emitter) and this reaction mixture was quenched with sodium metabisulphite.The counting was done with gamma counter to determine the labelling efficiency.[44]

20. Optimization of radiolabelling of theranostic nanoparticles.

The parameters like pH of reaction medium, reaction time amount of radioactivity added, amount of core etc. were optimised.[44]

21. *In vivo* bio distribution studies.

Animal protocol was approved by the ethics committee at BARC, Mumbai. The wistar rats were used for bio distribution studies. The animals were divided into two groups. One group was administered with radiolabelled formulation intranasal while other group was administered with intravenous route. The animals were sacrificed humanely at time points of 1hr, 4hr, 24hr and 48hr and major organs like brain, heart, lungs, stomach, liver, intestines, kidneys, liver and blood were isolated and counting of radioactivity was done. [45, 46]

D. On-going work.

- Cell line studies-confocal microscopy
- Bionanlytical method development for analysis of plasma and brain samples of animal studies
- Histopathology studies.
- Tumour regression study.

E. Conclusion.

From the present work involving development of theranostic nanoparticles it can be concluded that the developed formulation will serve as a diagnostic agent along with therapeutic agent due to its contrasting ability in MRI and drug carrying capacity. The lower particle size and high drug coating efficiency of the final formulation will help in treatment of brain tumours by enhanced retention and permeation effect. *In vitro* studies show mitochondrial targeting capability which can synergistically increase the anti-cancer effect of the formulation. Magnetic nature of the nanoparticles can serve as a targeting mechanism in presence of external magnetic field guidance. This can help in reduction of toxicity to the non-target organ which is the case with conventional chemotherapy.

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