6.1. Introduction

The influenza virus is a highly contagious pathogen that causes annual epidemics in the human population, and is much feared for its potential to generate new viruses able to jump to humans from different animal species and causing pandemics. Recently Papp et al., 2010 described their studies in which functionalized gold nanoparticles were used to inhibit the influenza virus. This is an orthomyxovirus containing a helical capsid with a genome of eight RNA segments. The capsid is covered by a lipid envelope containing mainly two virally-encoded glycoproteins, namely hemagglutinin (HA) and neuraminidase (NA) that forms spiky projections on the surface. The virus binds to the cell plasma membrane through an interaction between HA and sialic acid (SA) residues present on glycoproteins and lipids on the surface of the host cell. This is soon followed by a mechanism of receptor-mediated endocytosis that brings the enveloped virus particle inside the cytoplasm but surrounded by a second lipid bilayer besides the envelope, the endosomal one. Inside the late endosome, environment acidification triggers a conformational change of HA, which sets in motion a mechanism of protein (HA) mediated fusion of the endosomal membrane with the viral envelope ending with the release of the nucleoproteins and genome fragments into the cytoplasm (Palese et al., 2007).

The need to improved drug efficiency for both patient convenience and effective therapeutic uses has led to various areas of research including the development of magnetic carrier systems for drug delivery applications. Many drugs, particularly those associated with cancer therapy cannot be used effectively without the added complications of non-specific toxicities and severe side effects resulting from an attack on healthy cells. It is therefore assumed that a more effective method of delivery is that in which the majority of the drug is not released until the specified destination has been reached. In addition to reducing the side effects, the
required dosage can be reduced since the amount reaching the targeted site will increase. Among the possible delivery vehicles are magnetic carrying devices which involve the use of drug loaded magnetic nanoparticles that are transported through the bloodstream to the targeted site by an external magnetic field. These carriers may be composed of any material that is magnetic and bio-compatible as synthesized or through additional coatings. Unlike bulk magnetic materials, the potential of their nanoparticles as drug carriers is made possible through their size compatibility with cells, viruses, and genes (Mornet et al., 2004) in addition to the superparamagnetic properties which allow them to maintain magnetization only in the presence of a magnetic field.

The sesquiterpene alcohols (Z)-α-Santalol 1 and (Z)-β-Santalol together constitute over 80% of heartwood oil obtained from the well matured tree while heart-wood oil of 14 year old Indian sandalwood tree contains 44.7–46.7% (Z)-α-Santalol and 20.8–22.2% (Z)-β-Santalol, which is in the range of current international standards for the Indian sandalwood oil. Both (Z)α-Santalol and (Z)-β-Santalol are responsible for most of the biological activities of the sandalwood oil and have attracted increasing attention for their neuroleptic properties and chemo-preventive effects in in vitro and in vivo bioassay systems (Chilampalli et al., 2010).

One common method to increase the biocompatibility is to coat the iron particles with a biocompatible material. Several compounds and polymers such as Oleic acid (Lan et al., 2007), Pluronic-127 (Jain et al., 2005), PVA (polyvinyl alcohol) (Petri-Fink et al., 2005), PLGA (poly(D,L-lactide-co-glycolide) (Okassa et al., 2007), and poly(ethyl-2-cyanoacrylate) (Arias et al., 2001) have been used as coating materials. As well as increasing the compatibility, polymer coatings can serve to increase the hydrophilic nature of the particles in addition to providing an environment for drug loading. The goal of this chapter is to produce magnetic nanoparticles that
can be loaded with antiviral therapeutic compound such as β-Santalol and directed to the desired site of delivery.

6.2. Materials and Methods

6.2.1. Cell cultures and virus propagation

MDCK cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin; Stocks of influenza A virus (H1N1) were prepared by infecting the MDCK cells at a low multiplicity of infection (MOI = 0.01) for 1 hr at 37°C in 5% CO₂. Following virus absorption, unbound virus was removed and washed with phosphate buffered saline (PBS), and DMEM supplemented with 2% FBS to the cells. The virus was allowed to replicate for 6 days at 37°C in 5% CO₂, which is when complete cytopathic effect was observed. Virus was recovered from the cell supernatant and the cellular debris was removed from the viral suspension via centrifugation at 580 X g for 15 min. The stocks were preserved with 1% bovine serum albumin and stored at -80°C.

6.2.2. Materials

All starting materials including iron pentacarbonyl (Fe₂Cl₃), Decalin, PEG (Polyethylene Glycol), β-Santalol and PVA was purchased from Sigma-Aldrich.

6.2.3. Synthesis of Fe₂O₃ particles

The Fe₂O₃ nanoparticles were synthesized by simple one-pot hydrothermal method. In this experiment 3 mM of ferric chloride hexahydrate (FeCl₃·6H₂O) is used as source and 15 mM of urea (CH₄N₂O) is used as base medium were added to 40 ml deionized water. Then the solution is allowed to stirrer continuously for 45 min at room temperature to form a homogeneous solution. Then, this homogeneous solution is transferred to the 50 ml capacity
Teflon lined stainless steel vessel and kept in hot air oven at 140°C for 12 hr. After the reaction was completed, the solution was allowed to cool at room temperature and the precipitated is washed repeatedly more than five times in distilled water and three times in ethanol using centrifugation at 8000 rpm. The precipitate was separated and dried at 65°C for overnight to get final product Fe$_2$O$_3$ nanoparticles.

6.2.4. Synthesis of PVA coated Fe$_2$O$_3$

PVA-coated nanoparticles were synthesized using a similar synthesis procedure later by the addition of PVA. The detailed experimental procedure is as follows; PVA (0.56 g) was added to 10 ml solution of Fe$_2$O$_3$ nanoparticles (1mg/ml concentration). The solution was irradiated with ultrasound waves for 3 hr in open air at 30°C. The solution was centrifuged and washes several times with ethanol and dried overnight under vacuum. The as prepared sample is heated for 24 hr at 200°C.

6.2.5. β-Santalol conjugated Fe$_2$O$_3$ nanoparticles

The antiviral agent β-Santalol (0.25g) was chosen as a model drug. The loading was carried out by dispersing 6mg of PVA coated iron oxide nanoparticles in 8 ml aqueous solution of β-Santalol. The solution was irradiated with ultrasound waves for 3 hr in open air at 30°C. This experimental set up was stirred with the help of magnetic stirrer for 6 hr under room temperature and lyophilized for further use.

6.2.6. FT-IR characterization

The lyophilized Fe$_2$O$_3$ nanoparticles were grounded with KBr for FT-IR measurement by EQUINOX 55 Fourier Trans-formed Infrared Spectrometer (Bruker, Germany). Each KBr disk was scanned over a wave number region of 500–4000 cm$^{-1}$. 
6.2.7. XRD study

The method of X-ray diffraction (XRD) was used to investigate the material structure of Fe$_2$O$_3$ nanoparticles. The XRD analysis was conducted with a Philips PW 17291 powder X-ray diffractometer at a voltage of 40kV and a 25mA current. The scanning rate employed was 1°min$^{-1}$ over the 10–80° 2θ range.

6.2.8. Transmission electron microscope (TEM) study

The particle size and morphology of Fe$_2$O$_3$ nanoparticles were examined using a Philips CM120 TEM transmission electron microscope at a voltage of 80kV. The aqueous dispersion of the particles was drop-cast onto a carbon-coated copper grid and the grid was air dried at room temperature before viewing under the microscope.

6.2.9. β-Santalol Encapsulation Efficiency

β-Santalol 40 µg loaded Fe$_2$O$_3$ nanoparticles were dispersed in to 6 ml of phosphate buffer solution (PBS) and centrifuged at 12,000 rpm for 30 min. The supernatant was collected to measure the ultraviolet absorption at 272 nm. The loading efficiency and encapsulation efficiency of β-Santalol loaded nanoparticles were calculated as follows:

\[
\text{Loading Efficiency} = \frac{W_0}{W} \times 100\% \\
\text{Encapsulation Efficiency} = \frac{W_0}{W1} \times 100\%
\]

Where, \(W_0\) is the weight of β-Santalol enveloped in the Fe$_2$O$_3$ nanoparticles, \(W\) is the weight of Fe$_2$O$_3$ nanoparticles, and \(W1\) is the amount of β-Santalol added in the system.

6.2.10. In Vitro β-Santalol Release Study

The β-Santalol loaded nanoparticles were transferred to a beaker containing 100 ml of phosphate buffer (pH 7.4) by maintain temperature at 37°C with continuous stirring at 100 rpm. Sink condition was maintained by periodically removing 2 m sample and replacing equal volume
of buffer. The amount of β-Santalol release was analyzed with a spectrophotometer at 485 nm. A similar release study was carried out in acetate buffer (pH 4.5). The experiments were performed in triplicate for each of the samples.

6.2.11. Cytotoxicity assay

MDCK cells were seeded into 96-well tissue culture treated plates at a concentration of 1x10^4 cells/well in 24 hr post seeding, the MDCK cells were exposed to uncoated, β-santalol, PVA coated and PVA with β-Santalol coated nanoparticles were diluted to the various concentrations in DMEM and sonicated with a probe sonicator for 30 seconds prior to exposure. At time points of 24 hr and 48 hr post exposure a MTT assay was performed to determine cell viability.

6.2.12. Cellular uptake of Fe_2O_3 nanoparticles

The MDCK cells were seeded onto 13 mm glass cover-slips in a 24 well plate at a density of 1x10^4 cells per well in 1 ml of complete medium for 24 hr after which the growth medium was removed and replaced with the medium containing prepared Fe_2O_3 nanoparticles (0.1mg/ml medium). For control experiments, medium without nanoparticles was used. After 24 hr of culture, the cells were fixed with 1.5% glutaraldehyde buffered in 0.1m sodium cacodylate (HiMedia, India) (4°C, 1 hr). The cells were then post-fixed in 1% osmium tetroxide and 1% tannic acid (HiMedia, India) was used as a mordanant. Samples were dehydrated through a series of alcohol concentrations followed by further dehydration (dry alcohol). The cells were finally treated with propylene oxide followed by 1:1 propylene oxide-resin for overnight to evaporate the propylene oxide. The cells were subsequently embedded in Araldite resin, and ultra-thin sections (50 nm) cut with glass knives were stained with lead nitrate and viewed under Philips CM120 TEM at 80kV.
6.2.13. Virus growth inhibition assay

Confluent monolayers of MDCK cells in 12-well plates were washed with phosphate buffered saline (PBS) and then infected with influenza virus at 0.1 Multiplicity of Infection (MOI). The plates were shaker for 45 min at room temperature in particles free conditions for virus adsorption. The solution was removed and replaced with MEM containing uncoated and coated nanoparticles at 20 µg/ml concentration. Viruses were harvested at 12, 24, 36 hr post-infection, and the viral yield was estimated by plaque assay on MDCK cells. As a control, the infected cells incubated in nanoparticles free medium were included throughout the experiment.

6.3. Result

6.3.1. FTIR analysis

FTIR analysis was carried out to determine the presence of the β-Santalol coating on the surface of the Fe₂O₃ nanoparticles. Figure 6.1 depicts the FTIR spectra of (a) β-Santalol (b) β-Santalol conjugated Fe₂O₃ nanoparticles. The Figure 6.1 (b) spectra clearly show the presence of β-Santalol on the surface of Fe₂O₃ nanoparticles. The peaks at 2860, 2919, and 1702 cm⁻¹ that are associated with the -CH₂- symmetric, -CH₂- asymmetric, and CO bonds respectively in β-Santalol are also present in the coated oxides at similar shifts.

6.3.2. X-Ray diffraction

X-ray diffraction of as synthesized Fe₂O₃ nanoparticles was conducted to determine the crystal structure and crystalline nature of the particles. The powder X-ray diffraction pattern of Figure 6.2(a) clearly shows the PVA coated Fe₂O₃ nanoparticles are highly crystalline and all the peaks are assigned to the magnetite JCPDS file number 19-0629. No impurities were observed in this sample. The widening of the diffraction peaks are assigned to the nature of the nanosized
particles. Figure 6.2(b) shows the β-Santalol conjugated Fe$_2$O$_3$ nanoparticles. These results clearly show that the Fe$_2$O$_3$ nanoparticles are crystalline even after conjugation.

6.3.3. TEM

TEM analysis was conducted to understand the morphology and to determine the surface coating nature of the particles. TEM analysis of uncoated, PVA-coated and β-Santalol conjugated Fe$_2$O$_3$ nanoparticles are shown in Figure 6.3. The particles are equiaxed with an average particle diameter in the range of 50-60 nm. The unconjugated Fe$_2$O$_3$ nanoparticles are unagglomerate in nature are shown in figure 6.3a&b, whereas the PVA coated and β-Santalol conjugated Fe$_2$O$_3$ nanoparticles are agglomerate in nature (Fig 6.3c&d).

Most importantly, the particle sizes observed in both samples fall within the critical limits for drug delivery applications and are small enough to allow for size increase due to additional coatings or drug loading without reaching above the limits. The PVA-coated particles seem to be completely embedded in the PVA, thereby providing an environment for drug loading, protection from potential oxidation encountered once in vivo, and yielding a much more biocompatible material.

6.3.4. Encapsulation Efficiency

The β-Santalol loading and encapsulation efficiency of Fe$_2$O$_3$ nanoparticles with different β-Santalol concentration were shown in Table 6.1 and was found to be proportional to the β-Santalol concentration. The nanoparticles with 5mg/ml of β-Santalol showed highest encapsulation efficiency with 9.1 and 97.2% respectively.
Table 6.1: Encapsulation efficiency of Fe₂O₃ nanoparticles loaded with different concentration of β-Santalol

<table>
<thead>
<tr>
<th>β-Santalol Concentration (µg/ml)</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulation efficiency (%)</td>
<td>24.4</td>
<td>76.4</td>
<td>93.1</td>
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6.3.5. *In Vitro β-Santalol Release Study*

The ability of the carrier to release the goods efficiently at the desired site is an important feature of any delivery system. Figure 6.4 depicts the release of β-Santalol from the Fe₂O₃ nanoparticles in acetate buffer (pH 4.5) and phosphate buffer (pH 7.4). At the end of 9 hr incubation, 12% and 53.2% of β-Santalol was released in acetate and phosphate buffer, respectively. The release was characterized by a two stage process i.e., there was an initial burst release of β-Santalol and later a slow release of β-Santalol which is ideal for a drug carrier. This property was predominantly seen in phosphate buffer solution (pH 7.4) and less predominately in acetate buffer solution (pH 4.5) this again shows the ideal character of a drug carrier. This pH dependant release may help to improve efficacy of β-Santalol release into the system. This property will initiate a rapid release of β-Santalol from the nanoparticles after internalization. Such efficient release would ultimately result in improved antiviral activity against influenza virus. On the other hand, once β-Santalol encapsulated nanoparticles are taken up by host cells via endocytic process, a faster release may occur at lower pH aslo, i.e., surrounding the influenza virus, leading to significant improvement in antiviral therapy.

6.3.6. *Biocompatibility of uncoated and coated nanoparticles in MDCK cells*

After a 24 hr exposure, a little bit declines in cell viability was observed in MDCK cells exposed to β-Santalol, PVA coated and PVA with β-Santalol coated nanoparticles. In the case of
Fe$_2$O$_3$ nanoparticle alone treated group shows significant toxicity in MDCK cells. Treatments with uncoated Fe$_2$O$_3$ nanoparticle at 100 µg/ml resulted in only 20 % reduction in cell viability (Fig 6.5). There was no significant reduction in cell viability in the 100µg/ml dose treated groups of β- Santalol, PVA coated nanoparticles.

6.3.7. Cellular uptake of β-Santalol conjugated Fe$_2$O$_3$ nanoparticles

The cellular uptake mechanisms of prepared (coated and conjugated) Fe$_2$O$_3$ nanoparticles were confirmed by the transmission electron microscopic images. The images showed that the β-Santalol conjugated Fe$_2$O$_3$ nanoparticles are internalised within the MDCK cells after 24 hr (fig. 6.6). The β-Santalol conjugated Fe$_2$O$_3$ nanoparticles could be seen adhered on the surfaces of the cells, and also few particles have been endocytosed with in the cytoplasmic region of MDCK cells.

6.3.8. Inhibitory effects of coated and uncoated nanoparticles on viral replication

In order to explore the effect of β-Santalol conjugated Fe$_2$O$_3$ nanoparticles on virus yield reduction, the cells were infected with TCID₅₀ a concentration of influenza A/H1N1 (2009) virus was allowed to 30 min for incubation in CO₂ atmosphere. The various concentrations of coated and uncoated nanoparticles was treated with the cells infected with influenza virus. The inhibitory effect was observed in time dependent manner also, and throughout the virus infection rate was calculated as per incubation time 6 hr, 12 hr, 18 hr and 24 hr (Fig 6.7). This showed that viral load was significantly decreased when treated with β-Santalol conjugated nanoparticles whereas, the results showed a less significant value when treated with β-Santalol and nanoparticles alone. These results revealed that the antiviral effect is exerted not only on the initially infecting viruses and also newly propagated viruses.
6.4. Discussion

The need to improved drug efficiency for both patient convenience and effective therapeutic uses has led to various areas of research including the development of magnetic carrier systems for drug delivery applications. Many drugs, particularly those associated with cancer therapy cannot be used effectively without the added complications of non-specific toxicities and severe side effects resulting from an attack on healthy cells. It is therefore needed that a more effective method of delivery is that in which the majority of the drug is not released until the specified destination has been reached. In addition to reducing the side effects, the required dosage can be reduced since the amount reaching the targeted site will increase. Among the possible delivery vehicles are magnetic carrying devices which involve the use of drug loaded magnetic nanoparticles that are transported through the bloodstream to the targeted site by an external magnetic field. These carriers may be composed of any material that is magnetic and bio-compatible as synthesized or through additional coatings. Unlike bulk magnetic materials, the potential of their nanoparticles as drug carriers is made possible through their size compatibility with cells, viruses and genes (Mornet et al., 2004) in addition to the superparamagnetic properties which allow them to maintain magnetization only in the presence of a magnetic field.

The drug loading is attributed to the conjugation of –OH group in β-Santalol to the surface active –OH groups in PVA as shown in FTIR results (Fig. 6.1). The PVA concentration has a vital role in drug loading when the PVA concentration increases, the number of surface active –OH groups increases, which results in higher drug adsorption. The concentration of PVA also plays a pivotal role in the drug release property the drug released will be in a controlled manner in case of high percentage of PVA content used in the synthesis process. The
internalization of β-Santalol conjugated Fe$_2$O$_3$ nanoparticles in to the viral infected cells, the Fe$_2$O$_3$ nanoparticles will be degraded due its biocompatibility nature. The drug will be properly delivered to the tumor by drug-loaded superparamagnetic iron oxide nanoparticles (Moghimi 2002).

Currently, studies on magnetic iron oxide nanoparticles in viral targeted therapy have become hot subjects. Magnetic iron oxide nanoparticles have potential application prospect, and their superiority has come into being. The magnetic iron oxide nanoparticles will bring revolutionary changes for microbial therapy, and their application in medicine will certainly bring a new round of revolution in medical technology. However, studies on magnetic iron oxide nanoparticles are still in the experimental stage, and numerous problems await urgent solutions (Fu et al., 2006; Gao et al., 2008) Such as, how to increase the activity of functional groups on nanoparticle surface so as to enhance active targeting ability of nanoparticles and inhibit the viral replication system.