Chapter 5

Drug release profile and biocompatibility studies of silver nanobioconjugates
Chapter 5: Drug release profile and biocompatibility studies of silver nanobioconjugates

5.1 Introduction

Drug delivery systems need to penetrate into the biological system where the effective pharmaceutical ingredient exhibits its pharmacological effects. Nanotechnology provides a great platform to formulate the drug system very effectively. Nanodrug delivery offers targeted drug therapy for various diseases. Nanosystems offer wide possibilities in terms of structure and size. However, the increased surface area per unit volume of nanocarriers may increase the burst release of the drug, where the drug release rate depends on the rate of drug diffusion, the drug partition coefficient and the stability within the delivery system. Sustained drug release improves therapeutic efficacy and further decreases side effects (Loira-Pastoriza et al., 2014). The nanodrugs have distinctive characters to enhance and improve the drug with poor solubility, increase the retention and permeability, and deliver the drug to the target, which has helped to overcome the disadvantages of standard drug system used in cancer therapy (Chen et al., 2015).

Nanoparticles are small colloidal particles that are made up of biodegradable and non-biodegradable material, which are able to absorb, bind and carry other compounds such as drug molecules because of their large surface area. Various types of nanoparticles are involved in the drug delivery system, such as polymers, liposomes, micelles, niosomes, dendrimers, quantum dot, fullerene, gold, silver and diamond (Gan and Li, 2012; Panzarini et al., 2013; Gismondi et al., 2015).

Implementation of nano-based applications have enhanced the exposure level of nanoproducts to the humans. Some research has suggested that nano-size particles may pose a risk to humans.
Chapter 5: Drug release profile and biocompatibility studies of silver nanobioconjugates

(Hougaard et al., 2015). The European Commission has defined the nanomaterials for legislative purposes as "natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles, in the number size distribution, one or more external dimensions is in the size range 1nm - 100nm" (Pietroiusti and Magrini, 2014). Various researchers have mentioned about the Organization for Economic Co-operation and Development and its published guidelines for several validated and standardized in vitro and in vivo methods for analyzing the toxicity for nanoparticles in various fields (http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm) (Kuhnel and Nickel, 2014; Azqueta and Dusinska, 2015; Schlich and Hund-Rinke, 2015; Warheit and Donner, 2015).

The past few decades have witnessed the speedy and advanced growth in the field of nanotechnology using silver nanoparticles, which has lead to the production of silver nanoparticles to the tune of approximately 320 tons each year. Silver nanoparticles have been exploited in various nanomedical devices, for biosensing purpose in medical field, clothing, household products, food products and packing materials, jewellery, anti-smoking remedies, dental fillings, cosmetics and dietary supplements (Hadrup and Lam, 2014; Gliga et al., 2014).

Silver nanoparticles, in combination with hydroxyproline, induced the size of blood vessel and cartilage collagen fiber lattice size in the in ovo chicken embryos, resulting in stronger and longer lasting form of collagen fibers (Beck et al., 2015). Silver increased platelet aggregation and procoagulant activation in the thrombotic diseases both in vitro and in vivo (Jun et al., 2010).

Such widespread use of silver nanoparticles calls for and necessitates a thorough toxicology profile evaluation. The use of silver is not new to humans. Some studies have suggested that AgNPs induce oxidative stress, apoptosis and genotoxicity (Schultz et al., 2014). These effects would
be highly desirable if they occurred in cancer cells or other such diseased cells, but would be undesirable in normal cells. Experimental evidence has shown that certain plant extracts and their components exhibit a differential effect on non-cancerous and cancerous cells, triggering death in cancer cells and protecting normal cells (Kiruthika et al., 2013; Sumathi et al., 2013; Jambunathan et al., 2014; Sivaprabha et al., 2015).

Thus, it is presumable that the bioconjugation of silver with such herbal agents can target the toxicity of silver to cancer cells, at the same time enabling the anticancerous phytocomponents to be precisely delivered to the cancer cells. Testing this effect of the nanobioconjugates is, thus, essential.

The synthesis of nanomaterials from natural sources such as plants and fruits are the alternative choice to produce safe and non-toxic nanomaterials. Metallic nanoparticles have fascinated scientists because of their physical and chemical properties, which help them to be utilized in biomedical sciences and engineering. Among the various metals used to prepare nanoparticles, silver has the capacity to form various shapes such as spheres, discs, cubes, rods, platelets, rings, prisms and triangle, dumbbell and corn shapes, which exhibit varying physicochemical and biological properties compared with the standard metal. Due to the higher surface area, allowing large amount of atoms to interact with their surroundings, silver nanoparticles have become efficient vehicles to store and deliver medicines (Choi et al., 2011; Chernousova and Epple, 2013; Reithofer et al., 2014). The size and shape of nanoparticles have been shown to influence biocirculation, biodistribution, cellular uptake and overall drug efficacy (Truong et al., 2015).

Among the various antimicrobial agents available in the market, silver is probably the most potent and exhibits a strong toxicity toward a broad range of microorganisms, and simultaneously a remarkably low human toxicity. Silver has been proven to be safe for humans, plants and all multi-celled living matter. The toxicity has been observed due
to the oxidation of surface ions toward silver cations, which has been observed to affect basic cellular functions in a few mammalian cells (Dallas et al., 2011). Silver is non-toxic to human cells and possesses antimicrobial properties that have found uses in industrial processes as well as in the human body. Because of these characteristics, silver is ideally suited for a wide range of applications in consumer, industrial and medical products. Using natural sources of biosafe components in the synthesis of silver nanoparticles are the optional key to promote the nanodrug application in the biomedical field and improve cancer therapeutic efficacy.

In this phase of the study, the release of drugs from the nanobioconjugates under *in vitro* conditions was studied. The compatibility of the synthesized silver nanoparticles with human cells was also evaluated to determine the toxicity of nanoparticles using human blood cells under *in vitro* conditions.

### 5.2 Experimental procedure

The aim of this phase of the study was to evaluate the release of components from the silver nanobioconjugates and the toxicity level of silver nanobioconjugates.

#### 5.2.1 Drug release profile

The application of controlled drug release systems is to target the drug towards the specific tissues or in blood with the desired concentration and to maintain the release as long as possible to attain the maximum activity of the prepared drug (Dash et al., 2010). This is can be evaluated and validated only by the drug release profile study under *in vitro* conditions.

**Reagents**

- Dialysis membrane (molecular weight cut off between 12,000 to 14,000)
- PBS
Chapter 5: Drug release profile and biocompatibility studies of silver nanobioconjugates

- Sodium bicarbonate (0.2M)
- EDTA (0.01M)

**Preparation of dialysis bag**

The dialysis bag was pretreated and conditioned before using it for the experiment. The desired length of the dialysis bag was cut and immersed in 0.2M sodium bicarbonate containing 0.01M EDTA, and kept in a boiling water bath for 15 minutes. Then, the bag was allowed to cool and washed thoroughly in distilled water. The bag was then subjected to boiling in 0.01M EDTA for 15 minutes and washed thoroughly in distilled water. The prepared dialysis bag was then stored in distilled water at 4ºC until further use.

**Drug release study**

The silver nanobioconjugates (10mg) were dispersed in 1ml of PBS and packed into the prepared dialysis bag. Then the packed bag was dialyzed in PBS to monitor the release of silver ions across the membrane, over a period of 48 hours, which was measured spectrophotometrically at 420 nm for *Piper betle* and 440 nm for *Vitis vinifera* (Shimadzu-Bio Spec-nano, Japan), at one hour intervals upto 48 hours.

**5.2.2 Biocompatibility of AgNPs**

The biocompatibility of the synthesized AgNPs was monitored *in vitro* in terms of the extent of hemolysis, morphological changes in erythrocytes and whole blood clotting.

**5.2.2.1 Extent of hemolysis**

The uptake of nanoparticles into red blood cells is of great interest in nanotoxicology. Hemoglobin is one of the important and major blood protein in the human body. The fundamental perspective about the relative biocompatibility study using the blood protein (hemoglobin) is of significant importance for the assimilation of inorganic materials (Bhunia et al., 2015).
Reagents

- Sodium citrate - 3.8% (anticoagulant)
- Saline (0.9% NaCl)

Procedure

The experiment was carried out as described by He et al. (2009) with slight modifications. Blood was drawn from healthy volunteers, who were not on any medications. The blood was transferred into tubes containing sodium citrate at a ratio of 9:1 (blood:anticoagulant) and mixed gently. Red blood cells were obtained by centrifugation for 20 minutes at 2000g. The supernatant plasma was discarded and the erythrocytes were washed thrice with saline to remove the adhered serum proteins. The collected cells were resuspended in saline at a proportion of 1:5 ratio (centrifuged erythrocytes:saline). From this mixture, 100µl of cells were transferred into 1ml sample (50 µg nanobioconjugates dispersed in saline) and incubated at 37°C for 1 hour with mild shaking. After incubation, the cells were centrifuged at 3000g for 5 minutes to remove the debris and intact erythrocytes. The absorbance of the supernatant obtained was measured at 542 nm. The results were compared with the control samples, cells treated with saline as negative control and cells treated with water as positive control. The percent hemolysis was calculated as follows:

$$\text{Hemolysis (\%)} = \frac{\text{OD of test sample} - \text{OD (–) control}}{\text{OD (+) control} - \text{OD (–) control}} \times 100$$

5.2.2.2 Morphological changes of red blood cells

Cell morphology is key to investigating the maintenance of normal cellular functions. Any changes in cell structure can lead to the impairment or loss of cell function (Xu et al., 2012). Recording the changes in red blood cell morphology will indicate the extent of damage caused by a drug (He et al., 2009).
Chapter 5: Drug release profile and biocompatibility studies of silver nanobioconjugates

Reagents

- Sodium citrate - 3.8% (anticoagulant)
- Saline (0.9% NaCl)

In order to determine the effects of nanobioconjugates on the morphology of blood cells, 50µl of anticoagulant-treated human blood was incubated with the samples and saline respectively for 20 minutes at 37°C. After incubation, the changes in the appearance of the blood cells were examined by microscope. Whole blood diluted with saline was considered as negative control and was compared with the samples. The morphology of the red blood cells was observed using an inverted microscope (Metzer, India).

5.2.2.3 Measurement of whole blood clotting

The hemolyzing red blood cells (RBC) that were not trapped in the clot (formed on the surface of clot) were used to assess the whole blood clotting (He et al., 2009). A higher absorbance value of hemoglobin indicates a slower clotting rate.

Reagents

- Sodium citrate - 3.8% (anticoagulant)
- Saline (0.9% NaCl)
- CaCl₂ (0.1M)

Procedure

Blood was drawn from healthy individuals and transferred into citrate-containing tube, as mentioned in the earlier experiment. The silver nanobioconjugates dispersed in saline (10µl) were added into a 24 well plate. Saline was added to individual wells as a negative control. The clotting reaction was activated by adding 2.5ml of CaCl₂ to 25ml of blood. An aliquot (100µl) of activated blood was transferred to the wells containing the sample...
or saline and stored at room temperature for 30 minutes. At the end of the incubation time, the supernatants were carefully aspirated and 2.5ml of distilled water was added to the clot and incubated undisturbed for 5 minutes. The blood cells were lysed with the addition of water, which released the hemoglobin, that was measured at 542 nm.

5.3 Results

5.3.1 Drug release profiles

The drug release profiles of the nanobioconjugates prepared from the extracts of the *Piper betle* and *Vitis vinifera* selected for analysis, were recorded from 0 hour upto 48 hours at one-hour intervals. The profiles showed that the nanobioconjugates released steadily upto 14 hours, after which a plateau of release was observed (Figure 5.1 and 5.2). This pattern suggested that the AgNPs synthesized could release the components in a steady and sustained manner, avoiding toxic spikes.

**Figure 5.1**

**Drug release profile of silver nanobioconjugates synthesized from**

*Piper betle* leaves

The dotted line indicates the trend of release.
5.3.2 Effect of silver nanobioconjugates on the extent of hemolysis

Following the drug release profile, the toxicity of silver nanobioconjugates synthesized from *Piper betel*, eugenol, *Vitis vinifera* and resveratrol was tested on red blood cells from healthy human volunteers, to determine the biocompatibility. The extent of hemolysis for all the synthesized silver nanobioconjugates were found to be less than 0.6%. In contrast, 100% hemolysis was recorded in positive control (water) (Figure 5.3). Thus, the results showed that the synthesized nanobioconjugates from the extracts and the compounds were not toxic to human red blood cells.
Figure 5.3

Effect of silver nanobioconjugates on hemolysis

The values are Mean of triplicates

5.3.3 Effect of silver nanobioconjugates on the morphology of human blood cells

The non-toxicity of the synthesized AgNPs was further investigated based on the changes in the morphology of human red blood cells administered with the silver nanobioconjugates. The morphology of the red blood cells was observed using an inverted microscope and the photomicrographs are presented in Plate 5.1.

Exposure of RBCs to water caused complete hemolysis, as observed by a totally disrupted cell morphology. The cells in this treatment group showed disorganized structures and loss of membrane integrity. On the other hand, the RBCs subjected to AgNPs treatment showed normal morphology of the cells, which was comparable to the saline treated group. These observations further proved that the AgNPs were absolutely biocompatible in nature, and are safe for human use.
Chapter 5: Drug release profile and biocompatibility studies of silver nanobioconjugates

Plate 5.1

Effect of silver nanobioconjugates on the morphology of human blood cells

5.3.4 Effect of silver nanobioconjugates on whole blood clotting

The effect of silver nanobioconjugates from *Piper betle*, eugenol, *Vitis vinifera* and resveratrol on whole blood clotting was recorded as a measure of toxicity. All the four silver nanobioconjugates showed no significant differences in blood clotting, when compared with saline control (Table 5.1). This confirms that all the four nanobioconjugates effectively prevent cell clumping and clotting, reflecting their safety and biocompatibility.
Table 5.1

Effect of silver nanobioconjugates on whole blood clotting

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_{542}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.235 ± 0.02</td>
</tr>
<tr>
<td>PAgNP</td>
<td>0.234 ± 0.01</td>
</tr>
<tr>
<td>EAgNP</td>
<td>0.256 ± 0.03</td>
</tr>
<tr>
<td>VAgNP</td>
<td>0.252 ± 0.01</td>
</tr>
<tr>
<td>RAgNP</td>
<td>0.223 ± 0.01</td>
</tr>
</tbody>
</table>

The values are Mean ± S.D. of triplicates

Thus, all the above experiments revealed the sustained phytochemical release and confirmed the non-toxic nature of the synthesized silver nanobioconjugates. This detailed study articulated the high biocompatible nature of silver nanobioconjugates synthesized from *Piper betle*, eugenol, *Vitis vinifera* and resveratrol.

5.4 Discussion

There is an explosion of studies in nanomaterials in biological applications such as diagnostics, therapeutics, theranostics and targeted drug delivery. This has raised concerns about the risk of usage of nanomaterials and human health and safety. Thus, it becomes imperative to study the efficacy of the nanomaterials in releasing their cargo in the human system, as well as their safety, in terms of biocompatibility to human cells. Every attempt to synthesize nanoconjugates of different components used for therapeutic purposes, or human use in any form, should address the biocompatibility properties of the synthesized material, along with the potential pharmaceutical activity of the nanoparticles.
Chapter 5: Drug release profile and biocompatibility studies of silver nanobioconjugates

In the present study, the sustenance of the AgNPs of *Piper betle* leaf extract, *Vitis vinifera* seed extract, eugenol and resveratrol was assessed by the profile of drug release under physiological conditions. The biocompatibility of the nanobioconjugates was established *in vitro* using human blood cells.

The efficacy of the nanobioconjugates in releasing the organic phytocomponents of the extracts conjugated to silver was quantified as the amount of silver released from the nanobioconjugates. This was carried out only with the AgNPs prepared from *Piper betle* leaf extract and *Vitis vinifera* seed extract, which were considered as representative of their component phenolics also. The drug release pattern of all the four AgNPs revealed that there was steady release of the drug component upto 14 hours, after which there was a reduction in the release, probably due to the exhaustion of the materials. This observation showed that the silver nanobioconjugates synthesized in the present study could bring about sustained release of the phytocomponents. This strongly supports the druggability of the nanobioconjugates.

Salem *et al.* (2011) reported the continuous release of silver nanoparticles synthesized from tri-sodium citrate. An *in vitro* drug release study of AgNPs conjugated with azathioprine showed about two thirds of drug release within 24 hours period (Ram *et al.*, 2013). Bondarenko *et al.* (2013) observed the dissolution of casein coated AgNPs in test medium within 4 hours.

Similarly, Wang *et al.* (2015a) reported that methotrexate was released from the methotrexate-silicon nanoparticles during the first 12 hours, followed by a sustained release up to 96 hours. The silver nanoparticles synthesized from *Olax scandens* leaf extract showed a high release of silver ions in acidic environment under *in vitro* conditions (Mukherjee *et al.*, 2014). In another study, the *in vitro* release of miconazole from miconazole-AgNPs was found to be higher at acidic pH.
(GaneshKumara and Poornachandraa, 2015). In this context, the observation made in the present study that silver nanobioconjugates showed a steady release, suggesting that this nanobioconjugates have a strong potential to contribute as a drug delivery system, gains importance.

Most of the therapeutic drugs are based on oral or intravenous administration and the transit of the nanoparticles flow constantly in the blood. So, in order to ensure patient safety, a biocompatibility study was carried out. The bicompatibility of the AgNPs synthesized in the present study was tested in terms of the extent of hemolysis triggered, morphological changes to blood cells and coagulation pattern of whole blood. These are standard parameters directly indicative of toxicity of AgNPs. It is well acknowledged that, irrespective of the route of administration (oral or intravenous), silver nanoparticles enter the blood circulation and are then further distributed in the body (Laloy et al., 2014b). Thus, the blood cells are the first systems to encounter the AgNPs entering the human system, and take the brunt of the toxicity, if any.

The silver nanobioconjugates of both *Piper betle* leaves and *Vitis vinifera* seeds, as well as their phenolic components, eugenol and resveratrol, did not exhibit toxicity to the blood cells in all the three *in vitro* toxicity tests (hemolysis, morphology and coagulation). These observations clearly show that the AgNPs are fully bicompatible with the human system.

Several other studies have also been reported, wherein, lower toxicities were observed with the biologically synthesized nanoparticles than the chemically synthesized ones. The nanoparticles synthesized from *Euphorbia heterophylla* showed less toxicity to the human red blood cells, than the chemically synthesized silver nanoparticles (Borase et al., 2014b). Similarly, in another study, the silver nanoparticles synthesized from aloevera showed very low toxicity (Sadhasivam and Durairaj, 2014). The alcoholic extract of tulsi leaf mediated silver nanoparticles also showed very low red blood cell lysis when compared with conventional drug (Khatoon et al., 2015).
Venkatesan *et al.* (2014) observed, that the silver nanoparticles synthesized from *Rosa damascena* extract at various concentrations did not exhibit hemolytic activity against erythrocytes. *Zingiber officinale* extract capped silver nanoparticles did not induce any aggregation and hemolysis, which inferred a high compatibility towards blood (Kumar *et al.*, 2012). The nanoparticles synthesised from alpha amylase, aqueous leaf extracts of Ashoka and Neem did not show any significant hemolytic effect (Mishra *et al.*, 2013). AgNPs-glucan conjugates showed very low hemolysis at low concentrations, but the lysis increased in a concentration-dependent manner (Sen *et al.*, 2013).

In systemic injection, drug molecules in the blood stream come across a biological milieu include cells, protein and solutes, where the nanoparticles may bind to different molecules. Metal nanoparticles that interact with the plasma proteins can alter the protein conformation and inactivation of the factors or reduced availability to the components of the blood coagulation cascade, which may lead to prolongation or deficiency in the coagulation reactions. Various studies have suggested that silver nanoparticles reduce the adhesion of platelets to fibrinogen or affect the glycoprotein-fibrinogen interaction and inhibit platelet activation (Ilinskaya and Dobrovolskaia, 2013; Gamucci *et al.*, 2014).

In the present study, the effect of silver nanobioconjugates synthesized from *Piper betle*, eugenol, *Vitis vinifera* and resveratrol on whole blood clotting was monitored. The results proved that all the four nanobioconjugates did not influence the extent of whole blood clotting, reiterating their safety.

The gold nanoparticles synthesised from aqueous extract of pods of *Peltophorum pterocarpum* revealed anticoagulant activity (Raja *et al.*, 2015). The silver nanoparticles synthesised from wheat bran xylan showed resistance in clotting period when compared with the blood without any anticoagulating agent (Harish *et al.*, 2015).
The silver and gold nanoparticles stabilized and synthesised from glycosaminoglycans showed a significant effect in the blood clotting time compared with the control group (Kemp et al., 2009). ß-chitin conjugated nanosilver decreased the blood clotting capability when incorporated with ß-chitin-AgNPs than the ß-chitin alone (Sudheeshkumar, et al., 2010). Silver nanoparticles (60-70nm) treated rats showed no significant difference in the clotting time between treatment and control groups, except for high concentration of silver nanoparticles (Rezaei-Zarchi et al., 2012).

With the background of the above studies, the biocompatibility of silver nanobioconjugates from Piper betle, eugenol, Vitis vinifera and resveratrol reveals that they were not toxic and act as very good biocompatible materials, which can be applied in the biomedical applications such as anticancer activity. This property was also studied in the present study, which is documented in the next chapter.