CHAPTER 3

synthesis & assessment of biocompatibility of gold nanoparticles
3.1 Introduction

Nanomaterials have received considerable attention in recent times because of their potential applications in biology and medicine. Gold nanoparticles, which have interesting physicochemical properties [1-2], enjoy a long history dating back to Roman times and to the pioneering work of Faraday on the synthesis of stable aqueous dispersions of gold nanoparticles (gold hydrosols) [3]. It is of little surprise that there are innumerable recipes in the literature for the synthesis of gold nanoparticles both in aqueous [4-6] and organic medium [7-8]. In parallel with the development of new experimental processes for the synthesis of gold nanoparticles of controllable size, monodispersity and shape, the chemistry related to surface modification of gold nanoparticles has also evinced considerable interest. The demonstration that amine [9-11] and thiol [12] groups bind strongly to gold nanoparticles has enabled surface modification of gold nanoparticles with amino acids [13-14] and proteins [15-16], leading to important biomedical applications ranging from biodiagnostics [17], drug/DNA delivery [18-19], cell imaging [20], immunostaining [21] and biosensing [22] to electron microscopy markers [23]. The future use of gold nanoparticles in clinical applications is envisioned [24].

For any biomedical or other biological application, biocompatibility of nanoparticles is of paramount importance. A key factor in evaluating the biocompatibility of nanoparticles is the assessment of their potential cytotoxicity, attributable either to their size [25], shape (e.g. needle like carbon nanotubes [26-27]), chemical composition (e.g., heavy metals [28-29]) and physical properties (e.g., carbon nanotubes that have reached the lungs are significantly more toxic than carbon-black and graphite [26-27]) or due to their interaction with the cells (e.g., interaction of CdSe/ZnS particles with cells [30]). So far, most of the cytotoxicity studies on nanomaterials have focused on aerosols only [31] involving particle uptake by the lungs in live animal models. In order to minimize legal, moral and ethical issues on animal experimentation, in vitro cell culture systems provide excellent models for assessment of cytotoxicity of compounds/materials as first step in biocompatibility screening.

Cell culture as an experimental model offers two major advantages over animal models i.e. control of physiochemical environment and physiological
conditions. While the pH, temperature, osmotic pressure, oxygen and carbon di-oxide tension can be controlled precisely, the physiological conditions can only be kept relatively constant e.g. most of the cell cultures require supplementation of serum in medium whose composition may vary batch-to-batch. Cells in culture may also be advantageous as they are homogeneous in nature. They can be directly exposed to a reagent or material in time and dose dependent manner and they are economic as many replicates, variables and controls can be used simultaneously to perform experiments. Further, because experimental replicates are virtually identical, the need for statistical analysis of variance is reduced.

The cytotoxic effects of heavy metals [28-29], quantum dots [32-33] and gold nanoparticles in cell cultures have been studied in detail; the toxicity of gold nanoparticles inside the biological system has always been an issue of concern. Despite the scientific literature available on cytotoxicity and immunotoxicology of gold (I) [34-36] and gold (III) complexes [35-39] and recent reports on cytotoxicity of cationic and anionic functionalized gold nanoparticles [40], little attention has been focused on the immunological response of cells to gold nanoparticles. So far, most of the cytotoxicity studies of nanoparticles on cellular systems or in experimental animal models have been performed in the aerosols form [26-27]. It has been reported that nanotubes show signs of toxicity [26b]. The findings were confirmed in two independent publications by Lam et al. [26a] and Warheit et al. [27], which demonstrated the pulmonary effects of single walled carbon nanotubes after intratracheal instillation, in both rats and mice in vivo. Both groups reported granuloma formation and some intestinal inflammation. Lam et al. reported [26a] that if carbon nanotubes reached the lungs, they are much more toxic than carbon black and quartz.

The demand for nanoparticles continues to grow with the development of more biological applications. It is known that nanoparticles can transfect cells; however, effects of nanoparticles inside and outside of cells are unknown. As nanoparticles are commonly used and widely produced, the chances of unplanned events leading to their dissemination and accumulation in the environment may increase, and could lead to unforeseen changes in the biological systems. Therefore, the work presented in this chapter is an attempt to ascertain the biocompatibility of
gold nanoparticles as a gold standard for the assessment of biocompatibility of other types of nanoparticles.

3.2 Scheme of work

The first part of the chapter deals with the aqueous synthesis and characterization of biocompatible gold nanoparticles using sodium borohydride as the reducing agent. The second part of the chapter emphasizes on the selection of the treatment regimen of synthesized gold nanoparticles on cultured cells. The dose and time dependent assessment of toxicity of synthesized gold nanoparticles on different cell lines was conducted using MTT assay and trypan blue dye exclusion test. Further, RAW 264.7 cells were selected for stress induced cytokine studies in response to gold nanoparticles and their endocytosis. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) represent major class of endogenous cell signaling agents and cytotoxic effectors that are believed to contribute to cell death and apoptosis, and generation of ROS and nitric oxide species in response to gold nanoparticles has been discussed. Also, the effect of gold nanoparticles on expression of major cell structural proteins has been described. Though gold nanoparticles have been reported biocompatible, it would be prudent to test its effect on the functionality of differentiated cells and preferably organs as whole. In order to address this issue isolated murine islets have been used as a model system. Finally as an application in tissue engineering, gold nanoparticles have been immobilized onto the alginate hydrogel and their interaction with cells in co-culture system has been studied. The flow chart below describes the steps performed and materials and methods employed in this work.
3.3 Results and Discussion

3.3.1 Synthesis of sodium borohydride reduced gold nanoparticles

In this part of the chapter, synthesis and characterization of gold nanoparticles employing sodium borohydride as the reducing agent for chloroauric acid has been described. During the entire course of synthesis of nanoparticles for cell culture applications, all operations were performed under strictly sterile conditions to overcome the possibility of any microbial contamination [41]. Autoclaved ultra pure Milli-Q water was used throughout. Presented below are the details of characterization of sodium borohydride reduced gold nanoparticles.
**UV-Vis spectral and TEM characterization**

Once synthesized, the gold nanoparticles were characterized by UV-Vis spectroscopy and Transmission electron microscopy. Figure 3.1A shows the UV-Visible absorption spectrum of gold nanoparticles the curve corresponds to the spectrum of gold nanoparticles solution obtained by borohydride reduction of aqueous chloroaauric acid. A strong absorption band at ca. 527 nm is observed that corresponds to surface plasmon resonance of the gold nanoparticles. TEM image shown in Figure 3.1B represents the morphology of synthesized nanoparticles after dialysis against deionized water. The average morphology of the nanoparticles is quasi-spherical and after water evaporation the nanoparticles appear in close contact with each other. This is obvious due to the fact that the nanoparticles are not stabilized with a capping agent.

**Figure 3.1** Physicochemical characterization of sodium borohydride reduced gold nanoparticles. (A) UV-vis-NIR spectrum of the borohydride reduced gold nanoparticles after dialysis (B) TEM micrograph of the borohydride reduced gold nanoparticles.

### 3.3.2 Screening of medium for treatment

For the treatment of cells with nanoparticles, it is essential that the nanoparticles are prepared in aqueous solution and are stable in the treatment medium. Almost all metal nanoparticles synthesized by general wet chemical methods are prone to agglomeration in salt solutions and therefore it is a must to select the appropriate mode of treatment.

**Serum avoids agglomeration**

We screened different buffers including phosphate buffered saline (PBS), tris buffered saline (TBS) and respective culture medium with or without the
supplementation of 1% fetal bovine serum (FBS) in order to establish the treatment regimen. To assess the suitability of medium visibly by naked eye, we used indicator free medium (without phenol red) which is colorless. We suspended the gold nanoparticles in different media and assessed the visible clarity and color of the solutions and observed that presence of serum stabilizes the gold nanoparticles. In Figure 3.2 the test-tubes marked 1 contain $10^{-4}$ M dialyzed nanoparticles in deionized water. Same amount of nanoparticles were added in culture medium in such a way that the final concentration of medium remains constant. The medium was pre-supplemented with or without serum at a final concentration of 1% (v/v) serum in test-tubes marked 2 and 3 respectively. Test-tubes marked 4 contain only 1% serum. Since the medium without dye is colorless, the color imparted to the solutions results from the color of nanoparticles solution. In case of test-tube marked 3 color changes instantaneously after the addition of nanoparticles. Change in the colour is indicative of particle agglomeration. Hence, from this simple experiment it is clear that serum constituents stabilize the nanoparticles against salt induced aggregation and insults irrespective of the nature and method of nanoparticle synthesis. Figure 3.3 corresponds to the low and high magnification TEM images of gold nanoparticles obtained after incubation in medium supplemented with or without 1% FBS. Our observations are in agreement with the recent reports demonstrating that serum or its constituent proteins avoid agglomeration of particles [42-45]. The phenomenon of protection against agglomeration is instant and advantageous due to its applicability under in vivo situation. The observation is of vital significance since if the nanoparticles directly delivered in blood stream.

![Figure 3.2 Agglomeration of (A) borohydride reduced gold nanoparticles, (B) tyrosine reduced silver nanoparticles and, (C) curcumin reduced silver nanoparticles in culture medium. Equal volume of nanoparticles was suspended in water (1), DMEM with 1% FBS (2), DMEM alone (3), and 1% (v/v) FBS in water (4).](image-url)
These may be stabilized by serum proteins and may withstand the normal physiological salt concentration.

This in turn may help in diffusion of nanoparticles to the distant target locations in the body. The phenomenon also points towards the fact that there are no known antibodies against metals. Furthermore, since the nanoparticles can directly be treated in complete culture medium i.e. respective culture medium supplemented with 10 % FBS or New Born Calf Serum (NBCS) and antibiotics the effect of prolonged exposure times can be studied which would have not been possible with phosphate buffered saline (PBS) or tris buffered saline (TBS). Based on the above study, all the cell culture treatments were carried out using direct addition of nanoparticle solution to the complete medium.

**3.3.3 Biocompatibility of sodium borohydride reduced gold nanoparticles**

Growing evidence supports the potential of nanomaterials in the field of biology and medicine and thus extends their use beyond industrial applications. The applications in medicine include drug delivery [46-47], improved contrast agent for imaging [48-50], fluorescence biomarkers [51] chip based nanolabs capable of
monitoring and controlling individual cells [52], and as a vehicle in vaccine development [53].

When new nanomaterials are intentionally synthesized and used for biomedical applications their biocompatibility is the first and foremost important criteria to be evaluated. A key issue, in evaluating their biocompatibility, is assessing their potential cytotoxicity. In the current scenario, despite increased interest in the development of nanoparticles, very few studies have addressed their toxicity. Most of the studies on toxic effect of nanomaterials have been done either on the nanoparticulate materials in the form of aerosols [31] or in liposomes [54]. There are relatively very few studies that assess the toxicity of nanoparticles in aqueous medium. Therefore, we studied the cytotoxicity of gold nanoparticles suspended in aqueous medium using cell cultures as in vitro experimental model. The rationale behind selecting gold nanoparticles as the model system for our studies is the amenability of synthesizing these nanoparticles in various sizes and shapes. Besides, these nanoparticles can be easily characterized employing the techniques of UV-Vis spectrophotometry and Transmission Electron Microscopy (TEM). Also, gold from the ages is considered an inert element as it is nonreactive towards oxygen and sulphur at any temperature [55]. Furthermore, gold has long been used in traditional Ayurvedic and Unani medicine. In Ayurveda, gold bhasmas are used which are prepared by a combination of physical and chemical methods of nanoparticles synthesis.

**Gold nanoparticles are non cytotoxic**

In preliminary experiments we tested different cell lines in terms of their type, origin, morphology and functions for nanoparticles toxicity assessment. The selection was such that we tested primary cell cultures versus established cell lines: fibroblasts versus epithelial cells; cancerous versus non cancer cells; phagocytes versus cells with limited phagocytic activities; and human versus murine and fish cells. The cells were treated in a dose dependent manner for 48 h and viability of the cells was determined by trypan blue dye exclusion test. In order to avoid the possibility of bias, the cells were counted blindly. Figure 3.4 depicts the effect of gold nanoparticles on different cells tested and there was no apparent cellular toxicity on the tested concentrations except in case of NIH 3T3 cells where marginal toxicity
was seen at concentration as high as 200 μM. These findings are of significance in selective epithelial cell growth and in avoiding fibroblast contamination. Thus, gold nanoparticles could be employed in human skin epidermal cell cultures as well as in wound healing applications to curtail the unwanted growth of fibroblasts which would also prevent scar formation [56]. Our findings are in agreement with the recent report Pernodet et al. [57] who discusses the cytotoxicity issue in fibroblasts at higher doses of nanoparticles. Our results differ from Pernodet and co-workers only with respect to dosage, where we observed toxicity at a much lower dose than Pernodet et al. One possible explanation for this difference may be that we used sodium borohydride to reduce HAuCl₄ while they [57] used citrate reduced nanoparticles. Sodium borohydride is a potentially toxic biochemical while citrate has long been known to be biocompatible, is naturally present in plants and is taken as a food constituent. The second possible reason may be the size of nanoparticles used. Our average particle size is 3-4 nm as compared to 13-15 nanometers used in their study. Moreover, in a similar type of study performed on K562 leukemic cells, Murphy and colleagues [40b] found no toxicity at the same doses tested by us. It is known that reduction in size of nanoparticles leads to cytotoxicity [58]. Recently,
Tsoli et al. [59] have also demonstrated that 1.4 nm Au\textsubscript{55} radioactive gold nanoclusters show potential cytotoxicity in different cancer and non-cancer cell lines at much lower doses. Since, in the primary screening most of the cell types tested showed no sign of toxicity at the tested doses, we confined our further studies to fewer cell lines.

When considering the long term effects of nanomaterials, the adverse effects on cells should be taken into consideration. Cytotoxicity may not be the only effect that nanoparticles can induce and other morphological and functional impairments can also occur on treatment with nanoparticles. In order to pursue our further studies on interaction of gold nanoparticles with cells in detail, we concentrated on RAW 264.7 macrophage and NIH 3T3 fibroblast cells.

Macrophages are the principal immune regulatory cells of the body and form the first line of defense in response to foreign materials in many tissues including blood, lungs, brain, liver etc. In terms of nonspecific immune response, their main function is to phagocytose the foreign material and try to clear it. Macrophages have been widely used to study the interaction of nanomaterials with cells [60-62].

![Figure 3.5 Cytotoxicity studies of gold nanoparticles on RAW264.7 macrophage cells. MTT assay showing cell viability during exposure of cells to 10, 25, 50, and 100 µM colloidal gold for 24, 48, and 72 h, respectively.](image)
The biocompatibility of gold nanoparticles under in vitro conditions in RAW264.7 macrophage cells was examined in terms of the effect of gold nanoparticles on cell viability as well as cell proliferation by trypan blue dye exclusion test and MTT assay, production of reactive nitric oxide species (NOS) by Griess reagent and production of reactive oxygen species (ROS) by flow cytometry (fluorescence activated cell sorting, FACS) using DCFH-DA. To determine whether gold nanoparticles affect cell proliferation, an MTT assay (Fig. 3.5) was performed. Untreated controls and cells treated with increasing doses of gold nanoparticles for 24, 48 and 72 h were subjected to MTT assay for cell viability determination. After 48 h of gold nanoparticle treatment, RAW264.7 macrophage cells showed more than 90% viability up to 100 μM concentrations of gold nanoparticles (Fig. 3.5). After 72 h, there was a marginal decrease in the cell viability to 85% for 100 μM gold nanoparticle treatment. This slight decrease in cell number might be accounted for the stress caused to the cells because of depletion in the media nutrients for a prolonged exposure of 72 h. These results suggest that Au(0) nanoparticles have no detectable cytotoxicity up to 100 μM concentration of gold till 72 h of exposure in contrast to the previous reports that have shown the cytotoxic nature of Au(I) and Au(III) gold complexes [36-38]. Our results on non-cytotoxicity of borohydride reduced gold nanoparticles are in agreement with the conclusions of some recent studies on a range of gold nanoparticle preparations [40].

**Gold nanoparticles do not lead to enhancement of ROS and RNS**

The mechanism by which macrophages inactivate and destroy invading microorganisms and other foreign cells consists of three basic components. The first component is the respiratory burst of effector cells coupled with formation of oxygen derived toxic products. The second component is mainly the nitric oxide produced by nitric oxide synthase and the third component is lysis and degranulation of lysosomal granules with subsequent release of toxic substance onto the surface of target cells (the other infected cells of body). These three components may mutually cooperate in various ways. In case of nanoparticle endocytosis, the first two mechanisms that may be involved. Hence, to determine whether gold nanoparticles affect the production of reactive nitrite species (RNS) in the macrophage cells,
RAW264.7 macrophage cells were treated with 10, 25, 50 and 100 μM gold nanoparticles (blocks 1-4 respectively) for 24 and 48 h (Fig.3.6). Here, it is noteworthy that we did not attempt to include early time points as there is always a transient increase in RNS and ROS due to the process of nonspecific internalization. What we were interested in was the persistent long term effect of nanoparticles in the generation of nitric oxide and ROS. It is interesting to observe that when treated with 50 μM colloidal gold solution, there was no change in the RNS level up to 24 h in comparison with the untreated control; however after 100 μM gold nanoparticle treatment, we observed a reduction in the cellular RNS level. On the other hand, when the effect of gold nanoparticles on cellular RNS level was observed after 48 h of treatment, a concentration dependent reduction in RNS levels was observed. This data suggests a time and dose dependent reduction of RNS levels by gold nanoparticle treatment. The effect of gold nanoparticles on the production of reactive oxygen species (ROS) in macrophage cells was studied by flow cytometry using fluorescence activated cell sorter (FACS). RAW264.7 macrophage cells were treated with 10, 25, 50 and 100 μM gold nanoparticles for 24 h (Fig.3.7A) and 48 h (Fig.3.7B) and forward and side scatter (FSC/SSC) scatter-plots (left panels, Fig.3.7A and B) as well as fluorescence intensity (FL1-H) curves (right panels, Fig.3.7A and B) were obtained.

![Graph showing RNS levels with gold nanoparticle treatments](image)

**Figure 3.6** Nitric oxide estimation from the culture supernatants of RAW264.7 cells by Griess reagent following 24 h (*) and 48 h (△) of 10, 25, 50, and 100 μM gold nanoparticle treatment

In the FSC/SSC plots of control cells as well as gold treated cells after 24 h (left panel, Fig.3.7A) and 48 h (left panel, Fig.3.7B) of treatment, two different populations of cells were observed. With varying gold nanoparticle treatment, out of
two cell populations, the population depicted on the left side in each scatter plot (FSC/SSC scatter plots in Fig.3.7A and B) does not vary and hence the population diverging with increasing gold nanoparticle treatment (cell population on right side of FSC/SSC plots in Fig.3.7A and B) was gated for creating FL1-H curves.

The right panels of Fig.3.7A and B show the FL1-H curves obtained from the gated populations in respective FSC/SSC plots after treating RAW264.7 macrophage cells with 10, 25, 50 and 100 µM gold nanoparticles for 24 (curves 1-4, Fig.3.7A) and 48 h (curves 1-4, Fig.3.7B) followed by cell staining using DCFH-DA, an ROS-specific fluorescent dye. Unstained cells not exposed to gold nanoparticles were taken as the fluorescence control (curve 0, Fig.3.7A and B) while stained, gold-treated cells were taken as reaction control (curve 0, Fig.3.7A and B). It is clear from curves 0-4 in Fig.3.7A that there was no effect of different concentrations of gold nanoparticles (curves 1-4, Fig.3.7A) on the ROS level in macrophage cells upto 24 h of treatment (Fig. 3.7A) in comparison to untreated cells (curve 0, Fig. 3.7A). When treated upto 48 h (Fig. 3.7B) with various concentrations of gold nanoparticles (curves 1-4, Fig. 3.7B), upto 50 µM colloidal gold treatment (curves 1-3, Fig. 2D) the
gold nanoparticles do not seem to affect ROS levels. However, exposure to 100 \( \mu \text{M} \) concentration gold nanoparticles (curve 4, Fig. 3.7B) led to a reduction in ROS level compared to that of controls (curve 0, Fig. 3.7B). The insets in Fig. 3.7A and B show the mean fluorescent intensity from the three experiments done in quadruplets along with the error bars, arising from the respective samples indicating the statistical validity of the data, thus strengthening the dose and time dependent outcome of results.

The ROS data (Fig. 3.7A and B) correlates well with the RNS data (Fig. 3.6), which also showed a reduction in RNS levels at higher gold concentrations after prolonged exposure. Here it is noteworthy that other metals and metal oxide nanoparticles have recently been reported to increase ROS production in different cell cultures in time and dose dependent manner. Silver nanoparticles are found to be toxic at much lower concentrations and induce a significant amount of ROS production while Fe\(_3\)O\(_4\), Al, MoO\(_3\) and TiO\(_2\) had no measurable effect at lower doses but there was a significant ROS release at higher levels in rat liver derived BRL 3A cell line [63]. In another study, silica nanoparticles of two different sizes were exposed to human lung carcinoma cells and it was observed that irrespective of the particle size, SiO\(_2\) nanoparticles led to the production of ROS and cytotoxicity in a dose dependent manner [64]. However, decrease in ROS in a dose dependent manner has not been reported so far. It has been shown that Au(I) compounds inhibit the DNA binding activity of AP-1 and NF-\(\kappa\)B transcription factors, which in turn down-regulate the expression of a number of genes viz. IL-2 receptors and proinflammatory cytokines that in turn, would reduce the production of RNS and ROS [65a]. The similar mechanism of down-regulation of RNS and ROS might also be involved in Au(0) nanoparticles. However in contrast to Au(0) nanoparticles, Au(III) compounds have been reported [65b] to oxidize cellular self-proteins to convert them into non-self proteins, causing self peptide molecules to elicit immune response and hence giving rise to autoimmune diseases. Hence, a time and dose-dependent decrease in both RNS and ROS levels on Au(0) treatment indicate that gold nanoparticles exert, directly and/or indirectly, antioxidant effects on macrophages, which might eventually be helpful in treatment of various diseases including autoimmune diseases like rheumatoid arthritis.
Gold nanoparticles do not induce the secretion of stress induced proinflammatory cytokines

Macrophages are one of the principal immune effector cells and hence, in addition to cytotoxicity studies, the immunological response of RAW264.7 macrophage cells when exposed to gold nanoparticles was studied in terms of production of proinflammatory cytokines TNF-α and IL1-β, both at the mRNA (after 3 h) (Fig. 3.8C) and protein (after 24 h) (Fig. 3.8A and B) level. Neither the control cells nor the cells treated with gold nanoparticles up to 100 μM concentration expressed mRNA for either TNF-α or IL1-β (Fig. 3.8C).

To assess whether any additional effect of gold nanoparticles was exerted on the process of translation, production of proinflammatory cytokines was tested at protein level by ELISA (Fig. 3.8A and B). Neither TNF-α (Fig. 3.8A) nor IL1-β (Fig. 3.8B) were found to be expressed significantly after 24 h of incubation either in control cells (untreated) or in the cells treated with gold nanoparticles up to 100 μM concentrations. However, cells stimulated with bacterial LPS expressed significant amount of TNF-α (Fig. 3.8A) or IL1-β (Fig. 3.8B). Hence, the ELISA results obtained are in agreement with the RT-PCR results and suggest that gold nanoparticles do not elicit initial immunological response or production of proinflammatory cytokines TNF-α and IL1-β up to as high as 100 μM doses of gold nanoparticle. In the support
of our observations, Tsai et al. [66] have also shown that intra-articular administration of nanogold ameliorates the clinical course of collagen induced arthritis in rats in vivo. Gold nanoparticles exert anti-angiogenic activity and subsequently reduce macrophage infiltration and inflammation, which results in attenuation of arthritis by significant reduction in the level of TNF and IL-1 in the ankle joints of animals. Taken together, our in vitro results on cytokine release in response to gold nanoparticles demonstrate the therapeutic potential of the same.

In addition to cytotoxicity and immunological studies, the effect of gold nanoparticles on secretion of major cellular proteins involved in higher order cellular functions were also studied to find out whether gold nanoparticles cause any additional effects, particularly on the major biochemical pathways of the macrophage cells. The SDS-PAGE data of the protein profile of untreated RAW264.7 macrophage cells (lane 2) and of cells treated with 100 μM gold nanoparticles for 24 h (lane 3) are shown in the Figure 3.9. It is clear that there is no change in the profile of the major cellular proteins after gold nanoparticle treatment of macrophage cells upto 100 μM gold concentration and thus, little effect on the higher order cellular functions of macrophage cells.

**Figure 3.9** Polyacrylamide gel (12 %) electrophoresis profile of marker (lane 1), untreated RAW264.7 cells (lane) and cells treated with 100 μM gold nanoparticles for 24 h.

**Morphometry analysis**

Cellular cytoskeleton plays a crucial role in locomotion and cell migration, endocytosis, intracellular transport of proteins, cell shape and morphology, cellular defense mechanisms and signal transduction. Actin filaments (F-actin) are double
stranded polymers composed of monomer units of globular actin (G-actin). They are semi flexible with a high aspect ratio and a diameter of approximately 7 nm. F-actin is found dispersed throughout the cytoplasm, but most filaments are located just beneath the plasma membrane in the cortical region of the cell [67]. Berry and co-workers have studied the behaviour of cells in response to metal and metal oxide nanoparticles and nanoscale topologies using human fibroblasts as a model for cytoskeleton organization [68-72]. To check the dose and time dependent effect of AuNP's on fibroblast cells, we treated NIH 3T3 murine fibroblast cells in a time and dose dependent manner wherein NIH 3T3 cells were grown on cover glass and treated with 50 $\mu$M and 100 $\mu$M concentrations of AuNP. The cells were observed routinely under microscope and were fixed after 48h and 96h followed by F actin probing with phalloidin-TRITC. Actin filaments are known to regulate cell functions such as proliferation, adhesion and differentiation as well as internal architecture [73] Figure 3.10 summarizes the effect of AuNP on cells. The control cells (Fig. 3.10A) exhibited a well defined and organized morphology of typical fibroblast cell with prominent nucleus in the centre (blue) and actin filaments (red) throughout the soma. In the margins of cells intact stress fibers of actins are clearly visible. The cells when treated with different concentrations of AuNP for 48h, showed morphology comparable to the controls (Fig. 3.10B). The same was true for the lower concentration when cells were treated for a longer time (Fig. 3.10C). However, when the cells were exposed to higher concentration of nanoparticles for longer times (Fig. 3.10D) a disruption in the actin fibers was noted with the shrinkage in the cellular architecture in a fraction of cells. Though, a patchy pattern of F actin was noted (Figure 3.10D), there were significant number of F-actin stress fibers beneath the plasma membrane in the cortical region of the cell, indicating active spreading of cells (Figure 3.10D). The formation of actin dots rather than long stress fibers in the cortical region of cells (Figure 3.10D) suggests either a depolymerization process or lack of actin filament synthesis. When the cells were treated with gold precursor, there was a significant loss of stress fibers and dotted appearance was observed even at lower time points which at later stage resulted in total shrinkage of cellular cytoplasm eventually led to cell death. The dead cells detached from the cover glass and floated in the medium, and hence could not be stained at later time points.
In a similar study, Pernodet et al. [57] have also reported the actin dot formation and reduction in stress fibers at higher concentrations and longer time exposure of gold nanoparticles to fibroblasts. However, they have not used precursor control in their studies.

**Effect on the functionality of Islets: Thinking beyond cytotoxicity and ROS generation**

Islets of Langerhans are organelles present within the pancreas and are mainly responsible for production of insulin, glucagon, somatostatin and pancreatic polypeptide upon stimulation. Since their discovery in 1869 [74], isolation of islets [75] has promoted studies related to the understanding of the pathophysiology of diabetes and islet transplantation. Islet transplantation is a promising approach to the restoration of glucose homeostasis and the prevention of devastating secondary complications due to high blood glucose level in type I diabetes patients. They are miniature organ systems, retaining their architecture, differentiated state and ability of insulin secretion upon stimulation. Isolated islets (Fig. 3.11) represent an
ideal model system to study the effect of any test compound on the functionality of tissue in near in vivo scenario. Although, much is known about the gold insulin interaction and its biological application [76-77], to our knowledge there are no reports on the effect of nanoparticles on islet/organ functionality. With this view in mind we tested effect of increasing concentrations of nanogold particles on murine isolated islets in terms of insulin release and expressed as stimulation index. The insulin secretion in response to glucose stimulation was analyzed by commercially available insulin ELISA kit (Mercodia, USA) following manufacturer’s instructions. The stimulation index represents the ratio of insulin secreted at 5mM glucose stimulation to the insulin secretion at 16.5 mM glucose stimulation and is a measure of islet functionality.

<table>
<thead>
<tr>
<th>Nanoparticle Treatment concentration</th>
<th>Insulin release 1 U/ml at 5mM glucose</th>
<th>Insulin release 1 U/ml at 16.5mM glucose</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>48.71</td>
<td>78.42</td>
<td>1.57</td>
</tr>
<tr>
<td>25 μM</td>
<td>62.08</td>
<td>82.28</td>
<td>1.38</td>
</tr>
<tr>
<td>50 μM</td>
<td>64.65</td>
<td>80.88</td>
<td>1.24</td>
</tr>
<tr>
<td>100 μM</td>
<td>62.01</td>
<td>73.02</td>
<td>1.21</td>
</tr>
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It is evident from the table 3.1 that there is no substantial insulin secretion under the influence of nanoparticles as there is no increase in stimulation index. The absence of increase in stimulation index indicates that gold nanoparticles by themselves do not stimulate insulin secretion suggesting that they do not act as insulin secretagogue, further confirming their neutral role. The viability of the islets (as determined by trypan blue assay) was comparable to that of the controls, hence, the data suggest that the gold nanoparticles are biocompatible to isolated islets.

### 3.3.4 Immobilization of gold nanoparticles onto alginate hydrogel: step towards developing novel micro carriers

Alginate represents a family of polysaccharides composed of homopolymeric region of β-D-Mannuronic acid and α-L-guluronic acid interspread with mixed
sequences and has been widely used as an immunoisolation membrane for transplantation purposes [78] and as drug delivery vehicle [79] and cell based transplantation therapies [80].

The attachment of anchorage-dependent cells, such as fibroblasts, to material surfaces is a complex process involving cell attachment and spreading [81], focal adhesion formation, and extracellular matrix formation and reorganization [73, 82]. Cell attachment may occur through binding with specific adhesion proteins or, in the absence of such proteins, by direct interaction with the material surface [83]. The initial attachment of cell to a surface is by cytoskeletal-associated receptors in the cell membrane, of which integrins are probably the most important. This attachment process is dependent on the nature and conformation of adhesion proteins, such as fibronectin and vitronectin, present at the biomaterial surface. Attachment normally is usually followed by a reorganization of cytoskeletal actin, resulting in the flattening and spreading of the cell and formation of focal contacts, replete with clustered integrins which participate in cell signaling events regulating cell behavior. Figure 3.12 reveals that nanoparticle immobilization leads to aggregation of nanoparticles which in turn impart purple colour to the beads. The cells extensively and randomly spread with spindle morphology on the immobilized nanoparticle surface while all the cells on the alginate surface remain round and compact and tend to cluster following 5 h of cell culture. Interestingly, after 12 h of culture on alginate surface, only few cells were attached. However, average morphology was round and most of the cells were loosely attached.

The loosely attached cells could be detached from the beads either by shaking or incubating the beads in ice for 5 minutes. This phenomenon indicates the importance of alginates in case of immunoisolation device for islet encapsulation where adherence of cells to semi permeable isolatory barrier leads to clogging of the pores and ultimately leading to death of graft due to hypoxia and reduced nutrient supply across the membrane.

The factors that affect the cell adhesion include hydrophobic/hydrophilic property, electric charge, surface morphology, and the surface functional groups of a biomaterial [84]. Cell behavior is mainly mediated by interactions of the positive charge of the materials with cell membrane or by cellular uptake and subsequent activation of intracellular signal transduction pathways [84]. The weak negative
charge on the alginate surface inhibits the formation of initial cell contact due to the electrostatic repulsion interactions. However, there are other interactions, such as H-bond and hydrophobic interactions that exist between alginate and cells, because proteins and phospholipids components in cell membrane can form interactions with alginate through hydrophobic and H-bond interactions [85]. These interactions favor the formation of as loose cell contact on alginate surface.

On the other hand, nanoparticles immobilized on alginate beads due to gold and exposed lysine residues help in a quick adherence and on spreading of the cells. Gold is known to interact with thiol and amine groups while lysine being positively charged interacts via electrostatic interactions. It is seen from the figure 3.12E that the fibroblast cells adhere firmly to the modified alginate beads impregnated with nanogold particles and spread uniformly revealing their natural morphology. This feature of the modified alginate beads is of high significance for culturing anchorage dependent cells on large scale simulating micro carrier cultures similar to cytodex beads [86]. Since the alginate capsules are spherical in shape they provide a three dimensional surface for expansion and proliferation of cells leading to an increase in

Figure 3.12 Phase contrast images of uniformly constructed calcium alginate hydrogel microcapsules (A) after nanoparticles immobilization onto the surface (B) low magnification image of nanoparticle immobilized microcapsules with adhered cells (arrow heads) the size of microcapsule is around 800 µm as the grid in plate corresponds to 2 mm² (C) high magnification images of cellular morphology on as-prepared calcium alginate hydrogel (D) and nanoparticle immobilized hydrogels (E) after bead co-culture for 5 h.
the cell number due to increase in surface area. Thus our results present a novel and simple micro carrier system for large scale production of cells.

3.5 Conclusion

The nanoparticles synthesized by borohydride method are nontoxic, biocompatible and do not induce secretion of stress induced inflammatory cytokines and release of nitric oxide. The nanoparticles also do not stimulate intracellular reactive oxygen species level. Gold nanoparticles can be immobilized on to the surface of poly-L-lysine coated alginate hydrogel and support the growth of cells. These gold coated alginate hydrogel beads offer a novel model system for large scale cultivation of anchorage dependent mammalian cells for obtaining the cell product on industrial scale offering a new tool in biotechnology.
3.6 References


85. Zhu, A. P.; Fang, N.; Chan-Park, M. B.; Chan, V. *Biomaterials* 2005