Chapter 2

Preparation and characterization of selenium incorporated guar gum nanoparticles and its biological evaluation in H9c2 cardiomyoblast

2.1. Introduction

Recently the concept of nanoscience has been incorporated to the medical science for the amplification of the therapeutic potential of the material. A wide range of nanomaterials have been developed for biomedical applications due to their unique properties. The special physicochemical properties of nanomaterial make them entirely different effect on biological system compared to their macro and micro counterparts. There is high demand of antioxidants for control and management of heart diseases due to the importance of oxidative stress in the etiology of the same. Kim et al (2011) reported about the use of antioxidants nanoformulations against cardiac diseases like I/R injury, hypertrophy and myocardial infarction associated problems. For example, CuZnSOD protein encapsulated in biodegradable poly (D, L-lactide coglycolide) or PLGA nanoparticles (PLGA-SOD) has shown potential as a therapeutic agent for I/R injury. Other nanoformulated antioxidants studied in models of I/R injury are peroxalate nanoparticles, which instantaneously and specifically decompose H$_2$O$_2$, the most abundant ROS generated during I/R injury (Lee et al 2008; Lee et al 2007). The antioxidant micronutrient like cerium, Se, vanadium etc. have been reported to have therapeutic importance against heart diseases. Among this Se is having special position in biomedical sciences due to its role as an innate antioxidant system of body (discussed in chapter 1). Nanoparticles containing metals of biological importance are attracting much attention of present scenario because of their physical and chemical properties. Taking this into account, the present study was aimed to prepare and characterize SGG and study its interaction with H9c2 by assessing its effect on various vital parameters. For this we conducted batteries of in vitro experiments to evaluate its interaction with H9c2 cell lines. In this study GG was used to prepare nanoparticles through nanoprecipitation method with sodium selenite to decrease the toxicity and enhance the antioxidant property of selenite dietary supplement. The physicochemical properties of the nanoparticles were characterized by particle size analysis, transmission electron microscopy (TEM) analysis and X-ray diffraction (XRD) analysis. In order to see the interaction of SGG with cells we systematically investigated
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the effect of nanoparticle on H9c2 cells by analyzing various parameters like cell viability, apoptosis, DNA protection, ROS generation, mitochondrial transmembrane potential (ΔΨm) and alteration in cytoskeleton properties. This chapter also deals with comparative evaluation of the antioxidant potential of SGG, GGN and Se using in vitro cell free systems.

2.2. Experimental methods

2.2.1. Materials

Guar gum powder, mannanase enzyme from Helix pomatia, sodium selenite, Triton X-100, isopropanol, phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitrobluetetrazolium (NBT), 2-deoxy D-ribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), trolox, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium tripolyphosphate (TPP), dimethyl sulfoxide (DMSO), 2’,7’ dichlorodihydrofluorescein diacetate (DCFH-DA), acridine orange (AO), ethidium bromide (EtBr), 2, 3-diaminonaphthalene, 5,5’,6,6’-tetrachloro-1,1’,3,3’ tetraethylbenzimidazolyl carbocyanine iodide (JC-1), 4’,6-diamidino-2-phenylindole (DAPI), phallodin and pUC-18 plasmid DNA were purchased from Sigma Chemicals, USA. Ammonium molydbdate, ascorbic acid (AA), ferrous sulphate, methanol, hydrogen peroxide (H2O2) and potassium ferricyanide were purchased from Merck Specialities Pvt Ltd (India). Tris hydroxymethyl aminomethane hydrochloride (Tris-HCl), potassium hydroxide, and potassium persulphate were purchased from Sisco Research Laboratories, Mumbai (India). Ethylene diamine tetraacetic acid (EDTA), sodium phosphate and sodium hydroxide were purchased from SD Fine Chemicals Ltd (India). Dulbecco’s modified Eagle’s medium (DMEM) and foetal bovine serum (FBS) were from HiMedia Pvt Ltd India. All other chemicals and solvents used were of analytical grade.

2.2.2. Preparation and characterization of GGN and SGG

The GGN was prepared by nanoprecipitation method (Soumya et al 2010). Nanoprecipitation was carried out using the addition of a non-solvent (isopropyl alcohol) to the aqueous solutions of depolymerized sample previously mixed with surfactants (Triton X-100) and cross linker (TPP). Ionic crosslinking using polyanionic cross linker TPP was employed in this method. Vigorous vortexing and sonication were employed in
the procedure to prevent agglomeration of molecules. Triton X-100 was used to modify the surface properties of GG and to ensure the stability of nanoparticles. Besides GGN, SGG nanoparticles were also prepared by the same procedure. Briefly, 1% GG was depolymerised with mannanase enzyme with a pH of 5.2 in citrate phosphate buffer and incubated at 30 °C for 24 h. The hydrolysed suspension of GG was filtered through 0.2 µm syringe filters. The filtered suspension was stored in 4 °C for further characterization. Then through nanoprecipitation method GGN and SGG were prepared with depolymerised GG, 10% Triton X-100, isopropanol and 0.1% TPP. For preparing SGG sodium selenite was added to the solution containing 1% GG, 10% Triton X-100, isopropanol and 0.1% TPP. The solution was vortexed and sonicated for 10 min at room temperature, filtered with 0.2 µm syringe filters to get uniform nanoparticles. The average particle size (hydrodynamic diameter, Zaverage) of the prepared particles (GGN, SGG) were determined by photon correlation spectroscopy (PCS) using 3000 HSA Zetasizer (UK) equipped with He–Ne laser (633 nm). Photon-correlation spectroscopy requires the viscosity of the medium to be known and determines the diameter of the particle by Brownian motion and light scattering properties (Kreuter 1994). The results obtained by photon-correlation spectroscopy are usually verified by TEM. High-resolution transmission electron microscopy (HRTEM) is an imaging mode of the TEM that allows the imaging of the structure of a sample at an atomic scale (Williams et al 2009). Because of its high resolution, it is an invaluable tool to study nanoscale properties of the materials. For this study the morphology and particle size of the samples were analyzed using a high resolution transmission electron microscope, FEI, TECNAI, 30G2s-TWIN microscope. A thin layer of sample was coated on a carbon coated copper grid and dried under vacuum. This sample coated grid was used for the TEM analysis.

XRD of GG, pure sodium selenite and SGG were measured using X-ray diffractometer (XPERT Pro, Philips, Eindhoven, Netherlands) with nickel-filtered Cu-Kα radiation (0.154 nm). Qualitative analysis is possible by comparing the XRD pattern of an unknown material to a library of known patterns. The degree of crystallinity of samples was quantitatively estimated. A smooth curve which connected peak baselines was plotted on the diffractograms. The area above the smooth curve was taken as the crystalline portion and the lower area between smooth curve and the linear baseline in the
samples was taken as the amorphous portion.

2.2.3. Colloidal stability

The colloidal stability of the prepared nanoparticle was investigated by turbidity measurement. Therefore, the nanoparticles were mixed with DMEM medium both in the presence and absence of 10% FBS. The average particle sizes (hydrodynamic diameter, Zaverage) of the prepared particles (GGN, SGG) were determined by using a Zetasizer NanoZS (Malvern, UK).

2.2.4. Cell culture

Rat embryonic cardiomyoblast derived H9c2 cells were obtained from American Type Culture Collection (ATCC) Rockville, MD. Cultures of cardiomyocytes, usually from rat neonatal hearts are widely used to investigate the cellular and molecular changes. H9c2 cells mimic most of the characteristic features of adult cardiac myocytes and this is an ideal cell line to check the effect of drug on myocardium in in vitro system. Cells were cultured in DMEM supplemented with 10% FBS, 100 U penicillin/ml and 100 µg streptomycin/ml in 5% CO₂ at 37 °C. Cells were passaged regularly and subcultured to 80% confluence before the experiments. Fresh nanoparticle solutions were prepared and checked to ensure consistency of physical chemical and biological properties of nanoparticles. Nanoparticles were freshly prepared before use and vortexed thoroughly before being added to the cells. Experimental design consist of following groups unless otherwise specified (1) control cells (2) cells treated with sodium selenite alone (5, 25 and 50 nM) and (3) cells treated with SGG (5, 25 and 50 nM). Observations were made after 1, 6 and 24 h of incubation.

2.2.5. Evaluation of cell viability

Cell viability was determined by MTT assay. Cells in exponential growth phase were plated at 5×10⁴ cells per well in 24-well plate. Then, cells were exposed to various concentrations (5, 25 and 50 nM) of sodium selenite, and SGG for 1, 6 and 24 h and they were subjected to MTT analysis. For this 350 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The formazan crystals thus formed were dissolved in DMSO and the absorbance was read after 45 min in a microplate reader (Biotek Synergy 4, US) at 570 nm and percentage of viable cells were calculated.
2.2.6. Intracellular localization of SGG nanoparticles

Initial experiments were conducted to see whether H9c2 cells uptake nanoparticles. We found that both GGN and SGG emit autofluorescence at blue region (excitation: 360 nm; emission: 435 nm). For cellular uptake studies, cells were seeded in 96-well plate at a density of $5 \times 10^3$ per well and treated with various concentrations of SGG (5, 25 and 50 nM) for 1, 6 and 24 h and single concentration (600 µg) of macro guar gum (MGG) as reference. The cells of all experimental groups were subjected to DNA staining with AO (excitation: 502 nm; emission: 525 nm). Then the cells were subjected to fluorescent imaging by spinning disk microscope (BD PathwayTM Bioimager system, USA).

2.2.7. Estimation of intracellular Se concentration

Cells were treated with various concentrations of Se and SGG (5, 25 and 50 nM) for 24 h to check the influx of Se. For this 100 µl of cell homogenate were digested with 500 µl of HNO$_3$/ HClO$_4$ (4:1; v/v) at 190 ºC for 90 min. After cooling to room temperature, 500 µl of 5 M HCl was added and the open glass tubes were heated to 150 ºC for 30 min. Then 2 ml of 2.5 mM EDTA and 500 µl of diaminonaphthalene reagent was added at room temperature and the mixture were left at 55 ºC for 30 min. 1 ml portion of cyclohexane was used to extract the piazselenol and fluorescence was measured using a fluorimeter at excitation of 364 nm and emission of 520 nm (Wilkie 1970).

2.2.8. Nanoparticle interaction on plasmid DNA

To check whether nanoparticle prepared has any effect on DNA we conducted experiment on plasmid pUC 18. The reaction mixture consisting of plasmid DNA and various concentrations of SGG was subjected to agarose gel electrophoresis and visualized by EtBr staining. In addition, we also conducted study to see whether SGG protect DNA from hydroxyl radical induced damage. For this, the reaction was conducted at a total volume of 14 µl containing 2 µl of plasmid pUC 18 DNA (50 ng DNA/µl) in 5 µl of 5, 25 and 50 nM concentration of Se and SGG, 7 µl of Fenton’s reagent. The DNA (supercoiled, linear and open circular) was analyzed on 1% agarose gels and visualized. Ellagic acid was used as positive control.
2.2.9. DNA integrity

Briefly the cells in all experimental groups were stained with AO (excitation: 502 nm; emission: 525 nm) and EtBr (excitation: 510 nm; emission: 595 nm) to detect apoptosis and processed for fluorescent imaging to see alteration with various treatments. The working stain (100 µg/ml of AO and 100 µg/ml EtBr in phosphate buffered saline) was added to cells and was examined under spinning disc fluorescent microscope.

2.2.10. ROS generation with nanoparticles

Oxidative stress in response to nanoparticle incubation was measured by determining intracellular ROS generation. Intracellular ROS was determined by oxidative conversion of cell-permeable DCFH-DA to fluorescent 2’, 7’ dichlorofluorescein (DCF). For this H9c2 cells were seeded in 96-well plate and treated with different concentrations of Se and SGG (5, 25 and 50 nM) with different time duration. DCFH-DA stain in serum free medium was co-incubated with H9c2 cells at 37 °C for 20 min. After three washes, DCF fluorescence was measured by fluorimetry (excitation: 488 nm; emission: 525 nm) in multiwell plate reader and fluorescent imaging was done to detect the differences in the intensity of fluorescence emitted.

2.2.11. Alteration in Δѱm

The cells were seeded in 96-well plate in 200 µl of culture medium and treated with different concentration of Se and SGG (5, 25 and 50 nM) for 24 h. The experiment was done as per the protocol provided with the kit (JC-1 kit, Sigma). After respective treatments the cells were stained with JC-1 stain for 20 min at 37 °C and wash with growth medium. The shift of fluorescence was visualized under spinning disk microscope and fluorescence intensity was measured in multiwell plate reader. In normal cells, the JC-1 dye concentrates in the mitochondrial matrix where it forms red fluorescent aggregates because of the electrochemical potential gradient. Dissipation of Δѱm prevents the accumulation of JC-1 in the mitochondria and thus it is dispersed throughout the cell, leading to a shift from red (J-aggregates) to green fluorescence (JC-1 monomers) and visualized under spinning disk microscope. For JC-1 monomers, the fluorimeter was set at 490 nm excitation and 530 nm emission wavelengths and for J-aggregates, the fluorimeter was set at 525 nm excitation and 590 nm emission wavelengths. Valinomycin (1 mg/ml) was used as positive control.
2.2.12. Cytoskeleton integrity

Phalloidin staining was used to determine cytoskeletal integrity of the cells upon nanoparticle treatment. The cells from experimental groups were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized and dehydrated with cold 100% acetone for 3-5 min. Phalloidin stain (excitation: 488 nm; emission: 525 nm) was added and kept at room temperature for 30 min. Nucleus was counterstained with DAPI (excitation: 360 nm; emission: 450 nm) and visualized.

2.2.13. Antioxidant potential

2.2.13.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by the deoxyribose method (Halliwell et al 1987) with slight modifications. Different concentrations of samples were mixed with an adequate amount of potassium buffer (pH 7.4). To this freshly prepared 0.1 ml of FeCl$_3$ (200 µM), 0.05 ml of H$_2$O$_2$ (1 mM), 0.1 ml of EDTA (1.04 mM), 0.05 ml of deoxyribose (28 mM) and freshly prepared 0.05 ml of ascorbic acid (1 mM) were added and then the mixture was incubated at 37 °C for 1 h. To that 2% TCA (0.5 ml) and 1% TBA (0.5 ml) were added and the mixture was heated in a water bath at 100 °C for 20 min. Absorbance of the resulting solution was measured at 532 nm. The absorbance of blank was also measured under similar conditions using catechin as the standard. The IC$_{50}$ value was obtained from a dose response curve plotted between the percentage of inhibition and concentrations.

2.2.13.2. Total reducing power

Total reducing power (TRP) of the samples (Se, GGN and SGG) was determined colorimetrically using gallic acid as standard at the wavelength of 700 nm (Oyaizu 1986). Samples of different concentrations were mixed with distilled water, 200 mM phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated for 20 min at 50 °C. 10% TCA was added to the mixture, followed by vigorous vortexing for 10 min and the upper layer was mixed with distilled water and 0.1% of FeCl$_3$. The absorbance of the resultant solutions was read at 700 nm against blank. Increased absorbance indicates increased reducing power.

2.2.13.3. Metal chelating activity

The chelation of ferrous ions by the Se, GGN and SGG was estimated using
ferrozine (Rani et al 2010). Ferrozine can quantitatively form complexes with Fe$^{2+}$. In the presence of chelating agent the complex formation is disrupted resulting in the reduction of red colour of the complex. Measurement of colour reduction leads to the estimation of metal chelating activity. EDTA was used as the standard. The absorbance of red colour was read at 562 nm.

2.2.14. Statistical analysis

Results were expressed in mean and standard deviation (SD) of the control and treated cells from three independent experiments with duplicates (n=6). Data were subjected to one-way ANOVA followed by the Bonferroni test (Sokal and Rohlf 2009) to calculate the statistical difference among the groups using SPSS for Windows, standard version 11.5.1 (SPSS, Inc.) and significance was accepted at P≤0.05.

2.3. Results

2.3.1. Particle size measurements

In the present study GGN and SGG were prepared by nanoprecipitation method. Particle size analysed by PCS indicated the presence of fine spherical nanoparticles of size of ~41–132 nm with a polydispersity index (PDI) of 0.4 (Figure.2.1.a). In SGG the size of the nanoparticle had increased to ~69–173 nm range upon Se incorporation (Figure.2.1.b). This result is expected since selenite carried negative charges and electrostatically interacted with GG which would promote formation of nanoparticles through ionic cross-linking. Particle size is one of the most important parameters determining biocompatibilities and bioactivities of materials of therapeutic importance.

![Figure 2.1](image.png)

**Figure 2.1.** Particle size distribution of (a) GGN and (b) SGG. The prepared nanoparticles were subjected to particle size analysis. (a) GGN had an average particle size varies from 41 to 132 nm range and (b) SGG nanoparticle prepared by nanoprecipitation method shows an increase in particle size from 69 to 173 nm.
2.3.2. Transmission electron microscopic images of GGN and SGG

The TEM analysis of GGN revealed the presence of fine spherical nanoparticle of size 40 nm range with few larger particles. It was found that the sizes observed by DLS were larger than those determined by TEM images. This might be due to the fact that GG binds to the surface of the Se which in turn creates a layer and this has made the particles appear larger. TEM images also confirmed that upon Se incorporation the size of GGN had increased to 50–100 nm (Figure 2.2.a, b). An elemental composition analysis by TEM-EDX showed the presence of strong signals from the Se atoms together with signal of C, Na and O atom from SGG (Figure 2.2.c) confirming the presence of Se in SGG.

![TEM images of GGN and SGG](image.png)

**Figure 2.2.** TEM images of (a) GG showing spherical morphology with a size of 40 nm with few larger particles (b) SGG were also spherical in shape, upon incorporation of Se the particle size has been increased to the range of 50–100 nm (c) EDX spectrum of SGG show the presence of Se peaks, thereby confirming the presence of Se in SGG.
2.3.3. XRD characterization of nanoparticles

In nanoparticle preparation it is very important to control the particle size, shape and morphology. To determine the crystalline property of nanoparticles, SGG was characterized by XRD as it is an important analysis tool in nanomaterial science. Figure 2.3.a, b and c show the powder X-ray diffraction patterns of GG, sodium selenite and SGG respectively. XRD of GG (Figure 2.3.a) was amorphous because of its polymeric nature. XRD pattern of pure sodium selenite was compared with the standard JCPDS file data 32-1153. Peaks corresponding to sodium selenite had been observed in figure 3.c, confirming the incorporation of Se in SGG.

![XRD images of (a) GG (b) Sodium selenite and (c) SGG. GGN appears amorphous in nature in their morphology as per XRD data (Figure 3.a). While sodium selenite appears crystalline nature in the XRD pattern (Figure 3.b). The presence of major peaks of Se in SGG confirms the incorporation of Se in GGN (Figure 3.c).](image)

2.3.4. Behaviour of nanoparticles in cell culture medium

The agglomeration of nanoparticles in liquid medium is of great importance in nanomedicine. The size of the SGG in cell culture medium without serum was found to be 286.3 nm with a PDI value of 0.5 (Figure 2.4.a,b) while that in serum containing medium was 22.83 nm with a PDI value of 0.5 (Figure 2.4.a,a). The particle size observed in DLS was considerably decreased from the previous results due to the enhanced repulsive interparticle interactions, especially with proteins in serum containing medium.
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(Gil et al 2009). The multimodal peak obtained for GGN in solution for both serum containing and serum free medium indicates a broad distribution of particles in agreement with the primary DLS results (Figure 2.4b.a, b). The observed PDI value ranging from 0.1-0.5 indicates that SGG possess the ability to remain as primary particles in cell culture medium with and without 10% FBS, suggesting that the SGG is relatively non-toxic in solution and the particles are remaining as homogeneous in nature.

Figure 2. 4a. Particle size distribution for (a) SGG in serum containing DMEM (b) SGG in serum free DMEM

Figure 2. 4b. Particle size distribution for (a) GGN in serum free DMEM (b) GGN in serum containing DMEM

2.3.5. Morphological analysis of cells upon treatment with nanoparticles

The cytotoxicity of the SGG was verified for its biomedical application. H9c2
myoblast are spindle to stellate shaped that can be mono or multinucleated. The morphological examination of the cells treated with different doses of both SGG and Se showed normal cell morphology up to 50 nM for 24 h exposure (Figure 2.5).

**Figure 2.5.** Morphological examination of cells with nanoparticles. Images of H9c2 cells from different experimental groups under phase-contrast microscope (10x). (a) control cells; (b, c, d) cells treated with 5, 25 and 50 nM Se; (e, f, g) cells treated with 5, 25 and 50 nM SGG, respectively.

### 2.3.6. MTT assay

Nanomaterials are used increasingly in cosmetics, medical imaging, disease diagnosis and drug delivery. However more and more evidence indicates that reduction to nanoscale causes marked differences in properties compared with macroscale. In order to evaluate the cytotoxicity of the newly synthesized SGG and the bulk Se, H9c2 cells were treated with various concentrations (5, 25 and 50 nM) for various duration (1, 6 and 24 h). The results showed that 5 and 25 nM of Se and SGG nanoparticle for 1, 6 and 24 h of incubation were non-toxic. But higher dose (50 nM) of Se and SGG showed significant toxicity of 17.4% and 14.59% after 24 h incubation respectively (Table 2.1).
Table 2.1. Viability of H9c2 cardiac myoblast cells treated with sodium selenite, GGN and SGG

<table>
<thead>
<tr>
<th>Concentration</th>
<th>% toxicity</th>
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<tr>
<td></td>
<td>1 h</td>
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<tr>
<td>Sodium selenite (nM)</td>
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</tr>
<tr>
<td>5</td>
<td>1.73 ± 0.39</td>
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<tr>
<td>25</td>
<td>2.38 ± 0.43</td>
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<tr>
<td>50</td>
<td>9.05 ± 0.48</td>
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<tr>
<td>GGN (µg)</td>
<td></td>
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<tr>
<td>5</td>
<td>2.63 ± 0.37</td>
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<tr>
<td>25</td>
<td>4.44 ± 0.89</td>
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<tr>
<td>50</td>
<td>5.14 ± 0.30</td>
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<tr>
<td>SGG (nM)</td>
<td></td>
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<tr>
<td>5</td>
<td>1.79 ± 0.64</td>
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<tr>
<td>25</td>
<td>2.61 ± 0.93</td>
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<tr>
<td>50</td>
<td>10.87 ± 1.12</td>
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2.3.7. Uptake of SGG by H9c2 cell

To evaluate the cellular uptake of SGG and MGG we utilized autofluorescence property of GG. For this, cells were incubated with various concentrations (5, 25 and 50 nM) of SGG and MGG for 1, 6 and 24 h and counter stained with AO that stains the double and single stranded DNA of live cells which appears green in color and were subjected to fluorescence imaging by spinning disk microscope. We observed presence of fluorescence in the SGG treated cells (Figure 2.6A, 6B, 6C) whereas no fluorescent emission was observed in MGG treated cells (Figure 2.6A.b, 6B.b, 6C.c). This observation reveals the presence of SGG in cells not MGG. In addition, fluorescent data showed uptake of nanoparticle was dose and duration dependent. The maximal cellular uptake was found in 24 h exposure with high dose (Figure 2.6C).
Figure 2. 6A. Uptake of nanoparticle by H9c2 cardiac myoblasts for 1h incubation with various concentrations of SGG. (a) control; (b) MGG; (c, d, e) cells treated with 5, 25 and 50 nM SGG.

Figure 2. 6B. Uptake of nanoparticle by H9c2 cardiac myoblasts for 6 h incubation with various concentrations of SGG. (a) control; (b) MGG; (c, d, e) cells treated with 5, 25 and 50 nM SGG.
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Figure 2. 6C. Uptake of nanoparticle by H9c2 cardiac myoblasts for 24 h incubation with various concentrations of SGG. (a) control; (b) MGG; (c, d, e) cells treated with 5, 25 and 50 nM SGG.

2.3.8. Differential Se uptake by H9c2 cells

We examined the cellular uptake of Se from sodium selenite and SGG in H9c2 cell lines with diaminodihaloethane reagent. For this, cells were treated with different concentrations of sodium selenite (5, 25 and 50 nM) and SGG (5, 25 and 50 nM) for 24 h. Analysis showed that 25 nM of SGG was found to have more effective uptake of Se (7.2 nM) which was found to be significantly greater than Se uptake from sodium selenite (5.2 nM). Beyond 25 nM, concentration did not show any influence on Se uptake whether in SGG or sodium selenite (Figure 2.7). The more cell permeability of nanoparticle will enable to have more drug bioavailability at target site for better therapeutic property. This also reduces the dose of drug required for recovery and reduces the adverse effect of drug.
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Figure 2.7. In vitro cellular uptake of Se from sodium selenite and SGG. Se uptake study using diaminonaphthalene method shows that uptake of Se by cell is higher for SGG (red) than sodium selenite (blue). 25 nM of both Se and SGG showed maximum uptake of Se by cells.

2.3.9. Effects of SGG on apoptosis

In order to characterize the safety profile of the SGG we investigated whether SGG induce apoptosis in H9c2. To evaluate apoptosis inducing property of SGG, cells were stained with AO/EtBr and it was found that the exposure of H9c2 cells with different concentrations of 5, 25 and 50 nM of Se and SGG nanoparticle for 24 h did not cause apoptosis (Figure 2.8). SGG was effective to protect the cells from apoptosis at 50 nM for 24 h and this property of SGG will definitely help us to use this nanoparticle for therapeutic purpose against various disorders.
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Figure 2.8. Alteration in DNA integrity with Se and SGG. Photomicrographs of H9c2 cells under AO/EB staining to check induction of apoptosis with Se and SGG (20x); (c) merged images of control cells; (f, i, l) merged images of cells treated with 5, 25 and 50 nM Se; (o, r, u) merged images of cells treated with 5, 25 and 50 nM SGG.

2.3.10. DNA protection assay

Oxidative DNA damage has been implicated in various degenerative diseases. Sodium selenite is reported to inhibit oxidative DNA damage caused by iron (Fe$^{2+}$) in the presence of H$_2$O$_2$, in a cell free system, which contained plasmid DNA, Fe$^{2+}$ and H$_2$O$_2$ (Ramoutar and Brumaghim 2007). The effect of GGN and SGG on Fe$^{2+}$ dependent hydroxyl radical induced DNA damage of pUC18 plasmid was studied. The treatment of supercoiled (SC) DNA with Fenton’s reagent directed to the alteration of DNA to open circular form (OC). The addition of SGG nanoparticles to the reaction mixture substantially decreased the DNA strand scission and retained the SC form, thus
effectively protect DNA, in a dose dependent manner (Figure.9A). In addition we also checked whether it induce breakage or nick or ladder formation on plasmid DNA (pUC18) by agarose gel electrophoresis (Figure.9B). It did not affect plasmid DNA.

![Figure 2. 9A. DNA damage protection assay with Fenton’s reagent. (a) control (pUC 18 plasmid); (b) plasmid+Fenton’s reagent (c) positive control (ellagic acid 50 nM) (d, e and f) 5, 25 and 50 nM Se; (g, h, i) 5, 25 and 50 nM SGG. B. The effect of nanoparticle on plasmid DNA (a) control (pUC 18 plasmid); (b, c) positive control (ellagic acid 5, 50 nM); (d, e, f) 5, 25 and 50 nM Se; (g, h, i) 5, 25 and 50 nM SGG.]

2.3.11. Effect of SGG on ROS

Induction of oxidative stress is one of the common mechanisms of toxicity of nanoparticles (Soenen et al 2011). Oxidative stress occurs when generation of ROS exceed the capacity of antioxidant defense mechanism. It elicits a wide variety of physiological and cellular events including stress, inflammation, DNA damage and apoptosis. In the present study, attempts were made to evaluate ROS generation with various doses of nanoparticle to see whether they induce oxidative stress in H9c2 cells and it was found that treatment with various doses (5, 25 and 50 nM) of Se and SGG caused mild ROS generation (41%) after one hour and gradually reduced to (8%) in a span of 24 h (Figure.2.10C). It is interesting to note that the initial outburst of ROS did not cause any morphological alteration or toxicity on cells (Figure.2.10A). This temporary elevation of ROS (Figure.2.10A) may be due to over reaction of cells for self adaptation to the presence of the foreign material.
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Figure 2. 10A. Evaluation of ROS with various doses of Se and SGG after 1 h. (a) control cells; (b, c, d) cells treated with 5, 25 and 50 nM Se, respectively; (e, f, g) cells treated with 5, 25 and 50 nM of SGG, respectively; (h) The fluorometric analysis supports the microscopic data. B. Evaluation of ROS with various doses of Se and SGG after 6 h. (a) control cells; (b, c, d) cells treated with 5, 25 and 50 nM Se, respectively; (e, f, g) cells treated with 5, 25 and 50 nM of SGG, respectively; (h) The fluorometric analysis data. C. Evaluation of ROS with various doses of Se and SGG after 24 h. (a) control cells; (b, c, d) cells treated with 5, 25 and 50 nM Se, respectively; (e, f, g) cells treated with 5, 25 and 50 nM of SGG, respectively; (h) The fluorometric analysis data.

2.3.12. Alteration in ΔΨm of mitochondria

Mitochondria are the vital organelle which play significant role in the physiology of the cells and it is the centre of target for foreign particles interaction (El-Ansary and Al-Daihan 2009). In this study we verified the effect of various concentration of SGG on mitochondria of H9c2 cells. H9c2 cells are known for the high content of mitochondria to meet its metabolic need. Intact mitochondrion is very much essential for the normal well being of cells as it control many sensitive functions related to energy metabolism. Exposure of H9c2 to Se and SGG for different time duration (1, 6 and 24 h) did not cause much alteration in ΔΨm, as measured with JC-1 probe with an average ratio of red:green fluorescence (Figure.2.11A, 11B). But high dose (50 nM) caused some alterations in ΔΨm in the case of 24 h of incubation (Figure.2.11C).
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A

B

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Figure 2. 11A. Mitochondrial transmembrane potential with Se and SGG for 1 h. Images of H9c2 cells stained with JC-1 for mitochondrial study (20x). (a) control cells; (b) positive control valinomycin; (c, d, e) cells treated with 5, 25 and 50 nM Se, respectively; (f, g, h) cells treated with 5, 25 and 50 nM SGG, respectively; (i) relative fluorescence also showed a similar trend. B. Mitochondrial transmembrane potential with Se and SGG for 6 h. (a) control cells; (b, c, d) cells treated with 5, 25 and 50 nM Se, respectively; (e, f, g) cells treated with 5, 25 and 50 nM SGG, respectively; (h) relative fluorescence also showed a similar trend. C. Mitochondrial transmembrane potential with Se and SGG for 24 h. (a) control cells; (b, c, d) cells treated with 5, 25 and 50 nM Se, respectively; (e, f, g) cells treated with 5, 25 and 50 nM SGG, respectively; (h) relative fluorescence also shows a similar trend.

2.3.13. Effects of SGG on cell cytoskeleton

To check the effect of cellular uptake of nanoparticles on cytoskeleton organization of cardiac myoblast, F-actin component of cytoskeleton was stained using phalloidin. Staining revealed that there was no alteration in structure with Se and SGG treated group (Figure.2.12.b-g). All the groups had an intact filamentous network structure confirming the original structure. Nanoparticles upto 50 nM were safe in holding intact the mesh like architecture of the cells even at 24 h (Figure.2.12.d, g).
2.3.14. Total reducing power

To evaluate the antioxidant potential of Se, GGN and SGG various cell free assays like total reducing power, metal chelation and hydroxyl radical scavenging were checked. The high absorbance value of the test materials at 700 nm indicates potential reductive ability. TRP values of Se, GGN and SGG were compared with gallic acid as the standard. Reducing power (absorbance values) of Se, GGN and SGG was 0.028±0.004, 0.098±0.003 and 0.234±0.006, respectively (Table 2.2). From these the reducing power of SGG was found to be significantly higher than those of Se and GGN. In this assay, the yellow colour of the solution changed depending on the reducing power of each material. The data showed that the SGG have the significant ability to interact with free radicals to convert them to nonreactive species, thus terminating the radical chain reaction.

2.3.15. Metal chelation

Chelation of the metal ions is the main strategy to avoid ROS generation. Here,
the IC\textsubscript{50} of SGG for metal chelating activity was 3.6±0.05 µg/ml which was better than the positive standard EDTA (12.72±0.68 µg/ml) indicating the potential metal chelating capacity of SGG (Table 2.2). The IC\textsubscript{50} of the chelating effect of Se and GGN was 12.11±0.01 µg/ml and 4.1±0.1 µg/ml, respectively.

### 2.3.16. Hydroxyl scavenging activity

The IC\textsubscript{50} of Se, GGN and SGG for hydroxyl radical scavenging activity was 63.79±1.82, 11.97±1.13, 5.68±0.285 µg/ml respectively (Table 2.2). Catechin was used as the standard with IC\textsubscript{50} of 9.22±0.94 µg/ml respectively. Hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology (Li et al 2008). This radical has a capacity to join nucleotides in DNA and cause strand breakage that contributes to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz et al 2002). Here also SGG showed excellent hydroxyl scavenging activity compared to other materials and the positive control. These assays evaluate the scavenging potential of particles with respect to specific reactive radicals. Evaluation of antioxidant potential of the various test material revealed SGG exhibited better antioxidant activities like high reducing power, metal chelation capacity and hydroxyl radical scavenging activity compared to GGN and Se.

### Table 2. 2. Antioxidant chemical assays with Se, GGN and SGG.

<table>
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<tr>
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<th>TRP</th>
<th>Metal chelation</th>
<th>Hydroxyl RSA</th>
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<tr>
<td></td>
<td></td>
<td>IC\textsubscript{50} (µg/ml)</td>
<td>IC\textsubscript{50} (µg/ml)</td>
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<tr>
<td>GGN</td>
<td>0.098±0.003</td>
<td>4.1±0.1</td>
<td>11.97±1.13</td>
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<tr>
<td>SGG</td>
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<td>3.6±0.05</td>
<td>5.68±0.285</td>
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<tr>
<td>Se</td>
<td>0.028±0.004</td>
<td>12.11±0.01</td>
<td>63.79±1.82</td>
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<tr>
<td>GA</td>
<td>1.236±0.13</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>Catechin</td>
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Se: Sodium Selenite; GGN: guar gum nanoparticle; SGG: selenium incorporated guar gum nanoparticle; GA: gallic acid; RSA: radical scavenging activity. Each value represents mean ± SD (n=6).
2.4. Discussion

Nanoparticle characterization is indispensable for understanding and controlling of nanoparticle synthesis and applications. In the present study, the techniques used for these were particle size analysis, TEM and XRD. This chapter deals with preparation and characterization of SGG and its interaction with H9c2 by assessing its effect on various vital parameters. For this we conducted *in vitro* experiments to evaluate its interaction with H9c2 cell lines.

Although, biochemicals may often be used for the synthesis of nanomaterials, the biogenic synthetic route is frequently applied due to its ease and simplicity and also because no hazardous and toxic residues are released in the environment (Mukherjee *et al.* 2002; Mukherjee *et al.* 2001). There are reports that the reduction of sodium selenite (Na$_2$SeO$_3$) to Se nanoparticles by the biomolecules in the polysaccharide is by functional groups such as alcohol, aldehyde etc. (Husen and Siddiqi 2014). Important and challenging aspect of nanoparticle characterization is measurement under conditions that resemble *in vitro* or *in vivo* environment. It was shown in several studies (Ji *et al.* 2010; Maiorano *et al.* 2010; Metin *et al.* 2011; Fatisson *et al.* 2012; Allouni *et al.* 2009; Lordan *et al.* 2012; Safi *et al.* 2011; Zhang *et al.* 2009; Santander-Ortega *et al.* 2007) that stability of nanoparticles in different culture media can be severely reduced depending on ionic and protein composition consequently affecting nanoparticles characteristic and functionality in *in vitro* and *in vivo* applications. Literature also demonstrated that characterization of nanoparticles in relevant media is necessary for evaluation of toxicity (Fatisson *et al.* 2012). The stability of nanoparticles is a complex combination of nanoparticles surface properties, media compositions and nanoparticle concentrations, so it is essential to characterize the nanoparticles in physiologically relevant media which is crucial for understanding of their interaction with biological systems. Pavlin and Bergar (2012) reported about nanoparticles functionalized with polyacrilic acid are relatively very stable in different culture media, where level of aggregation depends on medium composition. Our study also the shows that the SGG particles showed more stability in serum medium than medium without serum. Compared with GGN in serum and serum free medium SGG particles have more stability. On the other hand there are several reports (Ji *et al.* 2010; Allouni *et al.* 2009; Zhang *et al.* 2009) identifying the increased stability of nanoparticles
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(TiO2) by the addition of the serum in the medium with the stabilization mechanism being again opsonization of the nanoparticle surface with proteins and peptides, which form stabilizing protein corona. This was similar to our results.

An important factor that usually contributes to nanomaterial based drug cytotoxicity is cellular uptake (Sohaebuddin et al 2010). GG is a non-ionic polysaccharide that is abundantly present in nature and has many properties desirable for drug delivery applications. Due to the presence of various functional groups on molecular chains polysaccharides can be easily modified chemically and biochemically, resulting in different kind of derivatives (Liu et al 2008). As they are highly stable, safe, non-toxic, hydrophilic, biodegradable and the cost of processing is very low, they are used as ideal material for drug delivery. Among polysaccharides GG is a potential candidate for drug delivery application due to its drug release retarding property and susceptibility to microbial degradation in the large intestine (Bayliss and Houston 1986). It is established fact that nanoparticles can efficiently intrude in cell by exploiting endocytosis machinery. But only specialised cells such as macrophages are capable of phagocytosis. On the other hand almost all cells internalize nanoparticles by pinocytosis. There are many factors like physicochemical properties like size, shape, surface charge and surface chemistry that was essential for modulating cellular uptake efficiency.

Se is a trace element with wide commercial applications, due to its special chemical and physical properties. Se nanoparticles attracted more attention due to their high bioavailability and antioxidant activities, low toxicity and novel therapeutic property (Li et al 2011; Yang et al 2012; Wu et al 2012; Zhang et al 2013). There are reports to suggest that nanoencapsulation of Se in polysaccharide like chitosan increase cellular Se level (Zhang et al 2011). Se in encapsulated stage desensitizes the cells to Se compounds and decrease damage to cells in contrast to application of native Se to the cells (Zhang et al 2011). This explains why more Se in GG encapsulated form is taken up by the cells. Another reason for the minimum toxicity with SGG is due to the fact that SGG are not metabolically available for the induction of any type of cell damage.

Induction of apoptosis is considered an important cellular event that can account for the cancer preventive effects of Se compounds. Apoptosis induced by supranutritional doses of seleno compounds are described in various types of neoplastic cells, including
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prostate, colon and liver cancer, leukemia and lymphoma. Se in encapsulated form at nanoparticle size upregulates selenoenzyme (Sanmartín et al 2012). DNA protection property of SGG will have therapeutic potential as in some metabolic disorders like diabetes, hydroxyl radical induced DNA damages are very common. From these results it is again clear that SGG prepared by nanoprecipitation is safe as well as it has therapeutic potential to use for biomedical research applications. There are reports that Se nanoparticles prevented DNA damage when cells were exposed to UV-light (Prasad et al 2013). In this study SGG inhibits oxidative DNA damage caused by iron (Fe^{2+}) which contained plasmid DNA. A mild increase in oxidative stress seen in cells act as a cell signaling mechanism required to trigger several responses to the foreign particle (Ruiz-Meana et al 2010). These results again confirm the suitability of SGG for biomedical application without any adverse effect. Normal cytoskeleton is essential to keep the morphology and physical structure of the body intact. One possible sign of cellular stress induced by uptake of nanosized materials is alterations to the cytoskeleton network (Soenen et al 2011).

There is not even a single report on SGG nanoparticle and investigation on interaction with cell and this is the first report in this regard. But there are reports of preparation of Se nanoparticles with hyperbranched polysaccharide in water (Zhang et al 2010). However, it was not prepared for any biological applications. In addition Se nanoparticles with spirulina polysaccharide had been prepared by some groups (Yang et al 2012) for evaluation of its anticancer activities and uptake of Se nanoparticle by cells. They reported enhanced cytotoxicity and uptake by melanocytes. In the case of GG, there are reports on silver nanoparticle preparation using polyacrylamide/guar gum graft copolymers for some chemical purpose (Abdel-Halim et al 2011). Green synthesis had also been exploited for preparation of biopolymer (GG) silver nanoparticle composite for application in optical sensor for ammonia detection (Pandey et al 2012). Overall results clearly reveal SGG prepared in this study exhibit potent therapeutic potential with respect to oxidative stress, apoptosis, DNA protection, $\Delta \psi_m$ and cytoskeleton. Moreover the cytotoxicity was minimum with SGG which is a great advantage of this particle for biomedical applications.
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To prevent the detrimental effects of free radicals in the human body, antioxidants gain increasing interest. In addition, there is a preference for antioxidants from natural sources due to low adverse effects and high tolerance to the human body (Abdalla and Rooze 1999). The methods for evaluation of the antioxidant status and oxidative damage are many and varied. The simplest ones are purely chemical in vitro reactions or tests in cell cultures. They can yield useful information about mechanisms of action; currently, there is no single antioxidant assay for quality grading because of the lack of standard quantification methods. Keeping these facts in mind, we checked the antioxidant potentials of Se, GGN and SGG employing various cell free assays like total reducing power, metal chelation and hydroxyl radical scavenging. These assays evaluate the scavenging potential of particles with respect to specific reactive radicals. Evaluation of the antioxidant potential of the various test materials revealed that SGG exhibited better antioxidant activities like high reducing power, metal chelation capacity and hydroxyl radical scavenging activity compared to GGN and Se.

Redox active metals catalyse oxygen radical formation thus causes tissue injury during I/R sequence (Spencer et al 1998). This concept is based on the basic chemistry of oxygen radicals in which trace amounts of transition metals are required to catalyst the formation ROS such as the OH⁻. Transition metals such as Fe²⁺ and Cu⁺ are present in the myocardium and can act as catalyst for the formation of oxygen free radicals during reperfusion after myocardial ischemia. There are reports to suggest that transition metal chelators such as desferrioxamine can reduce the production of such radicals and may thereby attenuate postischemic myocardial dysfunction (Spencer et al 1998). Studies investigating the role of transition metals as a catalyst for the formation of oxygen free radicals during I/R primarily focussed on iron; Fe²⁺ chelators results in improvement in myocardial functional and metabolic recovery after I/R, presumably by preventing oxygen free radical generation (Bolli et al 1990; Farber et al 1988; Maruyama et al 1991; Bolli et al 1987). Hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology (Li et al 2008). This radical has a capacity to join nucleotides in DNA and cause strand breakage that contributes to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz et al 2002). Gao et al (2002) reported the antioxidant properties of hollow
spherical nanoparticles of Se. The size of nanoparticles plays an important role in their biological activity as 5-200 nm nano-Se can directly scavenge free radicals in vitro in a size dependent fashion (Peng et al. 2007). Barnaby et al (2011) reported Se nanoparticles which is prepared using gallic acid exhibit good antioxidant activity. There are reports regarding the antioxidant property of GG (Iqbal et al. 2013). Based on these report we checked the antioxidant properties of Se, GGN and SGG and the results showed that SGG has better activity compared to native Se and GGN to scavenge free radicals.

Conclusion

The present study demonstrated that SGG can be successfully prepared under mild conditions via, nanoprecipitation method. Physicochemical properties, such as particle size, TEM, XRD data confirmed the nanoscale structure of the prepared material and proper incorporation of SGG. Elaborate investigations on the interaction of nanoparticle on biological system had been conducted on H9c2 and confirmed the safety of nanoparticle on biological system (H9c2). On the basis of these results SGG was found to be an ideal nanomaterial for further research with promising therapeutic properties especially for cardiac problems.
References


Preparation and characterization of SGG and its biological evaluation in H9c2 cardiomyoblast nanoparticles (Nano-Se) at supranutritional levels on selenium accumulation and glutathione S-transferase activity. *J Inorg Biochem* 101: 1457-1463.


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