SYNTHESIS, CHARACTERIZATION OF BOROSILICATE NANOPARTICLES AND INVESTIGATION OF THEIR CYTOTOXICITY AND GENOTOXICITY IN HUMAN CELL LINES

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ABSTRACT

SYNTHESIS, CHARACTERIZATION OF BOROSILICATE NANOPARTICLES AND INVESTIGATION OF THEIR CYTOTOXICITY AND GENOTOXICITY IN HUMAN CELL LINES

In this study, firstly, we aimed to synthesize silica and borosilicate nanoparticles by the Stöber method. We then investigated the biological response of bronchoalveolar carcinoma-derived cells (A549) and healthy bronchoalveolar cells (BEAS2B) against the silica and borosilicate nanoparticles, by evaluating cytotoxicity and genotoxicity.

The nanoparticles were synthesized by a modified Stöber method. To prepare borosilicate nanoparticles tri methoxy boroxine (TMB) was used as boron source to coat the surface of the silica nanoparticles. By varying the amounts of ethanol and ammonia the size of nanoparticles were tuned from 60 to 450 nm. We proved the presence of boron in the borosilicate nanoparticles by Energy-Dispersive X-ray (EDX), Fourier Transformed Infrared (FTIR) spectroscopy and an acid-base titration method.

Cytotoxicity and genotoxicity induced both silica and borosilicate nanoparticles were investigated for the cell lines of A549 and BEAS-2B. Silica and borosilicate nanoparticles in all three sizes and dosages up to 500 μg/mL did not induce cytotoxic effects in A549 cells with incubation time up to 72 hours. The same amount of particles did not result in any cytotoxicity in BEAS-2B cells for 24 hours incubation, but they showed cytotoxic effects when the incubation time was increased to 48 and 72 hours. Furthermore, nanoparticles with sizes of 60 and 100 nm showed no genotoxicity for A549 but for BEAS-2B cells, silica nanoparticles induced genotoxic effects contrary to borosilicate nanoparticles.

ÖZET

BOROSİLİKAT NANOPARÇACIKLARIN SENTEZİ, KARAKTERİZASYONU VE İNSAN HÜCRE HATLARINDAKİ SİTOTOKSİK VE GENOTOKSİK ETKİLERİNİN ARAŞTIRILMASI

Bu çalışmada, öncelikle, Stöber metodunu uygulayarak, silika ve borosilikat nanoparçacıkları sentezlemeyi amaçladık. Sentezlenen parçacıkların kanserli akciğer epitel hücrelerindeki (A549) ve sağlıklı akciğer epitel hücrelerindeki (BEAS2B) sitotoksik ve genotoksik etkilerini araştırdık.

Borosilikat nanoparçacıklarını sentezlemek için, bor kaynağı olarak tri metoksi boroksin (TMB) kullanılmış olup, boroksin ile silika nanoparçacıklarının yüzeyi kaplanmıştır. Kullanılan etanol ve amonyak miktarları ayarlanarak, parçacık büyüklüklerinin kontrolü sağlanmış olup, 60 ile 430 nm boyutlarında silika ve borosilikat naoparçacıklar sentezlenmiştir. Borosilikat nanoparçacıklardaki bor varlığını ve miktarı Energy-Dağılımlı X-ışını (EDX) spektroskopisi, Fourier Dönüşümlü Kırmızıaltı (FTIR) spektroskopisi ve asit-baz titrasyon metodu ile belirlenmiştir.

Silika ve borosilikat nanoparçacıklarının A549 ve BEAS-2B hücreleri üzerindeki sitotoksik ve genotoksik etkileri incelenmiştir. Silika ve borosilikat nanoparçacıklar sentezlenen tüm büyüklüklerde 500 µg/ml konsantrasyona kadar, 24, 48 ve 72 saatlik inkübasyonlarında A549 hücrelerinde sitotoksik etki göstermemişlerdir. Bu parçacıklar, aynı miktarlarda BEAS-2B hücreleriyle inkübe edildiklerinde 24 saat sonra sitotoksik etki göstermemişler, fakat inkübasyon zamanı 48 ve 72 saate çıkarıldığında sitotoksik etki göstermişlerdir. Ayrıca, 60 ve 100 nm'lik nanoparçacıklarla yapılan çalışmalar, A549 hücrelerinde parçacıkların tetiklediği genotoksik etkileri olmadığını, fakat BEAS-2B hücrelerinde borosilikat nanoparçacıklarının tersine silika nanoparçacıklarının genotoksik etkiler yarattığı ortaya konmuştur.

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CHAPTER 1

INTRODUCTION

1.1. General Remarks for Nanotechnology

Nanotechnology is a very broad science that deals with manipulating matter with structures sized between 1 to 100 nanometer in at least one dimension and also consisting of developing such materials and devices in this size (Lohmuller et al. 2011). Being a multidisciplinary field, nanotechnology, is an assortment of many sciences which are chemistry, physics, biology, engineering, medicine, molecular biology and genetic. In Figure 1.1, the nanometric scale is illustrated with samples of materials which are natural and manmade.

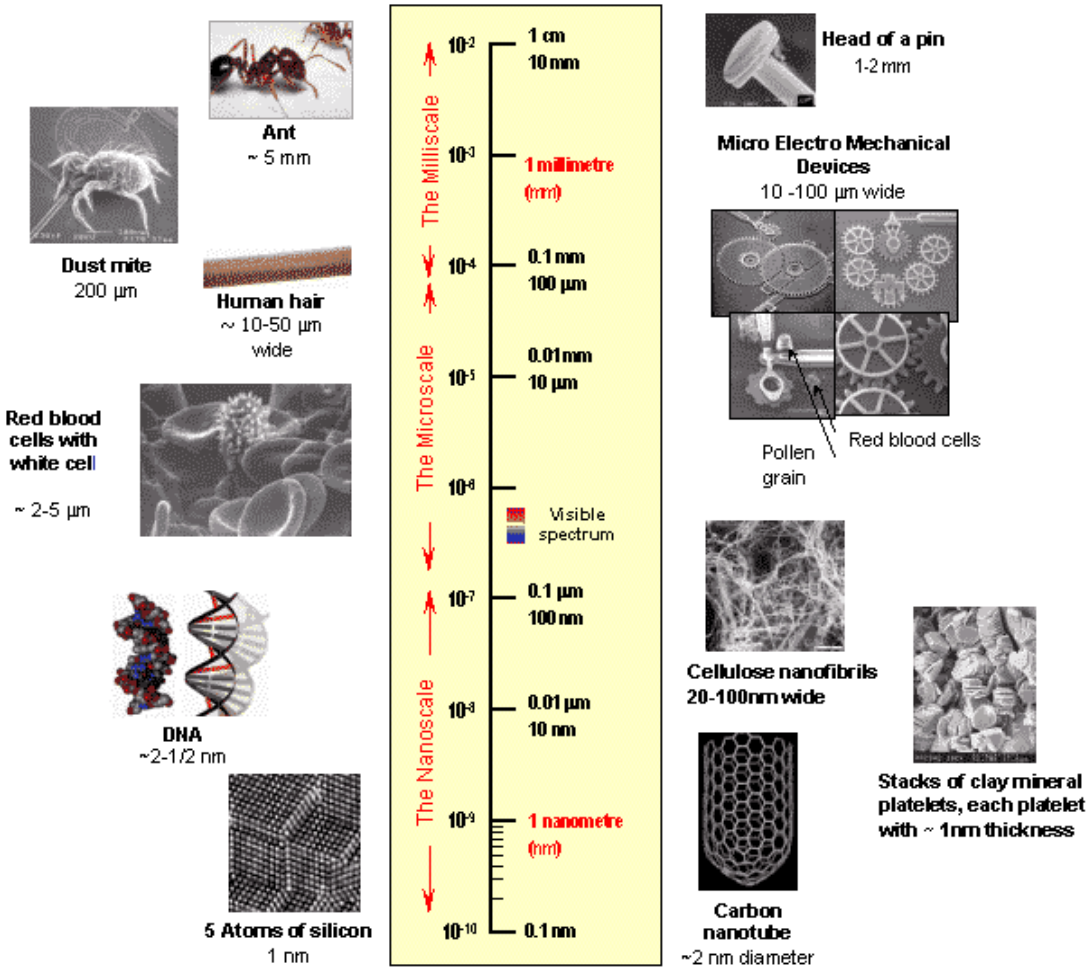


Figure 1.1. Understanding of nanotechnology
(Source: Töpfer 2009).

Nanotechnology exists on the borders between disciplines and technology domains. Several literatures called nanotechnology as a multidisciplinary field because multi-disciplined networked research is needed. It bounds up chemists, physicists, materials scientists, biologists, engineers and pharmacologists. Nanotechnology gives rise to breakthrough of development and application of nano-instruments in order to observe and manipulate materials in the nano-scale and the discovery of new nano materials such as nanotubes and also provides to enhance the building blocks of nano products. Therefore, nanotechnology introduces a precedent of science-based innovation, where an important revolution in analytical instruments, preceding discoveries and subsequent technological advancement stimulated the exploration of nano-scale structures and the development of nano-scale technologies.

In the 21st century, nanotechnology is thought as a technology that marks an era in terms of molecular and atomic engineering. There are a lot of examples about this

issue one of which is electronics, electrochemical systems especially for lab-on-a-chip devices that is comprised by precision engineering. Furthermore, it has the potential to produce advancing in biomedical applications in areas as diverse as gene therapy, drug delivery and novel drug discovery techniques. Many sciences and technologies are built upon nanotechnology and rather sophisticated. Both mechanistic version included materials science and microelectronics, having new or importantly improved mechanical, electrical and chemical properties or functions and a bio-mimetic version, more biotechnology inspired, having control of biological systems for achieving desired and designed outcomes, are comprehended by nanotechnology (Islam and Miyazaki 2010).

Nanotechnology as a human health care has so much benefit researches especially for medicine, communications, genomics, and robotics which are the prospect for the revolutionary. When desired to extend what that means, nanotechnology in drug delivery is so important the monitoring, repair, construction, and control of human biological systems at the molecular level, using engineered nanodevices and nanostructures One of the most significant issues is the proper distribution of drugs and other therapeutic agents within the patient's body (Sahoo et al. 2007).

So, nanotechnology has so many applications and demonstrated with its time line for prospective retainers that covers from textile, pigments to medicine and various devices used in engineering and also collaboration of these usages in Figure 1.2.

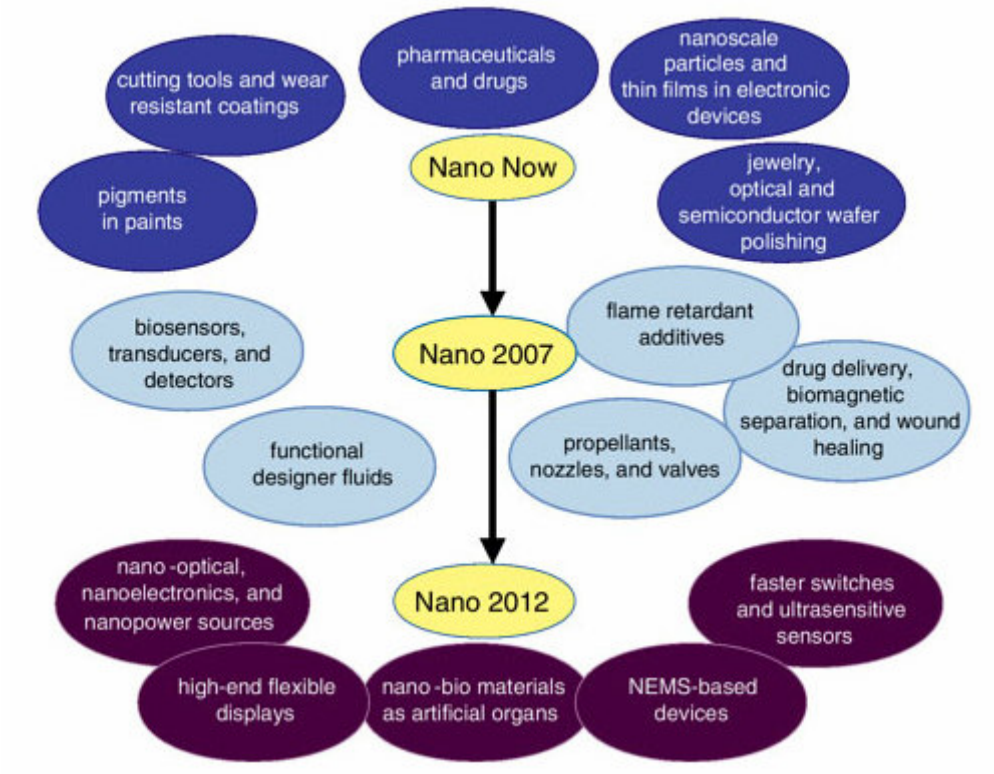


Figure 1.2. Current applications of nanotechnology and time line for anticipated advance (Source: STUPP 2002)

Nanotoxicology is coming up as a significant subdiscipline of nanotechnology. Nanotoxicology refers to the study of the interactions of nanostructures with biological systems with an emphasis on making clear the relationship between the physical and chemical properties (e.g. size, shape, surface chemistry, composition, and aggregation) of nanostructures with induction of toxic biological responses (Fischer and Chan 2007). In addition to this, nanotoxicology is so important because of the fact that interdisciplinary research now often consists of the toxicological and safety consideration of materials which are nanosized because of the plentifulness of nanomaterials in numerous medicinal applications and consumer goods (Philbert and Sayes 2010) in vitro toxicological studies have been increased explosively in publications compared to in vivo toxicological studies due to the fact that it has investigated the toxicity by inhalation, oral digestion and injection that required to test sample. That is to say, some animals whether small such as mice and rat or big mammal such as dog or monkey should be used. Thus, it brings about some entanglements due to the toxic effects of nanomaterials' on whole animals since all of the synthetic engineering. Furthermore, preparing in vivo test is ethically and administratively troublesome and individual research efforts have to work in partnership with the

institutional approval organization(s) such as IACUC (Institutional Animal Care and Use Committees). However, in vitro nanotoxicology has advantage over in vivo one because it is simpler to use various mammalian cells to test for viability or increase/decrease in a planned biological pathway against chosen engineered nanomaterials. For example, inorganic oxides including TiO_2, SiO_2, Fe_2O_3, carbon-based materials such as nanotubes, C60, and other nanoparticulates, and also semiconductor quantum dots and metal nanoparticles developed by different kinds of research groups. Some methods have been available for toxicology assays such as tetrazolium salt based assays, MTT and XTT, or WST. Now, a non-cytotoxic material can be easily identified due to the fact that various assay conditions brings about different results, the nature of the nanomaterial synthesis, and differences in physicochemical properties. Besides these, adsorption chemistry and physics of small molecules and biomolecules onto metal oxides have traditionally been a research topic in the physical sciences. Therefore, that provides to progress toxicology of nanomaterial (Suh et al. 2009).

1.2 The Purpose of The Study

The purpose of our study is to develop borosilicate nanoparticles and to evaluate cytotoxic and genotoxic response of healthy and carcinoma epithelial cell lines. To achieve these goals, colloidal chemistry will be used to synthesize the borosilicate nanoparticles. Biological response of the cells will be assessed by cytotoxicity and genotoxicity assays.

CHAPTER 2

PHYSICOCHEMICAL SYNTHESIS AND CHARACTERIZATION OF SILICA AND BOROSILICATE NANOPARTICLES

2.1. Introduction

Particles with size in the range of 1 to 100 nm are called as nanoparticles. They possess great surface to volume ratio because of their very small size. Monodisperse nanoscale colloids are one of the milestones of nanoscience and nanotechnology because of their well-defined dimensions and functional properties. Silica nanoparticles are the most functional colloidal material due to its ease of preparation and the silica nanoparticles can be modified easily because of their surface chemistry (Hartlen et al. 2008) such as adsorption, adhesion, chemical and catalytic properties. Therefore, surface chemistry of silica has gained too much attention for many practical applications because the surface chemistry of silica as an oxide adsorbent is directly related with the concentration and distribution of hydroxyl groups on the silica surface (Kim et al. 2009). Silica nanoparticles being inorganic shelling materials have many advantageous than organic as well as other inorganic materials. In particular, silica-based nanoparticles are resistant to microbial attack, stable in aqueous solutions, nontoxic, and biocompatible. In addition, they can be prepared easily with various sizes smaller than those obtainable for polymer nanoparticles. Also, since silica surface modification and bioconjugation chemistry is well established, silica NPs can be easily conjugated with a variety of biomolecules for binding to targets with specificity and high affinity, both of which are typically required by biological applications (Ma et al. 2009). In addition, silica nanoparticles have been used as electronic substrates, thin films, electrical/thermal insulators, emulsifiers, stabilizers, catalysis, pigments, pharmacy, electronic and thin film substrates and humidity sensors. The quality of some of these products is highly dependent on the size and size distribution of these particles (Rao et al. 2005; Rahman et al. 2007).

The common methods of synthesizing silica nanoparticles are the Stöber and microemulsion methods (Stöber et al. 1968; Yokoi et al. 2006).

2.2. Synthesis of Silica Nanoparticles

2.2.1. Synthesis of Silica Nanoparticles by Stöber Method

Monodisperse silica nanoparticles can be synthesized by the Stöber method involving hydrolysis and condensation of tetraethyl orthosilicate (TEOS) under alkaline conditions in ethanol. The size of silica particles are in the range of 40 nm to a few micrometers (Rahman et al. 2007). In the Stöber method, four reagents are mainly used: tetraethyl orthosilicate (TEOS), ammonium hydroxide (NH_4OH), ethanol (EtOH) and water (H_2O). TEOS is the silica source, NH_4OH is used as a catalyst and ethanol and water are used as solvents. Size of silica nanoparticles depends on solution chemistry of these reagents. In addition, pH, temperature and ionic strength play a significant role for size control and monodispersity of nanoparticles (Stöber et al. 1968; Ismail et al. 2010).

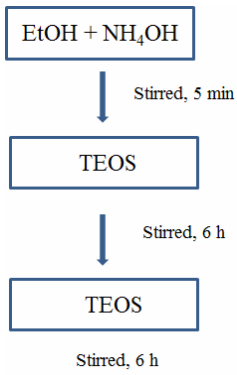


Figure 2.1. Flow chart of synthesis of silica nanoparticles.

The formation of silica particles can be divided into two stages. The first stage is the nucleation. The second stage is the growth process that starts with the condensation of the hydrolyzed monomers. There are two different approaches in order to describe particle formation and growth. The first approach is the addition of hydrolyzed monomers to the surface of the nuclei (Rao et al. 2005; Rahman et al. 2006). The second one is through a controlled aggregation (Matsoukas and Guleri 1988).

Nucleation leads to the formation of primary particles seed. Then, these primary particles will aggregate together to generate larger particles called as secondary particles. Formation of silica particles through hydrolysis and condensation reactions can be represented as seen in Figure 2.2.

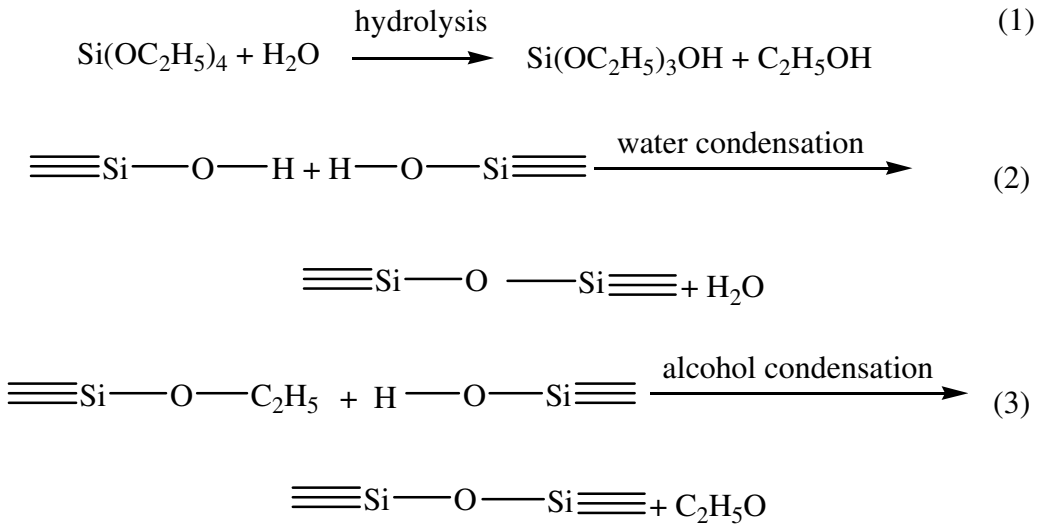


Figure 2.2. Reactions of formation of silica nanoparticles.

There are a lot of studies on synthesizing silica nanoparticles including optimizing the size of particles. Stöber et al. synthesized silica nanoparticles and controlled growth of monodisperse silica spheres in the micron size in 1968. Rahman et al. exhibited the effect of TEOS, ammonium hydroxide and temperature on the size of particle. Based on their observation, the concentration of TEOS was increased, the particle size slightly increased. Moreover, they demonstrated that the size of the particles increased sharply as the amount of ammonium hydroxide increased. According to their studies, as the temperature risen up, particle size decreased (Rahman et al. 2007). Rao et al. applied a different way to synthesize silica particles. They prepared silica nanoparticles by a sequential addition method. In this method, they mixed solvents, water and ethanol. Then, TEOS was added into the reaction flask and ammonium hydroxide was added finally (Rao et al. 2005). They investigated the effect of the parameters consisting of ethanol, TEOS, H_2O/TEOS, ammonium hydroxide and

temperature and agreed with the results reported by Rahman et al. (2007). Sharma et al. studied the usage of silica nanoparticles as the imaging agents such as dye doped silica nanoparticles and quantum dots. Sharma et al. considered that development of diagnostic tools and innovative therapies were very essential in order to understand the biological processes at the molecular level (Sharma 2006) by using fluorescent silica nanoparticles.

2.2.2. Synthesis of Silica Nanoparticles by Microemulsion Method

The other method to synthesize silica nanoparticles is the microemulsion method. In this method, there are mainly three reagents: water, surfactant and hydrophobic solvent such as oil. Microemulsion mainly allows size controlled production of particles.

Silica nanoparticles can be synthesized using a water-in-oil microemulsion method. Water-in-oil microemulsion can be defined as a thermodynamically stable, isotropic, and transparent solution of oil, water, and surfactant, wherein the water droplets are dispersed as nanosized liquid entities in a continuous domain of oil. The nanodroplets of water serve as a nanoreactor for the synthesis of these nanoparticles. This method has advantages in that it does not require extreme conditions of temperature and pressure and the particle size and shape can be simply controlled by varying microemulsion parameters. Because of the small size and the narrow size distribution of the products, the microemulsion method is preferred for the synthesis of nanodimensional particles. The microemulsion works well for smaller particles (< 100 nm). Various nanoparticles have been prepared using this method, showing its flexibility for the fabrication of different types and sizes of silica nanoparticles. (Bagwe et al. 2004; Jin et al. 2008; Meng et al. 2009).

Furthermore, Rahul et al. studied on the effect of ammonium hydroxide and water to surfactant ratio on the size of silica nanoparticles. They concluded that as the amount of ammonium hydroxide increased, the particle size decreased (Bagwe et al. 2004). Furthermore, Arriagada et al. synthesized silica in nanosize by using water-in-oil microemulsion method and investigated the effects of water to surfactant molar ratio

and ammonia concentration on particle size. They concluded that lower ammonia concentration provided smaller particle size but monotonically with increasing water to surfactant ratio. While the water to surfactant ratio was increased, the smaller particles were generated (Arriagada and Osseo-Asare 1999). Tapec et al. studied on the development of organic dye doped silica nanoparticles for bioanalysis and biosensors and they used two different silica sources to synthesize silica particles (Santra et al. 2001). In addition to these, Bagwe et al. interested in surface of silica nanoparticles synthesized by water in oil microemulsion. They determined the optimum balance of the use of inert and active surface functional groups including carboxylate, amine, amine/phosphonate, poly (ethylene glycol, octadecyl, and carboxylate/octadecyl (Bagwe et al. 2004). Hun et al. used silica nanoparticles for a different purpose especially for labeling. A sensitive fluoroimmunoassay for recombinant human interleukin-6 (IL-6) with Rubpy-encapsulated fluorescent core-shell silica nanoparticles labeling technique has been proposed by the microemulsion method (Hun and Zhang 2007).

2.2.3. Synthesis of Silica Nanoparticles Assisted by Amino Acid

An alternative method is developed based on a microemulsion method which uses amino acids such as L-Lysine and L-Arginine (Yokoi et al. 2006). L-Lysine is used as a catalyst instead of ammonia. It is a basic amino acid which provides the well-ordered arrangement of the silica nanoparticles with in a size range of 12-23 nm. It is biocompatible and L-form of Lysine is used due to being more stable than the D-form. Besides L-Lysine, another amino acid having amine groups could be used such as arginine. The hydrolysis and condensation reactions of TEOS as a silica source are used in the presence of L-Lysine (Yokoi et al. 2006). The main difference between the Stöber method and this method is the type of base. Yokoi et al. developed a different way to synthesize silica nanoparticles by using basic amino acids. So, well-ordered

silica nanoparticles may be synthesized with hydrolysis and condensation reactions of tetraethyl orthosilicate in the presence of basic amino acids, L-Lysine and L-Arginine.

2.3. Synthesis of Borosilicate Nanoparticles

Borosilicate nanoparticles have also many applications such as in the production of photonic bandgap devices with high optical contrast, contrast agents for ultrasonic microscopy, chemical filtration membranes (Parashar et al. 2008).

In the synthesis of borosilicate nanoparticles, formation of borosiloxane bonds takes important place. In the first step, alcohol is released, in the second step, water is released and in the third step, silanol and boroxol ring are formed (Tsvetkova et al. 2006). The mechanisms are shown in Figure 2.3.

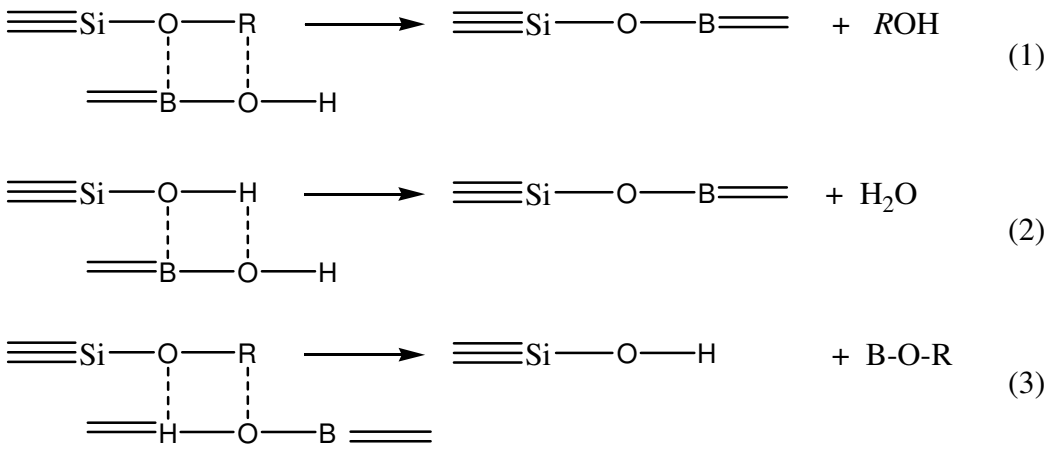


Figure 2.3. Reactions of formation of borosilicate nanoparticles (Source: Tsvetkova et al. 2006).

Boron source is tri methoxy boroxine (TMB). Reaction starts with the nucleophilic attack of highly electronegative oxygen atom of TEOS on the electron deficient boron of TMB resulting in formation of borosiloxane (B-O-Si) bonds with boroxol ring and boron methoxide/ethoxide as a side product. As seen in Figure 2.3, TMB acts as Lewis acid and TEOS acts as Lewis base (Parashar et al. 2008).

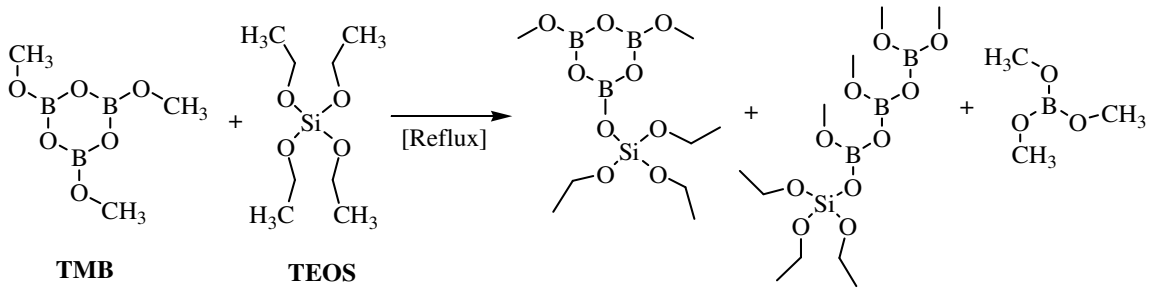


Figure 2.4. Proposed reaction of TMB and TEOS
(Source: Parashar et al. 2008).

Reports regarding to synthesis of borosilicate nanoparticles in literature are scarce. Parashar et al. proposed a mechanism of preparation of borosilicate NPs and studied on the effect of reaction temperature T and [B]/[Si] molar ratio on synthesizing borosilicate nanoparticles (Parashar et al. 2008). They investigated the optimum conditions to synthesize borosilicate nanoparticles. The temperature was varied between 20°C and 30°C and the [B]/[Si] was changed between 0.55 and 0.80 by means of mole percent. Moreover, they observed that if the temperature was not changed and the [B]/[Si] ratio was higher than 0.88, nanoparticles and boron oxide crystals were formed. However, if the [B]/[Si] ratio was lower than the 0.55, gel with separate phases of B_2O_3 and SiO_2 were generated. In addition to these, as the temperature increased above 45°C, micro and nanoparticles were formed. Compared to that, while temperature was lower than 10°C, agglomerated fused particles formed, by keeping the [B]/[Si] ratio between 0.55 and 0.80 (Parashar et al. 2008). There is also a report by Beckett et al. on TMB as an `oxygen transfer` reagent and its wage about formation of alkali-free borosilicate glass started with the synthesis of alkali-free borosilicate gel from $Si(OEt)_4$ and $(MeO)_3B_3O_3$ in non-aqueous solvents. The formation of the gel was achieved with the rapid transesterification/oxygen transfer with elimination of $B(OR)_3$. Then, alkali-free borosilicate glass was formed after removal of volatiles from the gel, followed by drying at 60°C for 12 hours and furnacing in air at 600°C for 20 minutes (Beckett et al. 2006).

2.4. Experimental

In order to characterize silica and borosilicate nanoparticles, Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS), Fourier Transformed-Infrared (FTIR) Spectroscopy, Energy Dispersive X-Ray (EDX) Spectroscopy were used. Titration method was also used to estimate the amount of boron. Phillips XL-30S FEG Scanning Electron Microscopy (SEM) which 5-30keV electron beam scans the specimen surface was applied to investigate the morphologies of the synthesized silica and borosilicate nanoparticles. EDX technique was used to investigate the chemical composition of silica and borosilicate nanoparticles especially for determining the presence of boron. The average particle size and size distribution based on intensity, volume and number distribution of nanoparticles were measured with Zetasizer 3000HS and Malvern Zetasizer Nano ZS by using DLS technique. XRD analysis was performed to determine whether the structure of silica and borosilicate nanoparticles was crystalline or amorphous. Diffraction pattern were recorded at 2□-range of 5-50°. FTIR was used to specify groups of Si-O-B, Si-O-Si, O-Si-O and B-O.

Tetraethyl Orthosilicate (TEOS 98%, Aldrich), ammonium hydroxide (NH4OH 28-30%, Aldrich), absolute ethanol (99.9%, Fluka) were used to prepare silica and borosilicate nanoparticles. To synthesize borosilicate nanoparticles, tri methoxy boroxine (TMB 95%, Aldrich) was used.

2.4.1. Synthesis of Silica Nanoparticles by Stöber Method

Silica nanoparticles were synthesized by using Stöber method based on the hydrolysis and condensation of alkoxy silane as a silica source. Particularly, we used tetraethyl orthosilicate (TEOS).

Briefly, 15 ml (0.257 moles) of EtOH was mixed with 1.2 ml (0.0312 moles) of NH_4OH and stirred for five minutes. Then, 1.2 ml (5.37 mmoles) of TEOS was added under N_2 and they were all stirred for 20 hours at room temperature. An additional, 245 µl (1.1 mmoles) of TEOS was added into the solution which makes approximately 18 mL total volume. All parameters were illustrated in Table 2.1.

Table 2.1. Amounts of precursor to prepare silica nanoparticles.

Sample name	EtOH, ml	NH_4OH, ml	TEOS, ml	Add. TEOS, µl	Size, nm
S1	20	1.2	1.2	245	60±6.5
S2	15	1.2	1.2	245	115±8
S3	15	2.4	1.2	245	430±7

2.4.2. Synthesis of Borosilicate Nanoparticles

Borosilicate nanoparticles were prepared by the Stöber method. Silica core was prepared and then tri methoxy boroxine (TMB) as a coating layer was added into the solution. Following the formation of silica core, 156.0 µl (1.1 mmoles) of TMB was added into the solution. The surface of the silica particle was covered by borosilicate shell (B-O-Si). Table 2.2 summarizes the amounts of reagents used.

Table 2.2. Amounts of precursor to prepare borosilicate nanoparticles.

Sample name	EtOH, ml	NH_4OH, ml	TEOS, ml	TMB, µl	Size, nm
S4	30	1.2	1.2	156	60±9.5
S5	15	1.2	1.2	156	126±15
S6	15	2.4	1.2	156	340±40

When TMB was added into the solution at the beginning of the silica preparation, borosilicate gel was formed instead of nanoparticles. Table 2.3 summarizes the amounts of reagents used.

Table 2.3. Amounts of precursor to prepare borosilicate-gels.

Sample name	EtOH, ml	NH_4OH, ml	TEOS, ml	TMB, µl	Add. TEOS, µl
S7	15	1.2	0.96	153	245
S8	15	1.2	0.25	60	245

2.4.3. Synthesis of Silica Nanoparticles Assisted by Amino Acid

Very small silica nanoparticles were also prepared by a modified microemulsion based method, the hydrolysis and condensation of tetraethyl orthosilicate (TEOS) in the presence of a mixture of octane and water as solvents, basic amino acid, L-Lysine, as a catalyst instead of ammonia (Yokoi et al 2006). The presence of amino acids yields well-ordered arrangement of the silica nanoparticles.

In a typical synthesis, 1.0 mmol (0.146 g) of L-Lysine was dissolved in 139.0 ml ultrapure water and 10.4 ml (7.3 g) of octane was introduced. They were stirred at 60°C under N_2 atmosphere for 20 minutes. The temperature of the reactions was controlled with an oil bath. 11.4 mL of TEOS was added dropwise through a syringe and the solution was stirred overnight at 60°C under N_2 atmosphere. In order to obtain silica nanoparticles in powder, the final solution was directly evaporated at 70°C. Figure 2.5 shows the experimental procedure of the silica nanoparticles assisted by L-Lysine.

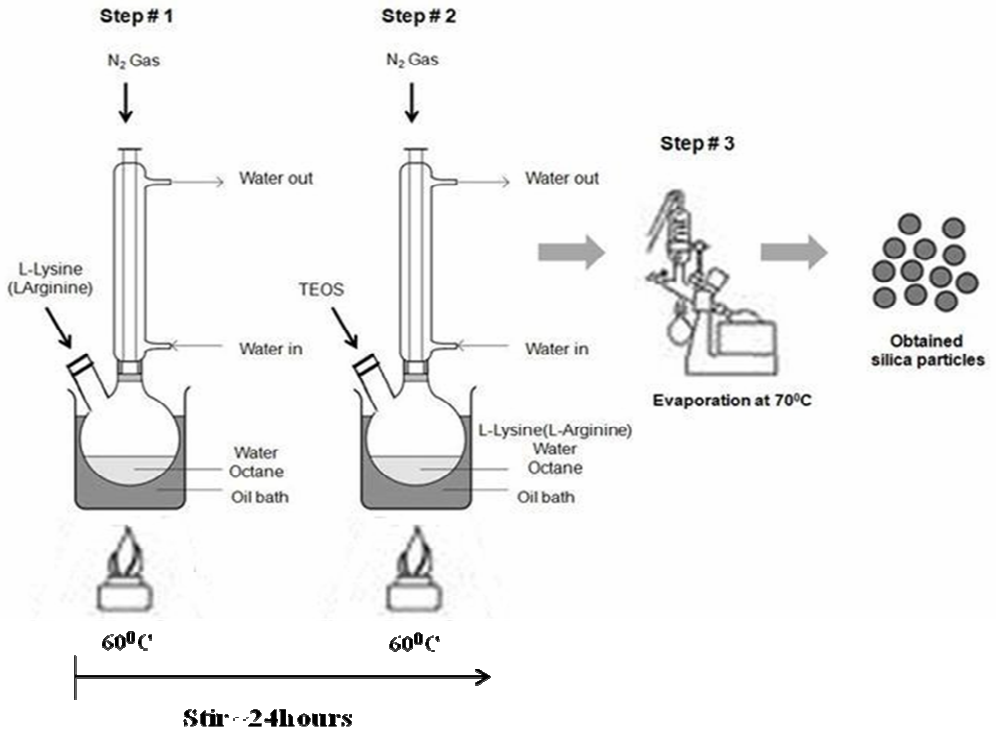


Figure 2.5. Experimental set up for synthesis of silica nanoparticles assisted by amino acids. (Source: Altin 2009).

2.4.4. Synthesis of Borosilicate Nanoparticles Assisted by Amino Acid

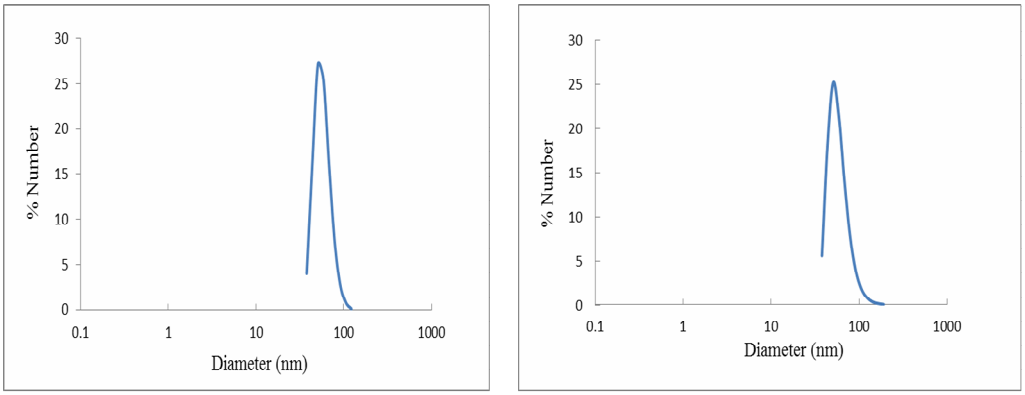
To synthesize borosilicate nanoparticles, TMB (trimethoxy boroxine) was added to the solution after synthesis of silica nanoparticles. The mole ratio of the TMB is the quarter mole of TEOS. The reaction was consisting of 139 ml of ultra-pure water; 10.4ml (7.3 g) of octane, 1.0 mmole (0.146 g) of L-Lysine, 11.4 ml (0.05 moles) of TEOS and 1.42 ml (0.01 moles) of TMB were used.

In order to purify both silica and borosilicate nanoparticles, following the synthesis, nanoparticles were first centrifuged and supernatant was removed. Then, 15 ml of EtOH was added and sonicated to disperse the particles and was centrifuged, again. That process was repeated for three times and particles were kept in dry.

2.5. Results

2.5.1. Measurement of Particle Size

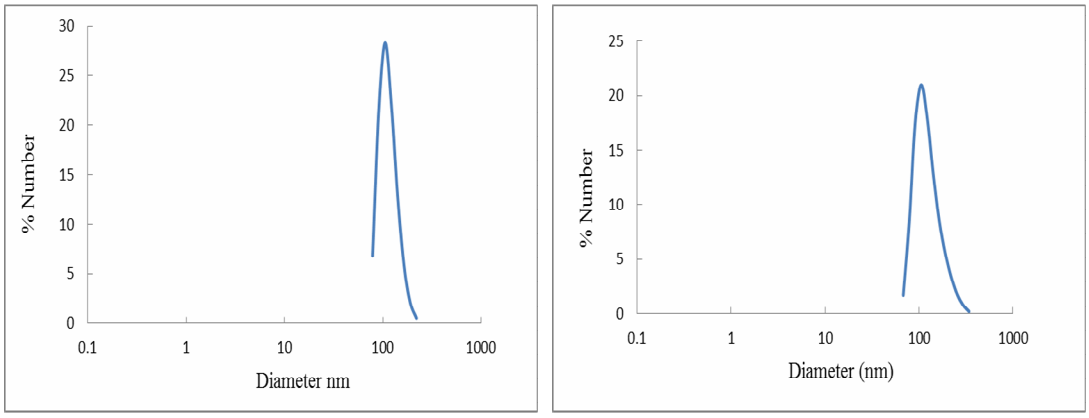
The size and size distribution of the silica and borosilicate nanoparticles were measured by using DLS technique. In Figure 2.6, silica nanoparticles, S1, were prepared by the reaction of TEOS, ethanol and ammonium hydroxide. Then, additional TEOS was introduced. However, TMB was added after the core formation of silica instead of additional TEOS to obtain borosilicate nanoparticles, S4.



S1 S4

Figure 2.6. Size distribution of the silica (S1) and borosilicate (S4) nanoparticles 60 nm in size.

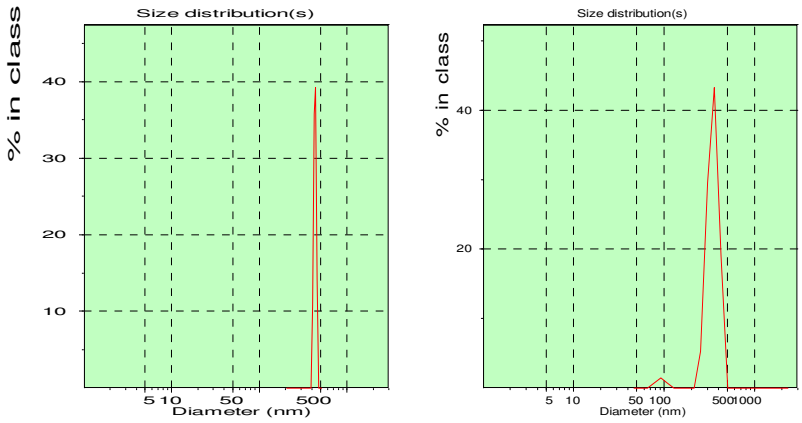
The size and size distribution of silica and borosilicate nanoparticles were analyzed by DLS, and particle sizes were 115 nm and 126 nm, as shown in Figure 2.7. Size distribution of the borosilicate nanoparticles was broader than the silica nanoparticles. TMB was introduced in the post coating process to synthesize borosilicate nanoparticles, S5.



S2 S5

Figure 2.7. Size distribution of the silica (S2) and borosilicate (S5) nanoparticles which were 115 nm and 126 nm respectively.

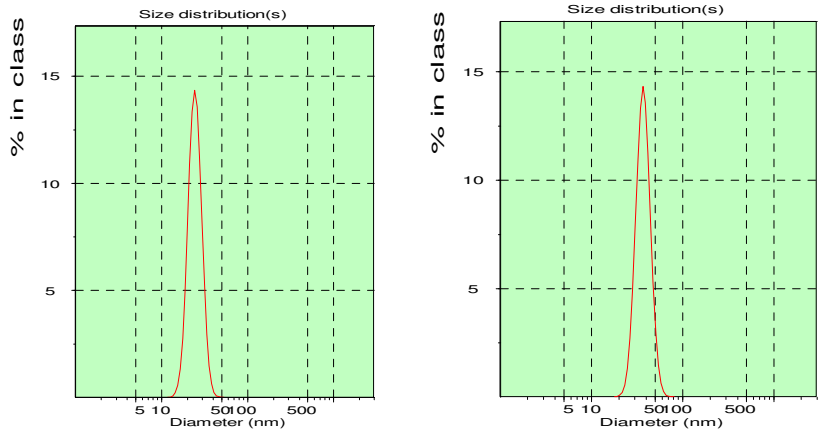
Figure 2.8 represents the size distribution of the S3 and S6 and the size of the nanoparticles were 430 nm and 340 nm respectively. S3 and S6 were performed as explained above. In these experiments, the concentration of NH_4OH was increased while keeping the concentration of the other reagents, TEOS and EtOH. Therefore, the bigger nanoparticles were obtained like the study performed by Rahman at al. (2007). We can understand from the Figure 2.8, borosilicate nanoparticles had broader size distribution compared to silica nanoparticles.



S3 S6

Figure 2.8. Size distribution of the silica (S3) and borosilicate (S6) nanoparticles that were 430 nm and 342 nm respectively.

Size distribution of the silica (S9) and borosilicate (S10) nanoparticles which were synthesized by using modified microemulsion based method were illustrated in figure 2.9. S9 had 25 nm in size and S10 had 35 nm in size. Thus, it was comprehended that borosilicate nanoparticles had larger size because boron source that was TMB was added after silica formation in this method.



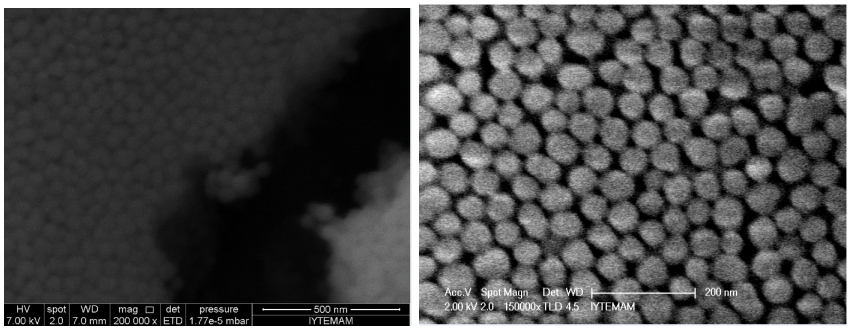
S9 S10

Figure 2.9. Size distribution of the silica (S9) and borosilicate (S10) nanoparticles by means of number which are 25 nm and 40 nm respectively.

2.5.2. SEM of Silica and Borosilicate Nanoparticles

Morphologies of the silica and borosilicate nanoparticles can be investigated with SEM. The size of the silica nanoparticles can also be measured by using this method.

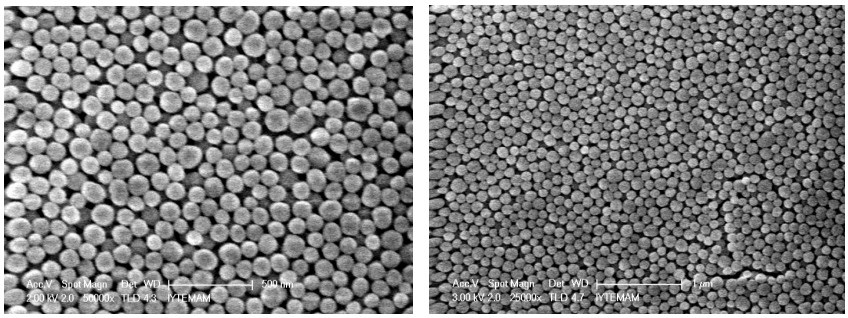
As seen in Figure 2.10, the morphologies of the silica and borosilicate nanoparticles were similar and both of them were spherical.



S1 S4

Figure 2.10. SEM images of silica (S1) and borosilicate (S4) nanoparticles.

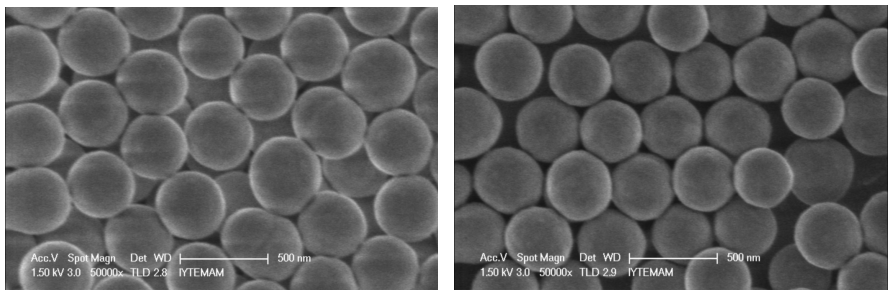
Size of the silica (S2) and borosilicate nanoparticles (S5), Figure 2.11, were determined by SEM analysis as 115 nm and 126 nm respectively.



S2 S5

Figure 2.11. SEM images of silica (S2) and borosilicate (S5) nanoparticles.

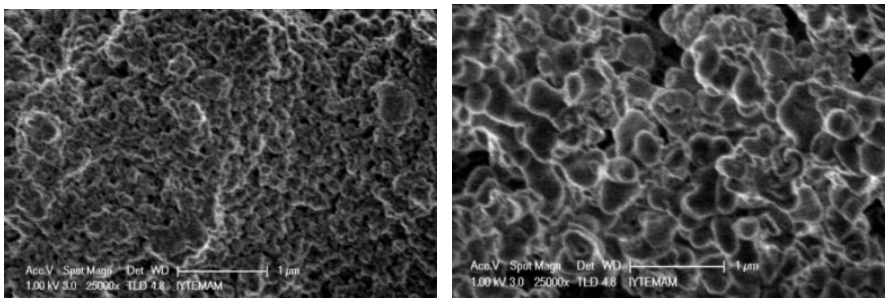
Images in Figure 2.12 were recorded by SEM, demonstrates that the size and shape of spherical silica and borosilicate nanoparticles were uniformly spherical and monodisperse.



S3 S6

Figure 2.12. SEM images of silica (S3) and borosilicate (S6) nanoparticles.

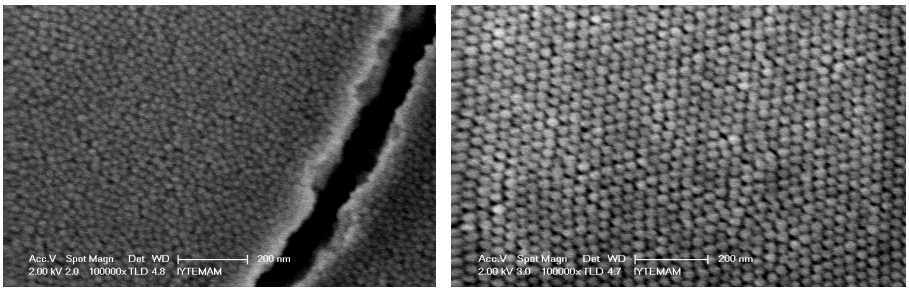
If the reagents that were TEOS and NH_4OH were mixed with EtOH in a separate flask and then TMB was added, borosilicate gels and nanoparticles were obtained simultaneously as seen in Figure 2.13. Although, different molar ratio of TEOS and TMB were used, borosilicate gels and nanoparticles were obtained.



S7 S8

Figure 2.13. SEM images of borosilicate gel, S7 and S8.

SEM analysis showed the silica and borosilicate nanoparticles as the assisted by an amino acid, Figure 2.14. Silica nanoparticles were smaller than the borosilicate nanoparticles because TMB was added after silica formation to synthesize borosilicate nanoparticles. It generated a layer on the surface of the silica nanoparticles.



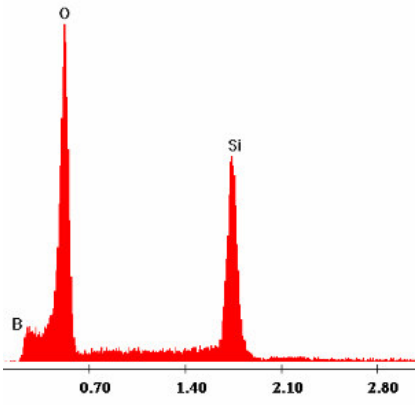
S9 S10

Figure 2.14. SEM images of silica, S9 and borosilicate, S10 nanoparticles.

2.5.3. EDX Analysis of Silica and Borosilicate Nanoparticles

EDX is an analytical technique used for the elemental analysis or chemical characterization of a sample. It is one of the variants of X-ray fluorescence spectroscopy.

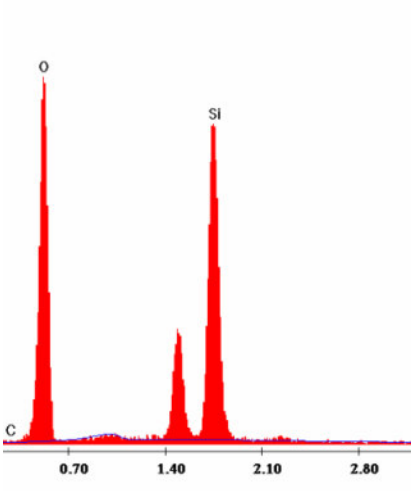
EDX analysis showed that borosilicate nanoparticles consisted of 12.3% Boron content as they were synthesized by Stöber method. That is to say, the 12.3 percent of sample included boron element as seen in Figure 2.15.



Element	At %
B	12.30
O	42.90
Si	44.80

Figure 2.15. EDX Analysis of borosilicate nanoparticles (S4).

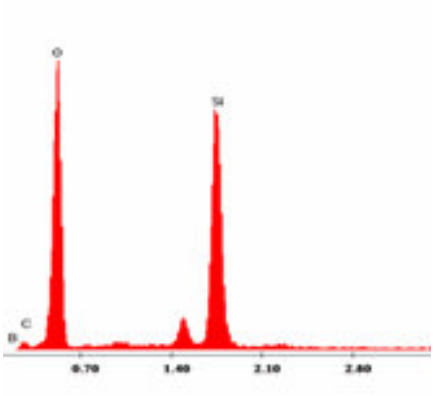
Figure 2.16 depicts that silica nanoparticles, about 100 nm in size, were lack of boron.



Element	At %
B	0.0
O	59.33
Si	38.27

Figure 2.16 EDX analysis of silica (S2) nanoparticles.

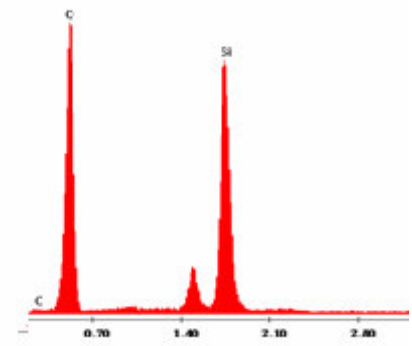
Figure 2.17 illustrates that the borosilicate nps about 100 nm in size contained 1.88% Boron according to EDX result.



Element	At %
B	1.88
O	56.72
Si	35.10

Figure 2.17. EDX analysis of borosilicate nanoparticles (S5).

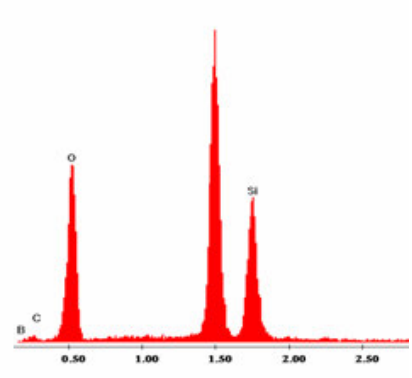
The silica nanoparticles about 400 nm in size did not contain any boron as seen in Figure 2.18.



Element	At %
B	0.0
O	62.34
Si	33.75

Figure 2.18. EDX analysis of silica (S3) nanoparticles.

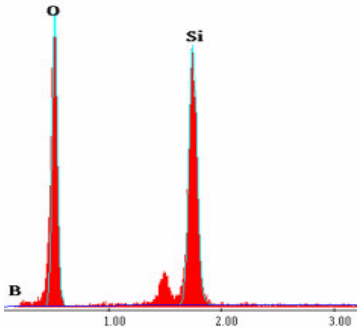
Boron content of the borosilicate nanoparticles was determined by EDX analysis that was 21.44 % as seen in Figure 2.19.



Element	At %
B	21.44
O	46.63
Si	25.02

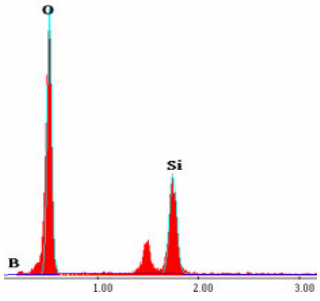
Figure 2.19. EDX analysis of borosilicate nanoparticles (S6).

As the TMB was introduced into the solution with TEOS before the post coating process, the borosilicate gels were obtained instead of borosilicate particles by Stöber method as seen in Figure 2.20 and Figure 2.21. S7 included 20% TMB and S8 included 80% TMB in terms of atomic percent.



Element	At %
B	11.11
O	57.70
Si	31.19

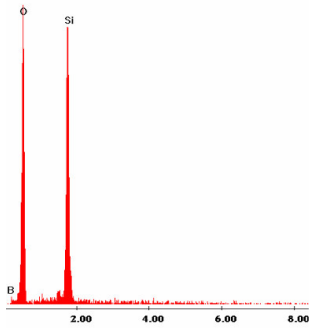
Figure 2.20. EDX analysis of borosilicate gels (S7).



Element	At %
B	5.81
O	73.90
Si	20.29

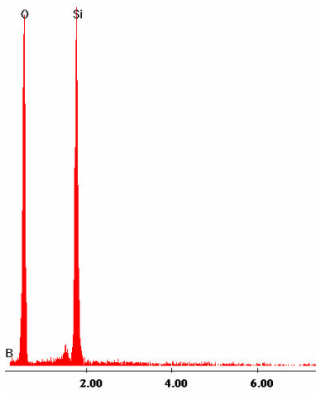
Figure 2.21. EDX analysis of borosilicate gels (S8).

If the reagents that were TEOS and NH_4OH were mixed with EtOH in a separate flask and then TMB was added, borosilicate gels and nanoparticles were obtained simultaneously as seen in Figure 2.22 and Figure 2.23. S11 contained 15.49 % Boron and S12 contained 7.77 % Boron. This was because, more TMB was used, two and half times, in S11.



Element	At %
B	15.49
O	48.98
Si	35.54

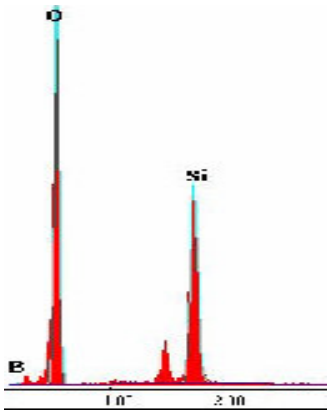
Figure 2.22. EDX analysis of borosilicate gels + nanoparticles (S11).



Element	At %
B	7.77
O	44.99
Si	47.24

Figure 2.23. EDX analysis of borosilicate gels + nanoparticles (S12).

In Figure 2.24, Boron source was added after silica formation in L-Lysine based method. Therefore, it was expected to cover the surface of the silica and contained 11.03% Boron.



Element	At %
B	11.03
O	54.37
Si	37.74

Figure 2.24. EDX analysis of borosilicate nanoparticles (S10).

2.5.4. FTIR Analysis of Silica and Borosilicate Nanoparticles

FTIR technique was used to specify the functional groups of particles. In our study, we especially intended to observe the B-O and B-O-Si bands.

The FTIR spectra of borosilicate nanoparticles about 60 nm in size was illustrated in Figure 2.25. The peaks at 1404 cm^{-1} and 796 cm^{-1} indicated the B-O and B-O-Si groups (Tsvetkov et al. 2006).

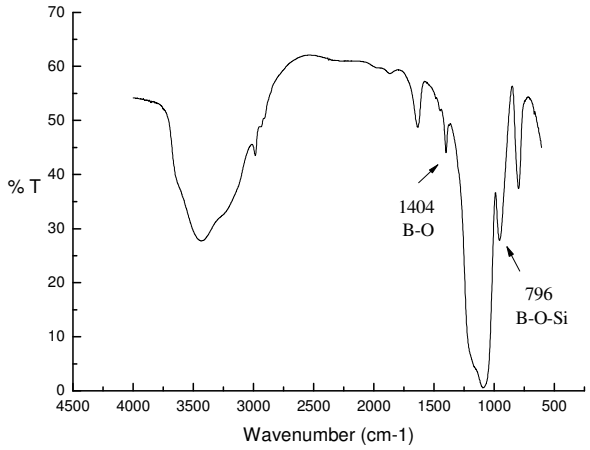


Figure 2.25. FT-IR spectrum of borosilicate nanoparticles (S4).

Result of FTIR analysis of the borosilicate nanoparticles with the size of 100 nm was represented in Figure 2.26. The broad peak at 3500 cm^{-1} indicated the $-OH^-$ group, 1627cm^{-1} showed the Si-O group. 1400, 951 and 798 cm^{-1} indicated the B-O stretching, B-O-Si stretching and O-Si-O vibrations, respectively.

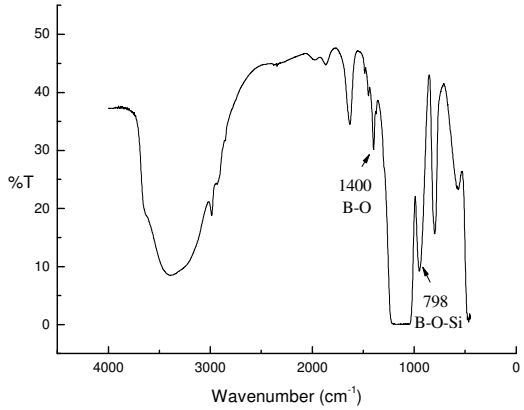


Figure 2.26. FTIR spectrum of borosilicate nanoparticles (S5).

In Figure 2.27, the particles which were about 340 nm in size were characterized. The peak at the 3382 cm^{-1} indicated the –O-H bond and 1630, 1400, 945 and 796 cm^{-1} showed the Si-O, B-O stretching, B-O –Si stretch and O-Si-O vibrations, respectively.

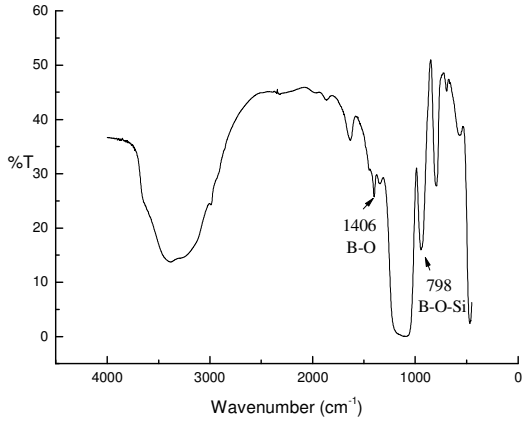


Figure 2.27. FTIR spectrum of borosilicate nanoparticles (S6).

Figure 2.28 is the FTIR spectrum of borosilicate nanoparticles prepared by the microemulsion based method. The spectrum was similar with the results of particles synthesized by Stöber method.

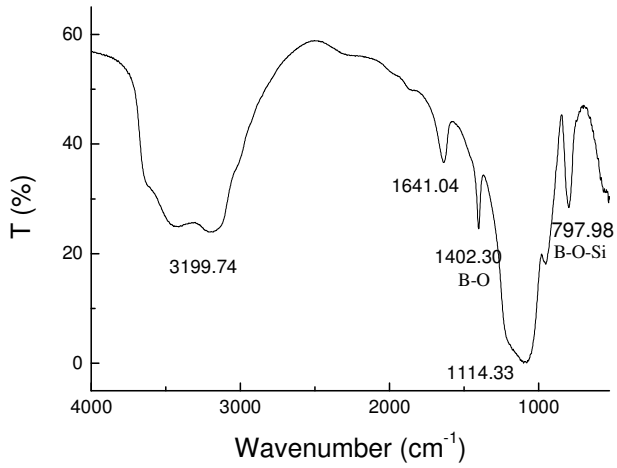


Figure 2.28. FTIR spectrum of borosilicate nanoparticles (S10) prepared by a modified microemulsion based method.

2.5.5. XRD and Acid-Base Titration Analysis of Silica and Borosilicate Nanoparticles

XRD was used to determine whether the structure of the substances were crystalline or amorphous. According to XRD result of borosilicate nanoparticles both 126 nm and 340 nm in size had amorphous structure not crystalline because the broad peak was observed at 23 degree of 2□.

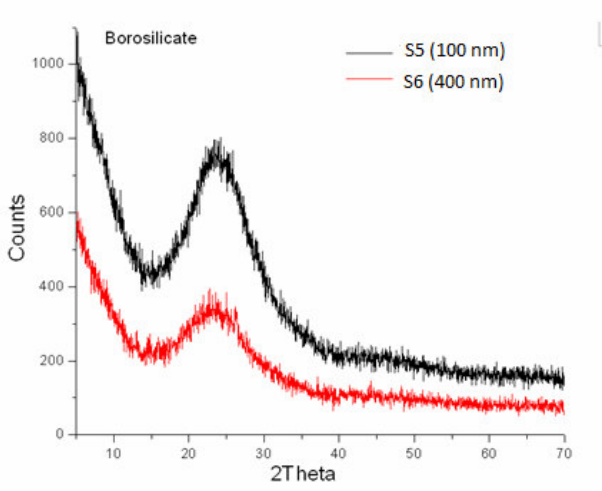


Figure 2.29. XRD result of the borosilicate nanoparticles that were 126 and 340 nm in size.

The presence of boron was also confirmed by acid-base titration method (Foote 1932). In this method, particles were firstly dissolved in HCl and titrated with NaOH.

Then, mannitol was added into the solution in order to release boron as boric acid. Thus, we could estimate the percentage of boron. We estimated the mole percent of boric acid of S4 as 13.85% and S5 as 1.62%.

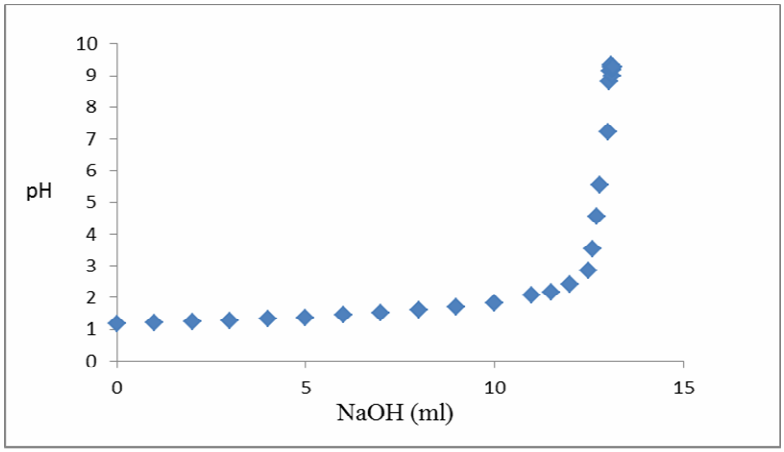


Figure 2.30. Titration result of Borosilicate nanoparticles (S4) including % 13.85 Boron content.

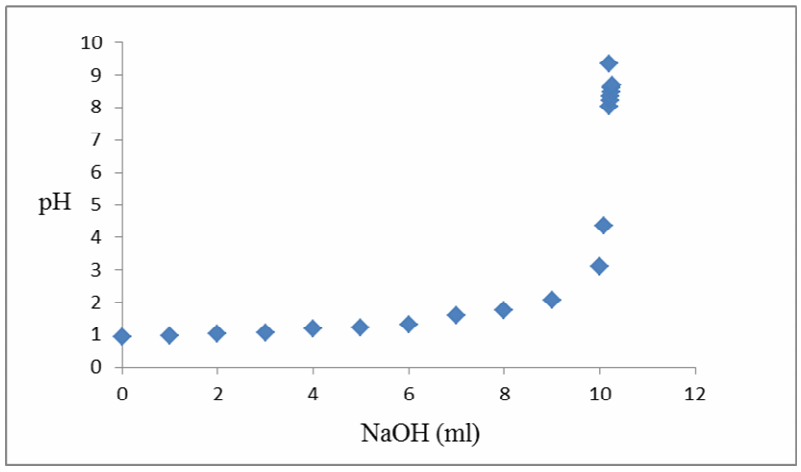


Figure 2.31. Titration result of Borosilicate nanoparticles (S5) including 1.62 % Boron content.

2.6. Discussion

Both silica and borosilicate nanoparticles and borosilicate gels were produced. This work was based on the Stöber method and the initial amounts of reagents were varied to obtain different size of particles.

We synthesized borosilicate nanoparticles by the Stöber method and used TMB as the boron source. The surface of silica nanoparticles were covered by TMB in contrast to Parashar et al. (Parashar et al. 2008). EDX and titration results showed clearly the presence of Boron in the nanoparticles. Parashar et al. initiated the reaction with TMB and TEOS adduct in order to synthesize borosilicate nanoparticles. B-O-Si linkage existed in whole parts of particles but we added TMB, after silica formation. Parashar et al. obtained borosilicate nanoparticles between 20°C and 30°C. Similarly, we prepared borosilicate nanoparticles at room temperature. They obtained these particles as the [B]/[Si] ratio was between 0.55 and 0.80 but we achieved borosilicate nanoparticles as the [B]/[Si] was 0.20. Parashar et al. obtained different borosilicate gels plus nanoparticles while altering the temperature and B/Si ratio. We comprehended that by using TMB during the core formation, gels were obtained instead of particles.

Silica particles were synthesized by the Stöber method. The particle size increased as the higher amount of NH_4OH used and agreed with the results reported by Rahman et al (Rahman et al. 2007) because as the ammonia is increased, also both the rate of hydrolysis and condensation reactions increases and so the intermediate [Si (OC2H5)4-X(OH)X] will be increased rapidly due to the high hydrolysis reaction; however, as it reaches the supersaturation region, the consumption rate of intermediate through condensation reaction is also relatively fast, which probably shortens the nucleation period. Therefore, the total number of nuclei formed will be less in numbers, and the final particle size of synthetic silica colloids will be relatively larger as exhibited in Figure 2.12. Moreover, it was indicated via Ismail et al (Ismail et al. 2010). Furthermore, the results of Rao et al. were convenient with our results in terms of effect of ammonia. However, we indicated that in the presence of large amount of ethanol, smaller particles were obtained contrary to Rao et al. Green et al. reported that NH_3 and H_2O and solvent, methanol and ethanol, provided the reaction rate which carries out the induction period before the onset of nucleation and they also found that particles synthesized in ethanol were larger than the size of those prepared in methanol (Green et al. 2003).

In addition to these, we synthesized silica nanoparticles assisted by amino acids with the method introduced by Yokoi et al. Moreover, we synthesized borosilicate nanoparticles with the help of this method. However, TMB that is the boron source was added into the solution after the silica formation like stober method. Since, the surface of the silica nanoparticles was covered with boron source.

One of our purposes is to develop a method to prepare borosilicate coated silica nanoparticles (borosilicate nanoparticles) by using the Stöber method. We carried on our study by preparing both the silica and borosilicate nanoparticles. We synthesized the particles with three different sizes approximately, 60, 100 and 400 nm, by varying the amount of some of reagents that were ethanol and ammonia. We found that there was no difference in terms of morphology of the both particles. In addition, we used EDX, FTIR and titration method to prove the presence of boron. These analyses exhibited that results were consistent with each other in terms of atomic per cent of boron in the samples.

CHAPTER 3

ASSESSMENT OF CYTOTOXICITY AND GENOTOXICITY

3.1. Introduction

Nanotoxicology is a new research area to study effect of nanomaterial on biological system and environment. Toxic effects can be allergy, inflammation, cytotoxicity, tissue damage and DNA damage etc. Cytotoxicity is the decisive of being toxic to cells, which means cytotoxic potential of a substance is determined by using some tests. Chemical substances can be given as examples for toxic agents. Cytotoxicity covers a big area because of evaluating the mutagenesis, malignant transformation, apoptosis, cellular pathology, and pharmaceutical toxicity because tumors will be caused only from living cells (Komissarova et al. 2005). Nanomaterials have been very instrumental to use them in cells. In addition, the easily changing physical properties of nanomaterials have been advantageous. Besides cytotoxicity, genotoxicity is also very important due to DNA damage and mutations stimulated by nanoparticles. It is necessary to assess biological response of cell against nanoparticles.

There are some assays to measure the cytotoxicity such as MTT (3-(4,5-Dimethylthioazol-2yl)-2,5-diphenyltetrazolium bromide and XTT(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide). The main principle of MTT is to observe reduction of the yellow colored tetrazolium salt MTT turned to purple formazan by detecting the cell growth. The color change is observed by the reduction of MTT or XTT to formazan because of enzymatic activity. These changes take place in mitochondria. As a result, mitochondrial activity can be observed in cells by using these assays. XTT is different from MTT in terms of requiring less time which enables direct absorbance readings. Reduction mechanisms of MTT and XTT are shown in Figures 3.1 and 3.2; (Mosmann 1983).

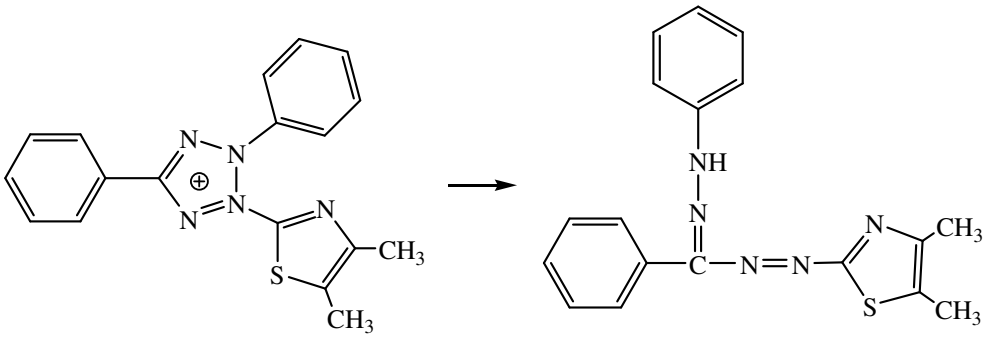


Figure 3.1 Schematic representation of the reduction of MTT to formazan
(Source: Dominic et al. 1988)

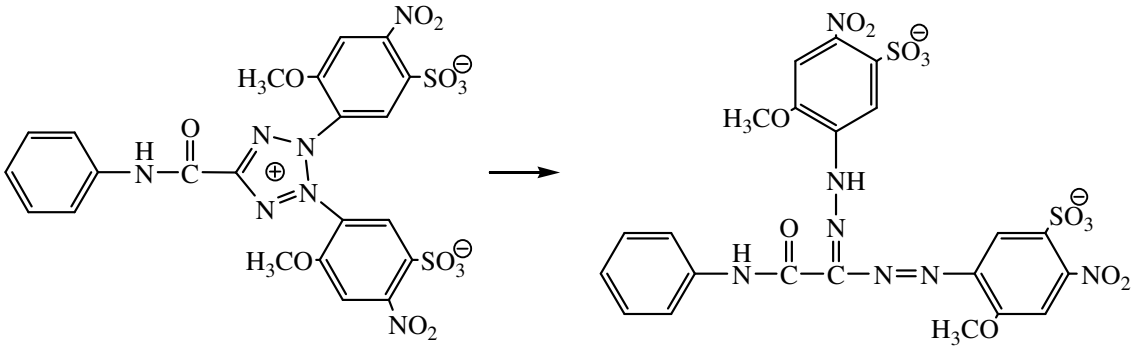


Figure 3.2. Schematic representation of the reduction of XTT to formazan
(Source: Dominic et al. 1988)

There are a lot of studies on cytotoxicity of silica nanoparticles. Lin et al investigated the cytotoxicity of 15 nm and 46 nm silica nanoparticles by using crystalline silica (Min-U-Sil 5) as a positive control in cultured human bronchoalveolar carcinoma-derived cells (A549) (Lin et al. 2006). They came to the conclusion that both 15 nm and 46 nm silica nanoparticles were nontoxic and there was not a significant difference for each particle. Moreover, SiO_2 nanoparticles were more cytotoxic than Min-U-Sil 5 (Lin et al. 2006). Akhtar et al. studied similar issue in vitro and in vivo. Akhtar et al. studied toxicity of 10 nm and 80 nm silica nanoparticles and measured the oxidative stress of particles and concluded that no size dependence of silica nanoparticles on the intracellular glutathione (GSH) level and the activities of glutathione metabolizing enzymes (Akhtar et al. 2010). Chang et al. reached a conclusion that silica nanoparticles are nontoxic at low dosages but cell viability decreases at high dosages (Chang et al. 2007). Fent et al. determined the uptake and

toxicity of fluorescent core-shell silica nanoparticles in early life stages of zebrafish (Fent et al. 2010). Napierska et al. investigated the effect of monodisperse amorphous silica nanoparticles on the viability of endothelial cells (EAHY926 cell lines) and used different sizes of particles (Napierska et al. 2009). Napierska et al. found that as the concentrations of particles (14, 15, and 16 nm in diameter) increased, it resulted in a 50 % reduction in cell viability. Brunner et al. investigated the in vitro cytotoxicity of oxide nanoparticles and compared to asbestos and silica. Brunner et al. also studied on the effect of solubility of particles and used human mesothelioma and a rodent fibroblast cell line for in vitro cytotoxicity tests using seven industrially important nanoparticles (Brunner et al. 2006). Ye et al. studied on myocardial cells (H9c2(2-1)) and they used silica nanoparticles with the sizes of 21 and 48 nm in these cell lines. Ye et al. had a conclusion that particles exhibited the toxicities at 0.1 and 1.6 mg/ml dosages with 12, 24, 36 and 48 hours exposure (Ye et al. 2010). Heikkila et al. determined cytotoxicity on undifferentiated human colon carcinoma (Caco-2) cell line by using ordered mesoporous silica in the size range of 1 to 160 nm. They considered the probability of using these microparticles in oral drug formulations. The concentration of samples varied from 0.2 to 16 mg/mL, and they were incubated for 3 and 24 hours with Caco-2 cells (Heikkila et al. 2010). Fisichella et al. used mesoporous silica nanoparticles (MSN) in HeLa cells and showed that LDH activity, WST-1 assay were reliable (Fisichella et al. 2010). Cytotoxicity studies indicated that these nanoparticles were non-toxic to HeLa cells up to a dose level of 50 µg/mL.

In this thesis study, A549 bronchoalveolar carcinoma-derived cells, and BEAS-2B, human bronchial epithelial cells, were used to assess cytotoxicity and genotoxicity of silica and borosilicate nanoparticles in the size range from 60 to 430 nm.

3.2. Experimental

We used MTT Assay, dimethyl sulfoxide (DMSO, 99% Sigma Chemical Co.), tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Chemical Co.) and XTT Assay, 2,3-bis(2-methoxy-4nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (Biological Industries Israel Beit Haemek LTD.) to assess the cytotoxic and genotoxic effect of silica and borosilicate nanoparticles.

Glassware and plasticware were cleaned by soaking them in dilute nitric acid (10%) and rinsed with distilled water prior to use.

To evaluate cytotoxicity and genotoxicity, Thermo Scientific Varioskan Flash multimode reader were used for the purpose of measuring toxic effect of nanoparticles.

3.2.1. Preparation of Cell Culture

Human lung adenocarcinoma epithelial cell (A549) and human bronchial epithelial cell (BEAS-2B) were used to evaluate biological response against the nanoparticles. A549 and BEAS-2B cell lines were kindly provided by Dr. Hasan Bayram, University of Gaziantep, Faculty of Medicine. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (BIO-IND), 1% (v/v) L-Glutamine, 1% (v/v) Penisiline-Streptomisin incubated at 37ºC in the dark with 5% CO_2 and 95% humidification and passaged when they reached 80-85% confluency. The medium for human bronchial epithelial cell (BEAS 2B) was RPMI including 10% fetal bovine serum (FBS) (BIO-IND), 1% (v/v) L-Glutamine, 1% (v/v) Penisiline-Streptomisin.

3.2.2. Treatment of Cultured Cells with Particles and Cell Viability Assays

Cell culture studies start with the cell thawing. Frozen cells were taken from -80°C, then, 1ml of medium are added into the thawing cells having volume of 1.5 ml. The medium includes RPMI or DMEM + Fetal Bovine Serum (FBS) 10 % + L-Glutamine (1% v/v) and Penisilin-Streptomisin (v/v 1%), and called the complete medium. After pipetting, 6.5 ml of the complete medium was added into the solution and it was centrifuged for 3 minutes and 3000 rpm. The supernatant is removed from the medium. 3 ml of the complete medium is added into the solution and pipetted. Finally, the solution is transferred to the 6 cm-plate and the cells were maintained at 37 °C in a humidified atmosphere, with 5% CO_2. If the confluency reaches to 80 % after cultivation, cells are passaged. The typical way of passaging started with washing the cells with 2 ml of PBS for two times after aspirating the medium and then, 0.5 ml of

trypsin was added to raise the cells and shaken gently. After that, it was incubated for a maximum of 5 minutes with 4 ml of the complete medium was added and pipetting. After that process, the cells are seed into 96 well plate. 5,000 A549 cells in 200µl medium were seed in every plate. However, as BEAS-2B cells were used, 10,000 BEAS-2B cells in 200 µl were seed and they were incubated for 24 hours. After 24 hours cultivation, nanoparticles were added into the cells with different dosages of 0.1, 1, 10, 100 and 500µg/ml for triplicate assays. The nanoparticles were diluted at the appropriate concentrations with the culture medium. Dilutions of nanoparticles were freshly prepared before each experiment. Then, cells were treated with the nanoparticles for 24, 48 and 72 hours. Cell viability was measured by MTT based on colorimetric assay. Four hours before the measurement of the optical density, MTT solution was added after aspirating the medium. MTT solution is included as 10 % in the solution for each well. Each well included 20 µl of MTT solution and 180 µl of complete medium DMEM or RPMI according to which type of cells used. After the 4 hours incubation, plates were centrifuged at 1800 rpm for 10 minute at room temperatures to avoid accidental removal of formazan crystals. The crystals were dissolved with 100µl DMSO and they were shaken gently for 5 minutes. Optical density (O.D) was measured by Varioskan reader. Results were represented as percentage viability and calculated by the following formula:

$$\% \text{ viability} = 100 - [\left(\frac{OD_s - OD_b}{OD_c - OD_b}\right) x\ 100 \tag{3.1}$$

OD_b indicated the optical density of blank, OD_s indicated the optical density of sample and OD_c indicated the optical density of control.

XTT has the same running principle as MTT. While applying XTT assay, 100 µl of activating reagent was dissolved in 5 ml of XTT solution. Then, 50 µl of solution was added for each well in 96 well plate including 200 µl of medium before 4 hours of reading of absorbance values which the cells were incubated for 24h, 48h and 72 hours. Results were calculated as in MTT.

3.2.3. Treatment of Cells with the Cleaved PARP [214/215]

Genotoxicity is the degree to which a physical or chemical agent damages DNA or causes mutation. Carcinogens, or cancer-causing agents, mutagens, or mutation-causing agents, birth defect-causing agents are called as genotoxins. Like cytotoxicity, there are some assays to measure genotoxicity. One of them is PARP 214/215 Elisa Parp Assay is one of the most abundant proteins exist in nucleus. It catalyzes the polymerization of ADP-ribose units from donor NAD^+ molecules on target nuclear proteins, resulting in the attachment of linear or branched polymers. Poly (ADP-Ribose) Polymerase (PARP, MW: 116 kDa) consists of three main domains: an amino N-terminal DNA-binding domain (DBD), an automodification domain and a carboxyl C-terminal catalytic domain (Chiarugi and Moskowitz 2002)

PARP has various roles in many molecular and cellular processes, including DNA damage detection and repair, chromatin modification, transcription, and cell death pathways.

The principle of the PARP [214/215] assay is that a monoclonal antibody specific for PARP has been coated onto the wells of the microliter strips provided. Samples, control specimens, and unknowns, are pipetted into these wells together with a rabbit (detection) antibody specific for cleaved PARP [214/215]. During the first incubation, the cleaved PARP [214/215] antigen binds simultaneously to the immobilized antibody and to the solution phase rabbit (detection) antibody. After washing process, a horseradish peroxidase-labeled anti-rabbit IgG-HRP is added. This binds to the detection antibody to complete the four-member sandwich. After the second incubation and washing to remove all the excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of cleaved PARP [214/215] present in the original specimen (Chiarugi and Moskowitz 2002).

While applying genotoxicity assay, there was no need to be sterile of studying region. Process started with preparing standard solution. So, standard was diluted. 2.3 ml standard diluent buffer was added and it was kept for 10 minutes at room temperature. The procedure was keeping on cell extraction. Firstly, medium was aspirated from 6 well plate. Then, PBS was added and shaken gently and this step was

repeated, aspirated and 500 µl of PBS was added. Secondly, the cells which were scratched were poured into eppendorf tubes. Then, cells were centrifuged at 4000 rpm, 4 °C for 3 minutes. The supernatant was removed and 500 µl cold PBS was added. Then, it was centrifuged for the second time and supernatant was removed. After that, 50ul of 2X Cell Lysate Buffer was added and they were kept in ice bath for half an hour.

Standard solutions were prepared. They were centrifuged at 13000 rpm, 4°C for 10 minutes and they were kept in an ice bath. Supernatant was kept in eppendorf tubes because dissolved proteins were in supernatant.

30 µl of A549 Control cells, approximately 400.000 cells, were taken in an eppendorf tube and 30 µl of BEAS-2B control cells were poured into another eppendorf tube. Then, 70 µl of standard diluent buffer was added into each solution. After that, 50 µl of each solution was added into each well and 50 µl of antibody was added into each well. Then, it was shaken for 3 hours at 400-600 rpm.

That process kept on with washing procedure. 2 ml of washing buffer was diluted to 50 ml with distilled water. 200 µl of washing buffer were added into each well and this process was repeated for four times.

3 ml of HRP diluent was poured into 15 ml-falcon tube. Then, 30 µl of anti-rabbit HRP was added. After pipetting, 1 ml of solution was poured into an eppendorf. Then, washing buffer was added into wells and 100 µl of prepared HRP solution was added into each well. The top of the wells were protected with a paper and kept in a dark room for a half hour. 96 well plate was reversed and washed with 200 µl of washing buffer for five times. Then, 100 µl of chromagen was added and kept in a dark room for half an hour. After that, 100 µl of stop solution which was at room temperature was added. Finally, it was read at 450 nm. The genotoxicity procedure could be summarized as seen in Figure 3.3.

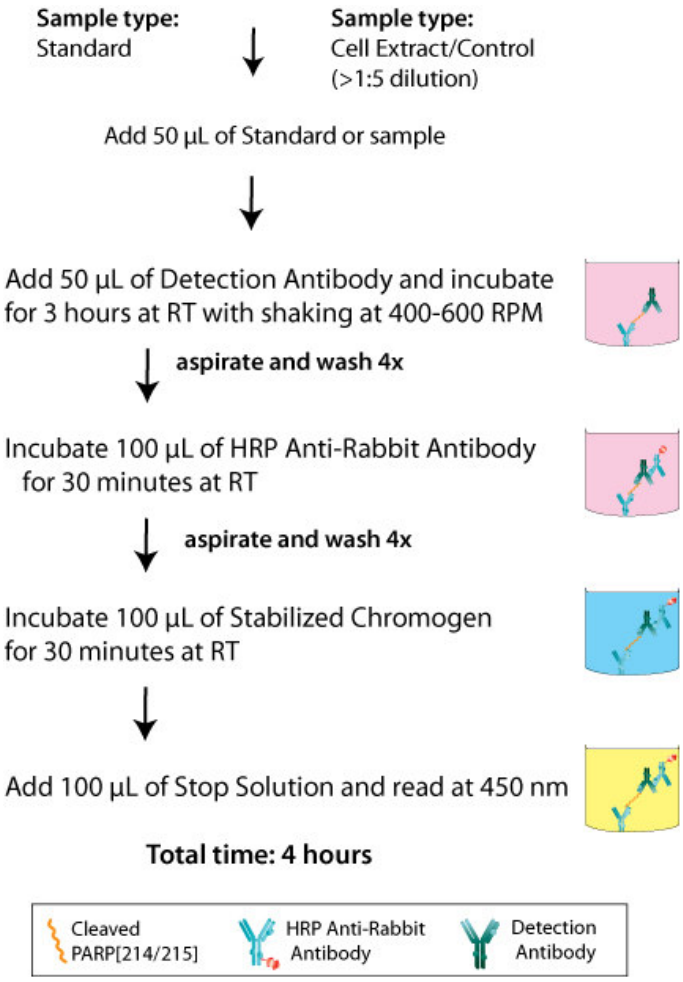


Figure 3.3. Schematic representation of the genotoxicity study
(Source: Chiarugi and Moskowitz 2002)

3.3. Results

3.3.1. Assessment of Cytotoxicity of Silica and Borosilicate Nanoparticles

In this chapter, we investigated the cytotoxicity and genotoxicity stimulated by silica and borosilicate nanoparticles for the A549 and BEAS2B cell lines.

As seen in Figure 3.4, dosages up to 500µg/ml of silica nanoparticles (size of 60 nm) were treated with A549 cell lines with incubation time between 24 and 72 hours. We found no cytotoxic effects induced by silica nanoparticles. Thus, cytotoxicity of silica nanoparticles was independent of the dosages and the incubation time.

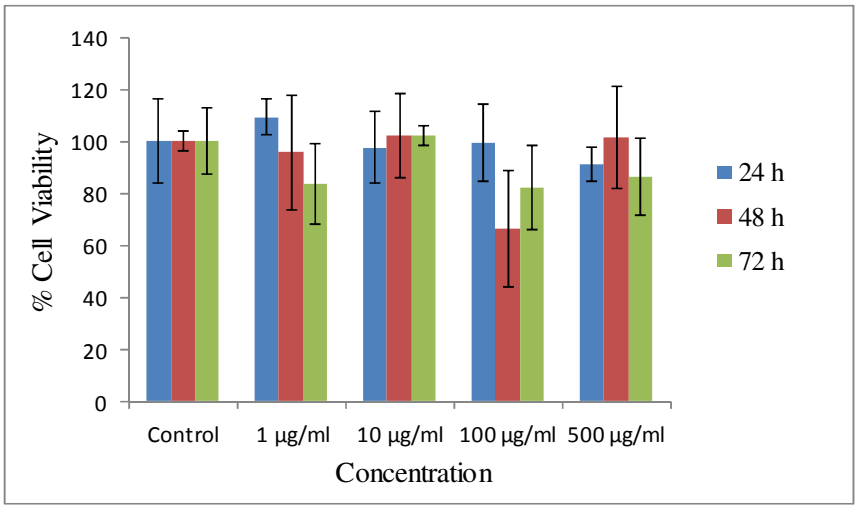


Figure 3.4. Cell viability results of 60 nm size of silica nanoparticles (S1) for A549 cell lines.

Similarly, Figure 3.5 depicts that borosilicate nanoparticles in size of 60 nm showed no cytotoxicity in A549 cells with dosages up to 500 µg/mL, and up to 72 hours of incubation period.

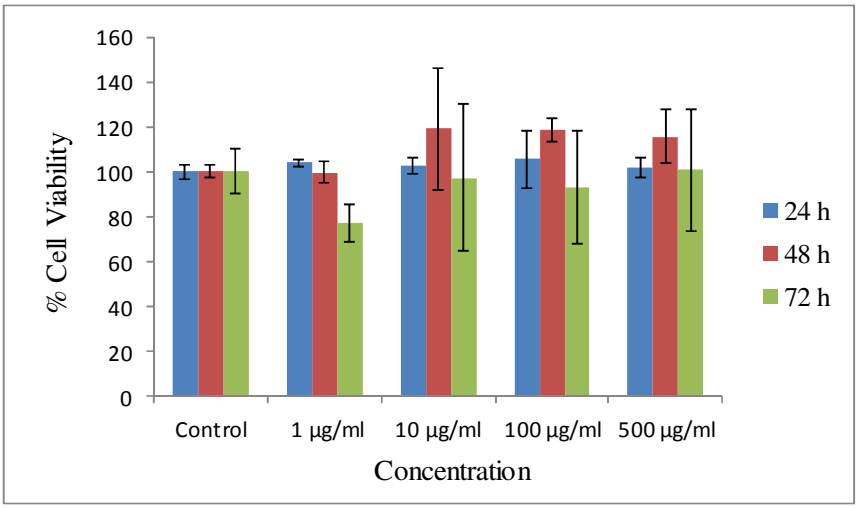


Figure 3.5. Cell viability assay for borosilicate nanoparticles (S4) with size of 60 nm for A549 cell lines.

Figure 3.6 illustrated that silica nanoparticles for the size of 100 nm were found to be nontoxic with 24 and 72 hours exposure times and dosages between 0.1 and 100 µg/mL.

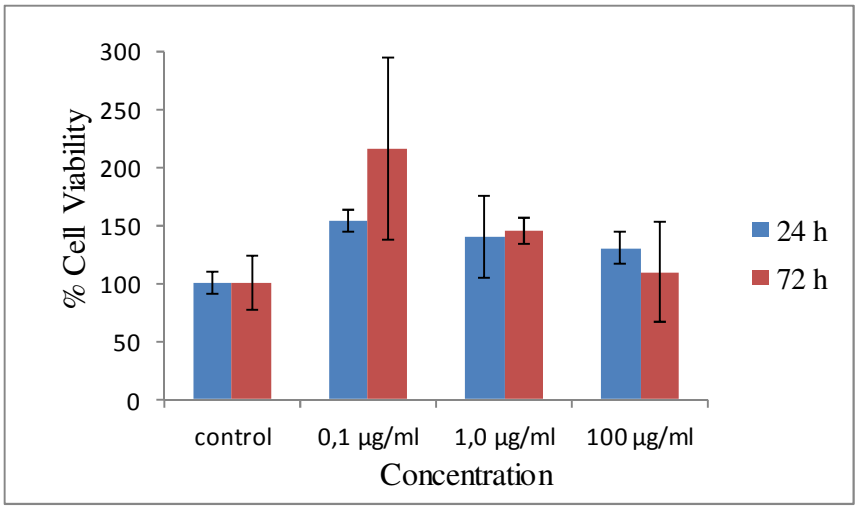


Figure 3.6. Cell viability of with dosages up to 100µg/mL and incubation time up to 72 hours silica nanoparticles (S2) for A549 cell lines.

Figure 3.7 illustrates that, A549 cells are viable for borosilicate nanoparticles about 100 nm in size in A549 cell lines up to 100µg/mL of dosages and 72 h of incubation.

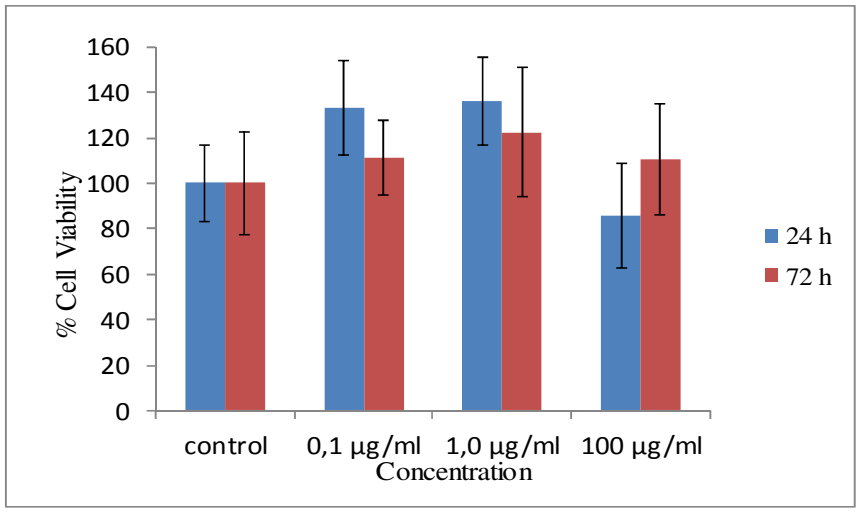


Figure 3.7. Cell viability of with dosages up to 100 µg/mL and incubation time up to 72 hours borosilicate nanoparticles (S5) for A549 cell lines.

Figure 3.8 shows that A549 cells are viable with incubation times of 24, 48 and 72 hours, as treated with the silica nanoparticles 430 nm in size, and dosages up to 500 µg/mL.

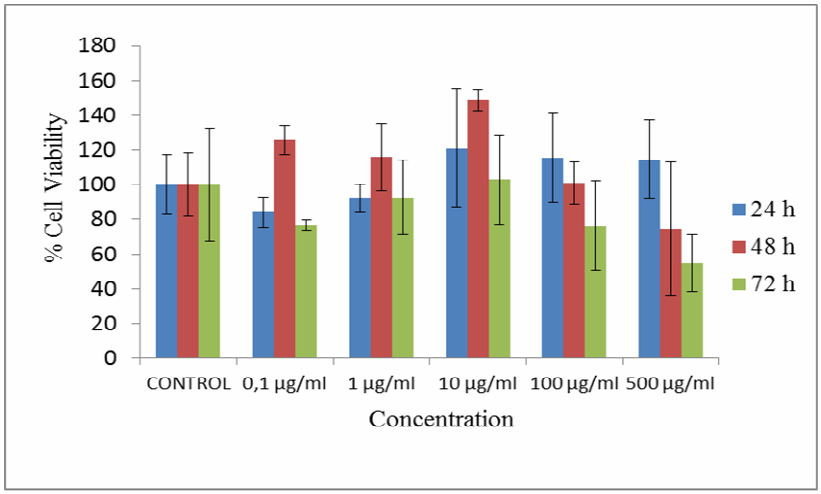


Figure 3.8. Cell viability assay for silica nanoparticles (S3) with size of 430 nm for A549 cell lines

As the dosages of borosilicate nanoparticles for 340 nm in size varied from 0.1 to 500µg/mL, there was no decrease in cell viability as seen in Figure 3.9.

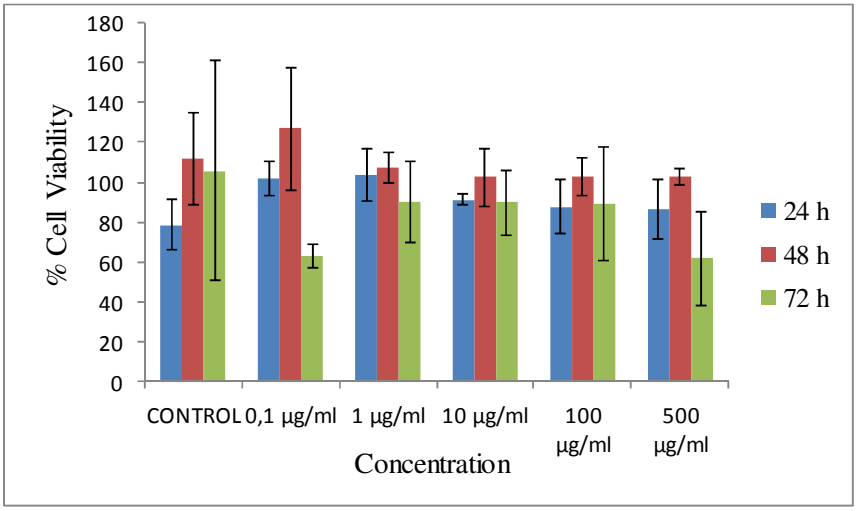


Figure 3.9. Cell viability assay for borosilicate nanoparticles (S6) with size of 340 nm for A549 cell lines.

Figure 3.10 showed that silica nanoparticles were nontoxic in BEAS-2B cells with 24, 48 and 72 hours incubation.

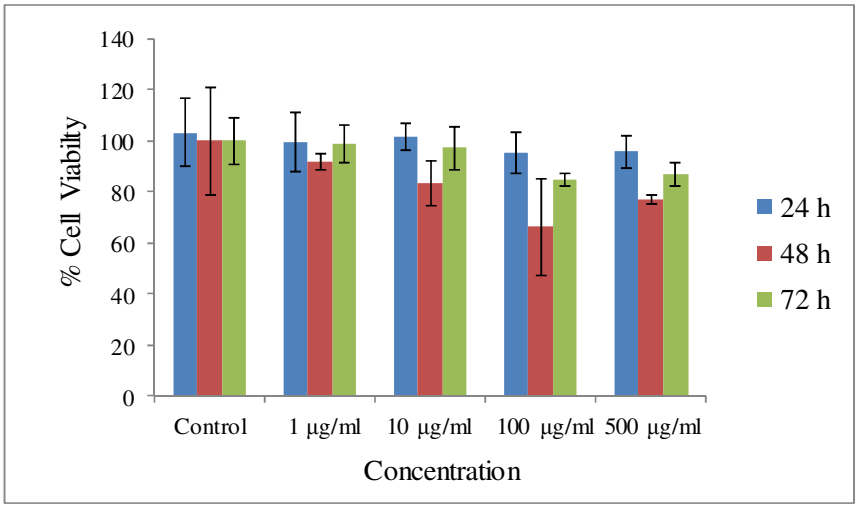


Figure 3.10. Cell viability assay for silica nanoparticles (S1) with size of 60.0 nm for BEAS-2B cell lines.

The borosilicate nanoparticles were nontoxic against BEAS-2B cells shown as Figure 3.11, for dosages up to500 µg/mL and up to 72 hours of incubation.

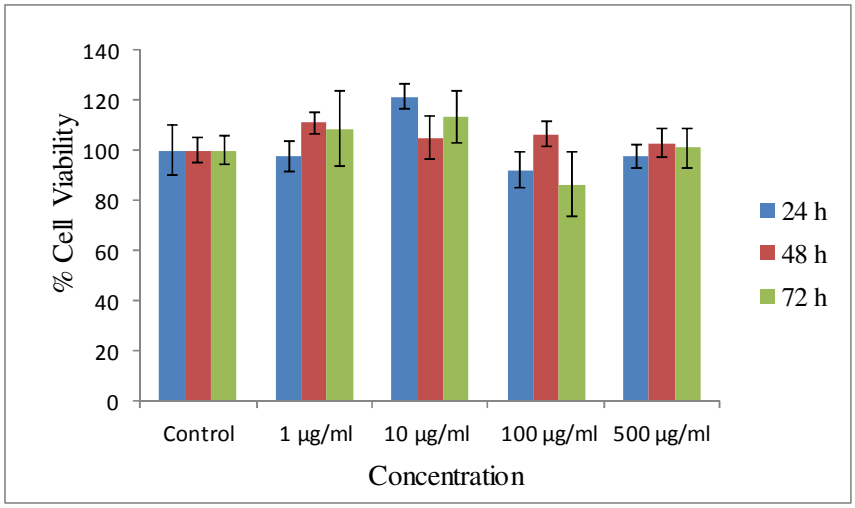


Figure 3.11. Cell viability assay for borosilicate nanoparticles (S4) with size of 60.0 nm for BEAS-2B cells.

We observed that silica nanoparticles with size of 115 nm had no toxic effect up to 72 hours exposure time and 100 µg/mL of dosages as seen in Figure 3.12.

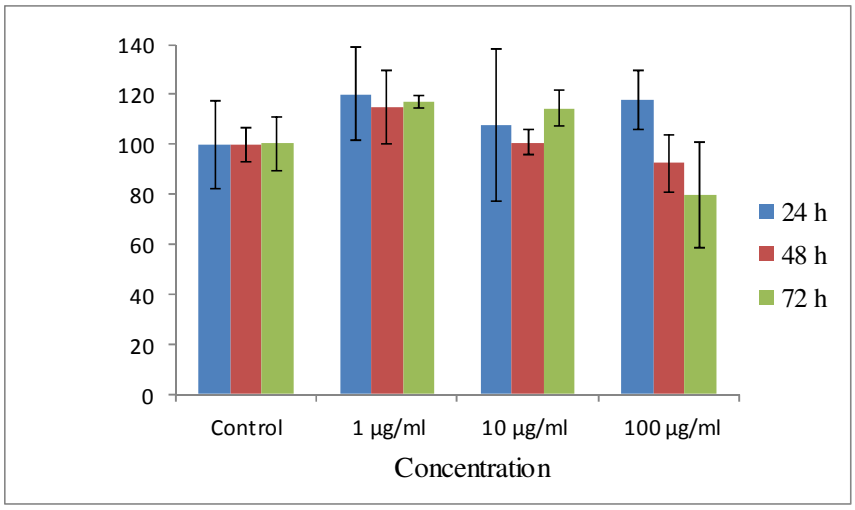


Figure 3.12. Cell viability assay for silica nanoparticles (S2) with the size of 115 nm for BEAS-2B cell lines.

In Figure 3.13, there was no difference between the control group and the cells with having borosilicate nanoparticles varying the concentrations from 1 to 100µg/mL in terms of cell viability. Thus, cytotoxicity of particles with size of 126 nm was independent of the dosages and incubation period.

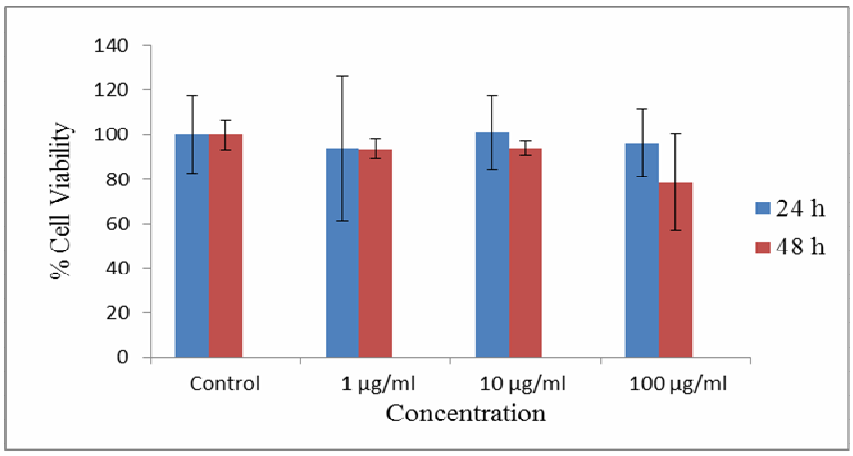


Figure 3.13. Cell viability assay for borosilicate nanoparticles (S5) with the size of 126 nm for BEAS-2B cell lines.

After 48 hours incubation time, cytotoxicity became apparent for the dosages of the silica nanoparticles in size of 430 nm at 100 and 500µg/mL for BEAS-2B cell lines as seen in Figure3.14.

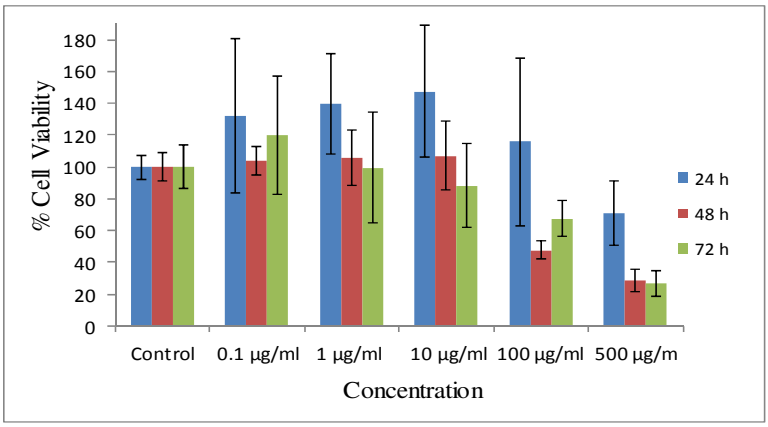


Figure 3.14. Cell viability assay for silica nanoparticles (S3) with size of 430 nm for BEAS-2B cell lines.

In Figure 3.15, as the cell viability reduces below 50%, cytotoxicities were induced by the borosilicate nanoparticles with size of 340 nm for BEAS-2B cell lines, when the incubation time is about 48 hours.

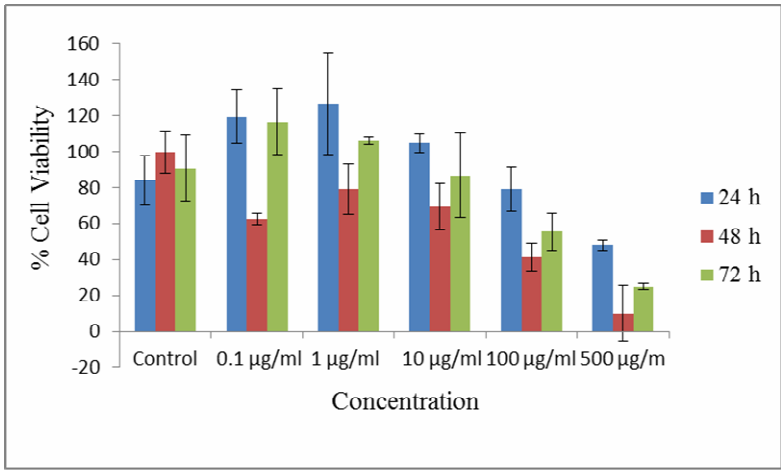


Figure 3.15. Cell viability assay for borosilicate nanoparticles (S6) with size of 340 nm for BEAS-2B cell lines.

3.3.2. Genotoxicity of Silica and Borosilicate Nanoparticles

Genotoxicity of silica and borosilicate nanoparticles is measured by the amount of DNA damage. In this study, the amount of DNA damage is determined by the PARP [214/215] assay.

We evaluated genotoxic response of A549 and BEAS-2B cell lines against silica and borosilicate nanoparticles. The dosage for genotoxicity was 500 µg/mL for both nanoparticles; in addition 1000 µg/mL was used for borosilicate nanoparticles. The amount of PARP expressed by the cells as a biological response was measured.

The amount of PARP was too low to make a clear assessment for genotoxicity induced by the nanoparticles. The results shown in Figure 3.16 points that genotoxicity in A549 cells is insignificant.

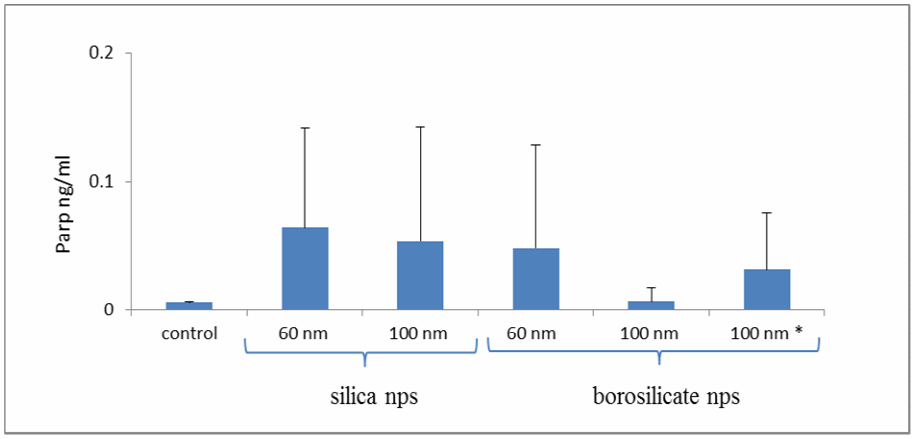


Figure 3.16. Amount of PARP expressed by A549 cells in the presence of silica (60 nm) and borosilicate nanoparticles (60 and 100 nm). The amount of both particles is 500 µg/mL and 1000 µg/mL* dosage for 100 nm borosilicate nanoparticles was also used.

The genotoxic response of the BEAS-2B cells against the silica nanoparticles was given in Figure 3.17. The particles induced genotoxicity for BEAS-2B cells. However, PARP assay up to 1000 µg/mL of dosage showed no genotoxicity at the borosilicate nanoparticles.

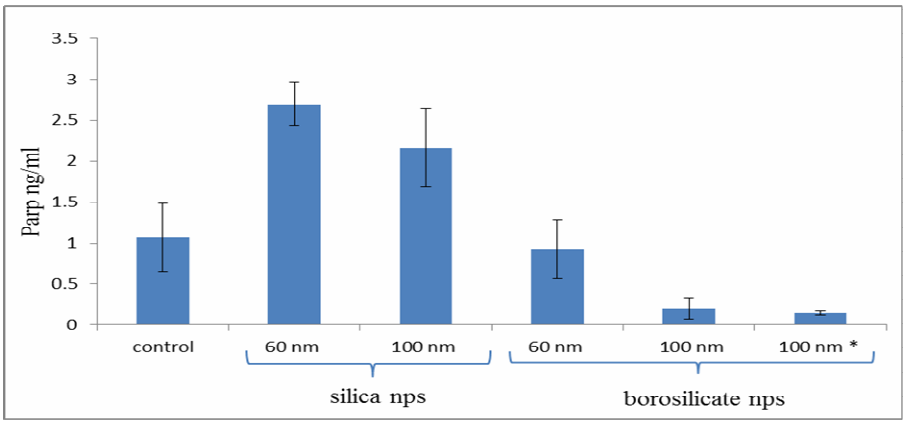


Figure 3.17. The amount of PARP expressed by BEAS2B cells in the presence of silica (60 nm) and borosilicate nanoparticles (60 and 100 nm). The amount of both particles is 500 µg/mL and 1000 µg/mL* dosage for 100 nm borosilicate nanoparticles was also used.

3.4. Discussion

In this study, we investigated the cytotoxic and genotoxic effects of silica and borosilicate nanoparticles for A549 and BEAS-2B cell lines. Cytotoxicity induced by both nanoparticles was measured by MTT and XTT assays. Synthesized both particles in all sizes, 60, 100 and 400 nm, were used for cytotoxicity studies. We used silica and borosilicate nanoparticles with dosages of from 0.1 µg/mL to 500 µg/mL dose and incubation periods of 24, 48 and 72 hours. Our results showed that both nanoparticles were nontoxic against A549 cells within the dosages with 0.1-500 µg/mL for all exposure times. Our results revealed that silica and borosilicate nanoparticles 400 nm in size became slightly cytotoxic at high dosages (500 µg/mL) for 48 and 72 hours incubation period as illustrated in Figure 3.15 and 3.16. Even though, they were nontoxic at low concentrations in BEAS-2B cells. In addition, both particles showed no cytotoxic effect in their all sizes and all incubation period as BEAS-2B cells were used.

Silica and borosilicate nanoparticles with the size of 60 and 100 nm were used for genetoxicity studies. Our results based on the PARP assays indicated that genotoxicity at the 500 µg/mL concentration of silica nanoparticles with the size of 60 and 100 nm for BEAS-2B cell lines. However, A549 cell lines were not affected by both particles and BEAS-2B cell lines were not damaged borosilicate nanoparticles.

Lin et al. evaluated the cytotoxicity of amorphous silica nanoparticles by using MTT assay. Lin et al. used 15 nm and 46 nm amorphous silica nanoparticles and Min-

U-Sil 5 used as a positive control with human bronchoalveolar carcinoma derived-cells (A549). The dosage of particles varied from 10 µg/mL to 100 µg/mL (Lin et al. 2006). They found dosage dependent cytotoxicity. Change et al. agreed with Lin et al. Change et al. induced the response of several normal fibroblast and tumor cells by altering doses of amorphous silica or composite nanoparticles of silica and chitosan. A cell proliferation assay showed that silica nanoparticles were nontoxic at low dosages but that cell viability decreased at high dosages (Chang et al. 2007). Ye et al. studied on myocardial cells (H9c2(2-1)) and they used silica nanoparticles with sizes of 21 and 48 nm and the incubation time was up to 48 hours. (Ye et al. 2010). Akhtar et al.'s observed that silica nanoparticles were not induced cytotoxicity. The cell viability fell down 70 % despite of consisting of 400 µg/mL silica nanoparticles with size of 10 nm and 80 nm for A549 cell lines incubated for 48 hours. In our case, we used silica nanoparticles having size of 60, 100 and 400 nm for A549 cell lines, the cell viability reduced to 60 % with 500 µg/mL of dosage. (Akhtar et al. 2010).

Cytotoxic and genotoxic effects induced by silica and borosilicate nanoparticles synthesized were evaluated for the particles with the size of 60 nm, 100 nm and 400 nm. Healthy and carcinoma cell lines were used for the assessment of the biological response. We found that silica and borosilicate nanoparticles are nontoxic up to 500 µg/mL in A549 cell lines and up to 72 h incubation times. However, they induce toxicity as the dosages reach to 500 µg/mL for BEAS2B cell lines for 48h and 72 h incubation period.

Genotoxicity of 60 nm and 100 nm silica and borosilicate nanoparticles have been investigated for both A549 and BEAS2B cell lines. The result showed that silica and borosilicate nanoparticles did not alter the amount of PARP for A549 cell lines. Borosilicate nanoparticles did not result in DNA damage for BEAS-2B cells. However, silica nanoparticles showed genotoxic effects for BEAS2B cells at 500 µg/mL dosages.

CHAPTER 4

CONCLUSION

One of our purposes is to develop a method to prepare borosilicate coated silica nanoparticles (borosilicate nanoparticles) by using the Stöber method. We carried on our study by preparing both the silica and borosilicate nanoparticles. We synthesized the particles with three different sizes approximately, 60, 100 and 400 nm, by varying the amount of some of reagents that were ethanol and ammonia. We found that there was no difference in terms of morphology of the both particles. In addition, we used EDX, FTIR and titration method to prove the presence of boron. These analyses exhibited that results were consistent with each other in terms of atomic per cent of boron in the samples.

Cytotoxic and genotoxic effects induced by silica and borosilicate nanoparticles synthesized were evaluated for the particles with the size of 60 nm, 100 nm and 400 nm. Healthy and carcinoma cell lines were used for the assessment of the biological response. We found that silica and borosilicate nanoparticles are nontoxic up to 500 µg/mL in A549 cell lines and up to 72 h incubation times. However, they induce toxicity as the dosages reach to 500 µg/mL for BEAS2B cell lines for 48h and 72 h incubation period.

Genotoxicity of 60 nm and 100 nm silica and borosilicate nanoparticles have been investigated for both A549 and BEAS2B cell lines. The result showed that silica and borosilicate nanoparticles did not alter the amount of PARP for A549 cell lines. Borosilicate nanoparticles did not result in DNA damage for BEAS-2B cells. However, silica nanoparticles showed genotoxic effects for BEAS2B cells at 500 µg/mL dosages.

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