Facile synthesis of mesoporous silica nanoparticles using modified sol-gel method: Optimization and *in vitro* cytotoxicity studies

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Abstract: The present study describes the synthesis of mesoporous silica nanoparticles using a modified sol-gel method. Various proportions of acetonitrile-water mixtures were utilized so as to optimize the reaction mixture for facile synthesis of mesoporous silica nanoparticles with controlled particle size for the very first time. After carefully adjusting the water and acetonitrile contents i.e. to 1:1 v/v ratio, a more uniform and small sized nanoparticles were achieved. The resultant particles were 140 nm in size having pore size of approximately 5.9 nm and were safe to be used in the cellular system, as confirmed by the *in vitro* cytotoxicity studies.

Keywords: Acetonitrile, mesoporous silica nanoparticles, tetraethyl orthosilicate, *in vitro* cytotoxicity, cetyltrimethyl-ammonium bromide.

INTRODUCTION

The last two decades have seen tremendous advancement in nanomaterials which exhibits controlled drug release and targeting owing to their unique physicochemical features (Singh *et al*., 2014, Jortner and Rao, 2002, Khan *et al.* 2017). The major goal of engineering smart nanomaterials is to prepare nanomedicines that are capable of enhancing drug's bioavailability at the target sites there by reducing the dosing frequency and side effects that are often associated with a large variety of drugs (Davis *et al*., 2008, Rizvi and Saleh, 2018). Recently, inorganic nanoparticles made of silica, alumina, titania, zirconia, silicon nitride and silicon carbide have been reported to be successfully used in biomedical and pharmaceutical applications (Singh *et al*., 2014). Nonetheless, mesoporous silica nanoparticles (MSNs) for drug delivery have attracted many researchers since the first report on MCM-41 type MSNs in 2001. Mesoporous structures, possesses large surface area as well as pores with diameter in the range of 2 to 50 nm and have shown great potential in catalysis, biological and biomedical applications (Tang *et al*., 2012). Mesoporous silica nanoparticles offer a variety of advantages over other nano-carriers, such as being porous, easy surface functionalization, biocompatibility, low toxicity and easy to manufacture with relatively low cost procedures (Kim *et al*., 2011, Wu *et al*., 2013, Florek *et al*., 2017). MSNs have been well exploited in the pharmaceutical industry as a versatile carrier that offers tuneable surface properties along with sufficient porosity for encapsulation of a wide variety of drugs (Vivero‐Escoto *et al*., 2010, Zhang *et al*., 2010, Lu *et al*., 2007, Li *et al*., 2017), proteins (Slowing *et al*., 2007, Pan *et al*., 2012, Deodhar *et al.,* 2017), genes (Slowing *et al*., 2008, Kim *et al*., 2011, Zhou *et al*., 2018) and enzymes (Lee *et al*., 2009, Chen *et al.,* 2013, Llopis-Lorente *et al*., 2017) for their delivery in different clinical situations (Tarn *et al*., 2013, Mamaeva *et al*., 2013). MSNs with a large number of pores and greater surface area for accommodating a large drug's pay-load not only control the drug release at a specific area of interest, but also specifically target the cells where the drug release is necessary, thanks to the ease of surface functionalization (Li *et al*., 2004, He *et al*., 2010, Vivero-Escoto *et al*., 2010, Wen *et al*., 2017).

Among the various challenges in MSNs synthesis, the control over size, porosity and shape of the nanoparticles are the most important ones. There are several methods available for synthesizing MSNs including flame spray pyrolysis, chemical vapour deposition, sol-gel process, micro-emulsion and few others; however, sol-gel process remains the most popular because of its ability to control size, porosity and shape of the nanoparticles (Singh *et al*., 2014, Brinker and Scherer, 1990). The sol-gel technique, a term first coined by Graham while he was working on silica sols (Graham, 1864) and is also known as soft template method. It offers a low temperature synthesis of purely inorganic or organic-inorganic materials (Tang *et al.,* 2012). Earlier study reported by Kolbe demonstrated formation of silica nanoparticles by reacting tetraethyl silicate in aqueous-alcoholic mixtures in the presence of some bases and cetyltrimethylammonium bromide as liquid crystal template (Kolbe, 1956). The reaction proceeded slowly and resulted in fine and spherical silica particles. However, Stöber and co-worker could not

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replicate the product as described in above mentioned study, so they proposed drastic adjustment to the experimental conditions. These involved the use of various alcohols such as methanol, ethanol, propanol and *n*-butanol, either in pure form or in alcoholic, aqueous alcoholic and saturated alcoholic ammonia mixtures. Their results indicated that smallest nanoparticles were obtained when methanol was used as the solvent, whilst the size range increased with increasing alkyl chain length of alcohol. Thus, their study demonstrated importance of solvents in controlling the size and shape of the silica nanoparticles (Stober *et al*., 1968). Later studies by (Grün *et al.,* 1997) and (Zhang *et al*., 2004) also demonstrated the use of aqueous alcoholic mixtures in the formation of tuneable silica nanoparticles. Although the use of aqueous alcoholic mixtures in the previous studies produced small sized particles, yet controlled growth of silica nanoparticles remained an issue, which led to the polydispersity in the resulted MSNs.

Here, we proposed an alternative method for synthesising monodispersed MSNs using acetonitrile-water mixtures for the very first time as the reaction media in the presence of potassium hydroxide as the base, cetyltrimethylammonium bromide (CTAB) as structure directing surfactant and tetraethyl orthosilicate (TEOS) as silica precursor using one-pot one-step synthesis method. The prepared MSNs were extensively characterized using a combination of analytical tools including X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), Nitrogen adsorption desorption analysis, size and zeta potential measurement, and finally the nanoparticles were tested for their safety in cells using *in vitro* cytotoxicity studies.

MATERIALS AND METHODS

Materials

The silica precursor, namely tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), potassium hydroxide (KOH), acetonitrile (HPLC grade) were procured from Dae-Jung Chemicals (City, Korea) and were used as received. Deionized water prepared at in-house facilty was used throughout the experiments.

Preparation of MSNs

The mesoporous silica nanoparticles were synthesized by modifying the previously reported method (Nunes *et al*., 2002). Briefly, different sol-gels were prepared by systematically varying the acetonitrile to water ratios (1:0.2, 1:0.4, 1:0.6, 1:0.8, and 1:1 in the first set of experiments; 0.2:1, 0.4:1, 0.6:1, 0.8:1 and 1:1 in the second set of experiments). Each mixture (100mL) was poured in a 250mL volumetric flask and the mixture temperature was maintained at 35°C.The pH of the mixture was adjusted to 9.0 by adding 1mL of 0.5M potassium hydroxide drop wise. Then, 0.5g (0.014M) CTAB was added to the mixture under continuous stirring at 1500rpm on a magnetic stirrer until a clear solution was achieved. After complete mixing of surfactant, 5mL of TEOS was slowly added using a 10mL syringe under continuous stirring for 1 hour at 35°C. After approximately ten minutes, the solution turned opaque indicating the start of the reaction and formation of white gel. The white gel was filtrated by using 0.2µ Sartorius filter under nitrogen pressure, washed thrice with deionised water and dried overnight in a desiccator at room temperature. Finally, the dried mass was calcined at 500°C for 6h to completely remove the surfactant template (Yoon *et al*., 2007). In the third set of experiments, 0.2g (0.0055M) CTAB was used and the acetonitrile was systematically varied in acetonitrile-water mixtures as given in the second set of experiments. The formulations were designated as MSN1 to MSN16 in this study.

Characterization of MSNs

Morphology of MSNs

MSNs surface, morphology, and approximate size was investigated by using scanning electron microscopy (SEM) (VEGA3, TESCAN). Mesoporous particles were carefully transferred to specimen holder having double adhesive tape. The samples were gold coated and micrographs were taken at different resolutions.

Size and zeta potential measurement

Particle size and zeta potential of optimum sample was measured using Zetasizer Nano ZS (Malvern, MALM1127001,UK). Suspensions having the concentrations of 100mg/L were prepared using water as dispersant. A minute quantity of 0.1M $HNO_3$was used for the pH adjustment. Afterwards measurements were recorded at 25°C.

Wide angle X-ray diffraction

X-ray diffraction patterns of selected sample was obtained using an X-Ray diffractometer (Malvern Panalytical X'pert PRO, UK) equipped with a CuKα radiation source operating at 30 mA and 30 kV. Data was recorded from 2θ angle of 5° to 50° at a step size of 0.02° and scanning speed of 4°/min.

FTIR spectroscopy

FTIR spectroscopy was used to study any possible interactions between various components employed for synthesis of the mesoporous particles and identify silicate group in optimal particles. FTIR spectra, over the spectral range of 500-4000cm^{-1}at 2cm^{-1} resolution, were acquired through a Nicolet IS7ATR-FTIR spectrometer (Thermo Scientific, USA).

Nitrogen adsorption desorption analysis

The micromeritic properties including surface area, pore volume and pore size of optimized MSN were determined by nitrogen adsorption desorption using Gemini VII 2390

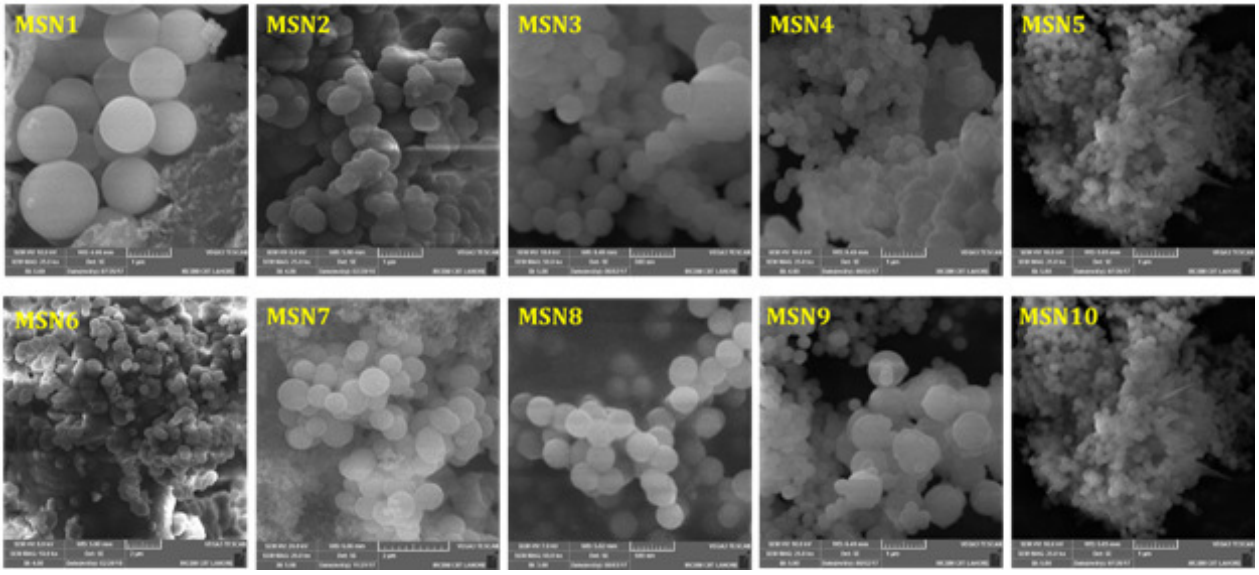

Fig. 1: SEM images of various mesoporous silica nanoparticles.

surface area analyzer (Micromeritics Instrument Corp., Georgia, USA) operating at -196.15°C. The sample was degassed at 200°C for 24 h prior to analysis. Barrett-Joyner-Halenda (BJH) and Brunauer-Emmett-Teller (BET) procedures were applied on adsorption-desorption data to characterize pore characteristics (Brunella *et al*., 2016).

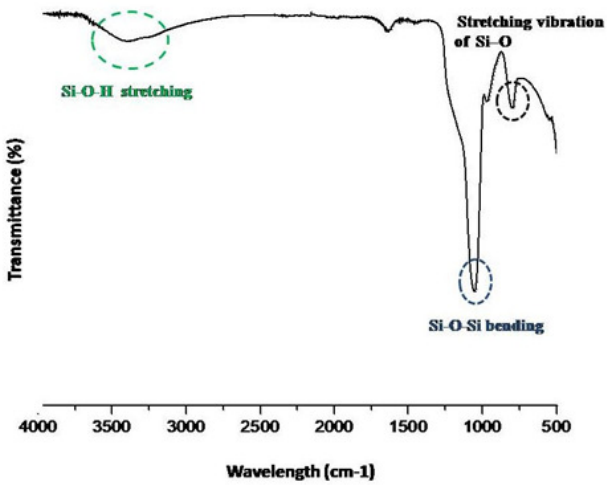

Fig. 2: FTIR spectrum of mesoporous silica nanoparticle.

In vitro cell viability studies

Human hepatocellular carcinoma (HepG2) cell line was obtained from American Type Culture Collection (ATCC; Manassas, and grown by University of Lahore). Cells were maintained in DMEM supplemented with 10% (v/v) FBS in a 95% (v/v) humidified atmosphere and 5% (v/v) CO_2 at 37°C. Cells were seeded near confluence a day before incubation with MSN5. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to measure succinate dehydrogenase mitochondrial activity as an indicator of cell viability/proliferation (Mosmann, 1983). Different concentrations of MSN5 (50- 400µg/mL) was added to cell medium for 24 hours. Afterwards, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37ºC for additional 4hours. Then the medium with MTT was aspired carefully and formed formazan crystals in each well were solubilised in 100µl of dimethyl sulfoxide (DMSO) and then absorbance of dissolved formazan crystals were measured at 570 nm using micro plate reader (Patel *et al*., 2009). The percentage cell viability was determined using following formula.

$$\% \text{ cell viability} = \frac{\text{Abs. of treated cell} - \text{Abs. of blank}}{\text{Abs. of Control} - \text{Abs. of blank}} \times 100$$

RESULTS

Morphology of MSNs

SEM images revealed that most of the formulations resulted in spherical MSNs, whilst the size and shape was dependant on acetonitrile-water proportions. However, no MSNs were possible when we reduced the CTAB concentration from 0.5g to 0.2g in the reaction mixture (SEM Data not shown).We obtained smallest particles with spherical shape for MSN5 and MSN10 formulation, which were synthesized when acetonitrile to water content were equal (1:1). However, no clear trend in particle size was observed when we progressively altered the acetonitrile contents. It is noteworthy that, no MSNs were obtained when only water was used as the reaction media. Based on morphological study using SEM, MSN5 was designated as optimized formulation, and was used for further characterization.

FTIR spectroscopy

The synthesised MSNs were investigated for identification and characterization of functional groups present on silica particles using FTIR spectroscopy in the spectral range of 500-4000 cm^{-1}, as exemplified in the fig.

2. Mesoporous silica nanoparticles showed typical absorption bands of the silicate at 797.97, 1053.78, 1636.88, 3396.49 cm^{-1}, which are usually assigned tosiloxane bond (797.97) (Wardhani *et al*., 2017), Si-O-Si bending (1053.78cm^{-1}) and silanol (Si-OH) symmetric stretching (3396.49) (Sevimli and Yılmaz, 2012, Liu *et al*., 2015) and bending vibrations at 1636cm^{-1}(Liu *et al*., 2015, Wardhani *et al*., 2017).

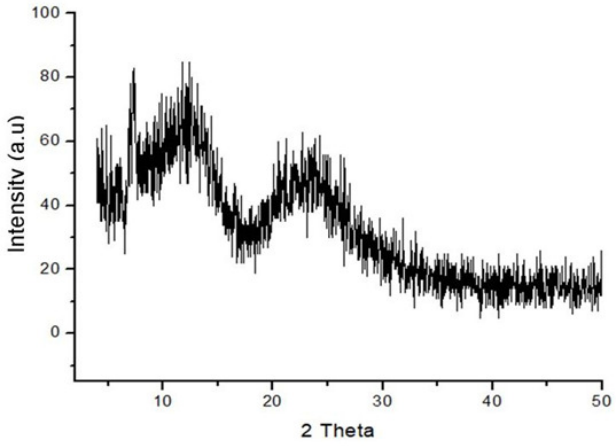

Fig. 3: XRD pattern of selected mesoporous silica nanoparticles (MSN5).

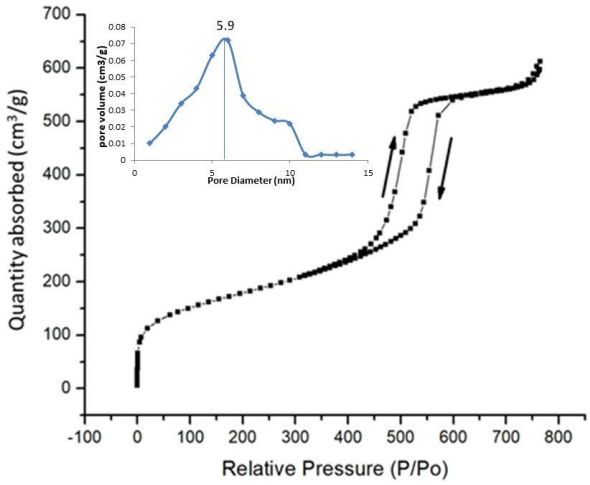

Fig. 4: N_2 adsorption–desorption isotherms of pure MSN 5. Inset picture shows corresponding BJH pore size distribution curve.

Wide angle X-ray diffraction

Wide-angle X-ray diffraction was used to investigate the amorphous and crystalline nature of optimum formulation MSN5. In the XRD analysis the mesoporous silicate nanoparticles gives three diffraction peaks and is considered as a finger print for the mesoporous silica particles (Lai *et al*., 2003, Slowing *et al*., 2006). The X-ray diffraction patterns confirmed the amorphous nature of the prepared SiO_2 samples as shown in fig. 3. Two broad peaks observed at 25° and 10° confirms the amorphous structure of MSN (Ghani *et al.,* 2017, Huo *et al*., 2014), as depicted in fig. 3.

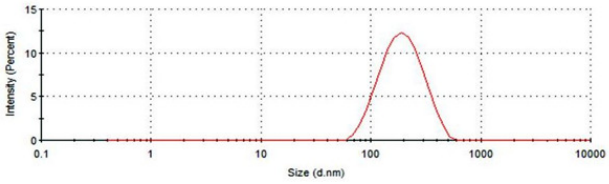

Fig. 5: Particle size distribution of the silica nanoparticles (by intensity) produced from Zetasizer Nano Analyzer.

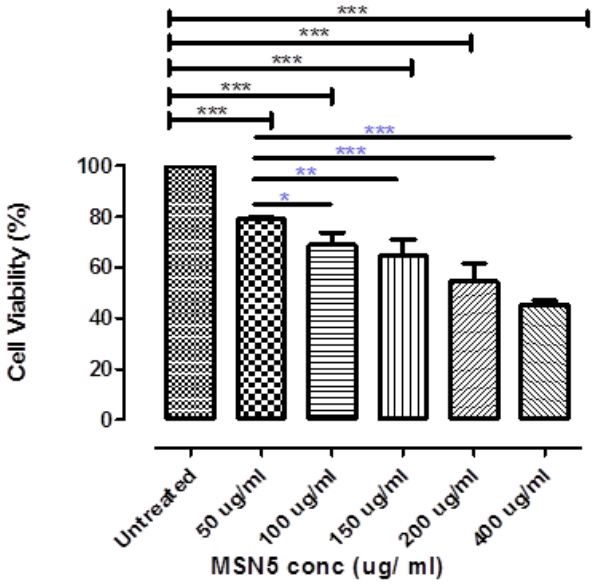

Fig. 6: Cell viability after exposure of silica nanoparticles for 24 hours. Black asterisk show comparison between untreated and MSN5 treated cells. Blue asterisk shown comparison between different treatment groups of MSN5 Where * = p<0.05 and *** = p<0.001. When compared with untreated significant reduction in cell viability was observed in MSN5 treated cells. When compared between MSN5 treatments groups, no significant changes were observed at 50 and 100µg/ml, while increasing the concentration resulted in significant reduction in cell viability.

Micromeritic properties using N_2 adsorption – desorption analysis

To confirm the porous nature of MSN5, nitrogen adsorption/desorption isotherm was acquired using a surface area analyzer working on BET principle. The resultant isotherm exhibited type IV isotherm properties of mesoporous materials according to the IUPAC classification (Sing, 1982) as shown in the fig. 4, which is associated with mesoporous materials where multilayer adsorption of molecules is followed by capillary condensation. Furthermore, the hysteresis loop (the two branches of isotherms) obtained here are almost vertical and nearly parallel during nitrogen gas uptake, thus

referred to type H1 hysteresis loop (Sing *et al.,* 1985). BET and BJH principles were applied on the data obtained from the nitrogen adsorption/desorption and corresponding pore properties were estimated. The inset picture in fig. 4 describes the pore width distribution. The optimized mesoporous nanoparticles (MSN5) exhibited BET surface area of 344.9 m^2/g, total pore volume of 0.218 cm^3/g and the pore width of 5.9 nm.

Particle size analysis

Particle size and zeta potential of synthesized MSN5 was investigated with DLS (Malvern, MALM1127001,UK). Hydrodynamic diameter of MSN5 was 140nm with 0.214polydispersity index (PDI).The PDI of optimized formulation is below 0.5, which indicates uniform particle size distribution and stable dispersion, thus increasing its worth for drug delivery applications (Das *et al*., 2014, Khan *et al*. 2015).

On the other hand, zeta potential can predict particle surface charge, stability and possible cellular interaction. The optimized formulation (MSN5) exhibited zeta potential of -10.2mV, which indicated stable formulation and is insufficient magnitude to keep the particles segregated, thus aiding towards stability of particles in the suspension form (Das *et al*., 2014).

In vitro toxicity testing

In vitro cell viability (MTT assay) studies were conducted on human derived hepatoma cell line (HepG2) using five different concentrations (50, 100, 150, 200 and 400µg/mL) of MSN5.Cell viability was lower in the formulation-treated HepG2 cells with high concentration of MSN5 particles (Figure 5).Apparently, MSN5 synthesis with equal amount of acetonitrile and water is nearly nontoxic and biocompatible. Percent toxicity induced at50, 100, 150, 200 and 400 µg/mL of MSN5 was 20.47 ± 1.13, 27.11± 5.56, 30.00±7.48, 39.63± 8.17 and 53.31± 1.86µg/mL respectively indicating dose-dependent cytotoxic pattern.50% inhibitory concentration (IC_{50}) of MSN5 towards HepG2 cells after 24 h of exposure was calculated to be 376.7 µg/mL showing relatively non-toxic nature of MSN5.

DISCUSSION

Here we report a facile one-pot one-step synthesis method of MSNs by reacting TEOS with positively charged CTAB, facilitated by acetonitrile-water mixture at various proportions under basic conditions in order to obtain MSNs of controlled size and shape. The proportion of acetonitrile and water was systematically varied in order to investigate the effect of solvent/co-solvent content on the prepared MSNs. Fifteen experiments were conducted in three different sets to optimize acetonitrile-water mixtures for the preparation of MSNs of desired properties. In the first experimental setup, we progressively increased the proportion of water in acetonitrile to water mixtures. In the second experimental setup, acetonitrile contents were progressively increased while keeping water content constant. In first and second experimental setups, the quantity of CTAB was fixed at 0.5g. We were also interested to study the effect of CTAB concentration on the MSNs preparation. Therefore, third experimental design involved the use of 0.2g CTAB with acetonitrile-water mixtures in which acetonitrile contents were progressively increased. In all cases, the CTAB quantity was well above the critical micelle concentration (CMC), which is 0.001 M (Bakshi, 1993). Generally, presence of additives such as co-solvents may affect the micellization process of various surfactants and can increase or decrease the CMC as reported previously (Bakshi, 1993, Jalali *et al*., 2000). Acetonitrile is known to have effects on CMC of CTAB (Jalali and Gerandaneh, 2011), however, the quantities of CTAB used in our study were sufficiently higher than its CMC to produce spherical micelles.

Above critical micelle concentration, the surfactant monomers self-aggregate to form spherical micelles. The negatively charged silica almost instantaneously interacted with the positively charged micelles in the basic conditions, which resulted in successful formation of mesoporous silica nanoparticles of various sizes, depending upon the composition of acetonitrile-water mixture. More interestingly, the reaction speed increased with increasing the acetonitrile content as observed through the opaqueness of the reaction media upon addition of the TEOS. The reaction proceeded with hydrolysis followed by condensation of TEOS at the polar head region of CTAB micelles in the presence of KOH, which served as the catalyst. The various acetonitrile-water mixtures dictated the overall morphology of MSNs, as depicted by the SEM images (Figure 1), because of slight variation in micelle formation in these mixtures, which ultimately led to the variations in the arrangement of the silica precursor at the top of micellar template (Vazquez *et al*., 2017).

FTIR spectra confirmed the presence of silicate in prepared mesoporous silica nanoparticles. Similar FTIR results of mesoporous silica nanoparticles are reported previously (Maleki and Hamidi, 2016, Wardhani *et al*., 2017) which complements our findings. XRD further confirmed the formation of MSNs particles and their amorphous nature as these particles show two broad peaks at 10° and 25° as reported in literature. Furthermore, these particles were uniform in size and show characteristic type IV isotherm and a clear type H1 hysteresis loop which show particles developed are regular and uniform with average pore size of 5.9nm. H1 hysteresis loop is frequently associated with porous materials, agglomerates or highly arranged spherical particles having cylindrical pore geometry, facile pore connectivity and narrow pore size distribution (Sing *et al.*, 1985). Nanoporous structure coupled with large surface can accommodate high

concentrations of drug thus they can be used for preventing premature degradation, enhancing solubility and release its payload in control release manner.

Zeta potential not only foresees particle surface charge but also reflects stability and possible cellular interaction. In general nano formulations having charge between -10mV to +10mV are considered neutral while one with greater than +30 mV or less than -30mV are referred as strongly cationic and anionic respectively. As, most of the cellular membranes have negative charge on them so, their zeta potential will affect membrane permeation. Generally, particles with cationic charge react quickly with biological components and display more toxicity by cellular membrane disruption (Clogston and Patri, 2011). The obtained size with low polydispersity and suitable charge indicates that our prepared MSN can be used as drug carrier (Mohseni *et al.*, 2015). During in vitro cytotoxicity testing our particles showed dose dependent toxicity (Figure 5) where at 50 µg/mL particles exhibited approximately 80 percent cell viability. It decreased afterwards and possibly could also be due to i) high concentration of particles and ii) direct physical contact with monolayer cell line (Khan *et al.* 2015). In one of studies researchers developed ZnO and SiO_2 particles and both of them exhibited concentration-dependent cytotoxicity as indicated by cell viability assay (Sahu *et al.*, 2016).

CONCLUSION

In this study, we have successfully developed mesoporous silica nanoparticles by employing various water and acetonitrile ratios via a simple sol-gel method with appreciable yield. Formulation having equal proportions of water and acetonitrile (1:1) gave uniform and mono dispersed particles with an average size of 140 nm and narrow PDI of 0.214. Nitrogen adsorption isotherms confirmed porous nature of prepared particles, which were approximately 5.9 nm in size with a large surface area (344.99 m^2/g). *In vitro* cytotoxicity testing showed compatibility of these particles with cells in high enough concentration. Precisely, due to its high yield, large surface area, fine particle size combined with relatively economical and fast production make it attractable carrier for pharmaceutical applications such as drug solubility enhancement, controlled release and targeting. Furthermore, it is recommended to evaluate in vivo performance of these particles to ensure their safe usage in human beings.

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