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Exploring the potential of amine-functionalized mesoporous silica nanocarrier to conjugate guide RNA for gene delivery

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Abstract

Sugarcane is a key cash crop, with nations such as Brazil, India, and China being the leading producers. The trade of sugar and related products contributes significantly to these economies. Sugarcane is the primary feedstock for producing sugar and ethanol in the sugarcane processing industry, yielding sugarcane bagasse fly ash (SBFA) as a byproduct. This byproduct comes primarily from the combustion of bagasse (the fibrous residue after juice extraction) and contains silica (SiO2), alumina (Al2O3), and other minerals. The use of sugarcane waste materials reduces the need for new raw resources and lowers the environmental impact. Turning waste into valuable nanoparticles also helps manage waste better and reduces the amount that is burnt or dumped in landfills. The fly ash was treated with an alkaline solution (e.g., sodium hydroxide) to extract silica. This process helps dissolve other minerals, leaving a silica-rich residue. After extraction, the silica was purified through acid washing to remove impurities. Mesoporous silica nanoparticles were created using the sol-gel method and 3-aminopropyltriethoxysilane (APTES) was added to them functionally to conjugate sgRNA. The synthesized mesoporous silica nanoparticles were characterized for size and shape, functional groups, and crystallinity using various instruments such as Transmission Electron Microscope (TEM), Fourier Transform Infrared Spectroscopy (FT-IR), Dynamic Light Scattering, Brunauer-Emmett-Teller (BET) Analyzer, and X-Ray Diffractometer (XRD), respectively. Furthermore, synthesized mesoporous silica nanoparticles were bioconjugated with sgRNA of OsEPF1, a gene negative regulation of stomatal development, which in turn, helps reduce yield loss in rice due to drought and heat stress.

Keywords

sugarcane bagasse; mesoporous silica; sol-gel; sgRNA; bioconjugation

Introduction

Mesoporous silica nanomaterial was lighted on 1992 by Kresge and his co-worker from alumina-silicate gels using the liquid crystal template mechanism. Mesoporous silica nanoparticles (MSNs) have the potential to develop climate change-resilient crop varieties due to their unique properties and ability to deliver agrochemicals, nutrients, and genetic materials efficiently. Since the majority of agricultural crop residues, like rice straw, rice husk, corncob,

bamboo leaves, and coconut husk, particularly those from cereal crops, are silica-enriched. Silica nanoparticles (SiNPs) were preferred among the several kinds of nanoparticles synthesized from agro-wastes. Mesoporous silica nanoparticles (MSNs) are made up of silica (silicon dioxide, SiO2), characterized by a network of pores with diameters typically between 2 and 50 nm. Because of the Si-O bond, mesoporous nanoparticles based on silica can tolerate external stimuli, including mechanical stress and deterioration (1). This has drawn a lot of interest because of its textural qualities, which includes tunable pore diameter, narrow pore size distribution, chemical inertness, and high surface-to-volume ratio (2). The mesoporous material has various pore geometrics which include cage type, bi-continuous cubic and 2D hexagonal evincing the pore opening gating mechanism for targeted delivery (3). Several methods can be used for the synthesis of MSNs which includes hydrolytic sol-gel process (4), chemical etching (5), template (6) and microwave-assisted methods (7). In the sol-gel process, silica precursors are polymerized to form a porous structure, in the through templating methods, a template molecule is used to create pores that are later removed, leaving behind the mesoporous structure. By adjusting the temperature, surfactant concentration, silica source, and pH of the reaction mixture, the pore parameters can be regulated during the synthesis process. These mesoporous silica nanoparticles find their applications in areas such as drug delivery, gene delivery, catalysis, adsorption, and sensing.

The Stober method is the conventional method of synthesizing MSNs where powdered sodium carbonate (Na2CO3) is mixed with quartz sand powder and heated around 1300℃ before subjecting to sulfuric acid (H2SO4) treatment to form sodium silicate (Na2SiO3). This method requires high temperature, which makes high capital expenditure and also leads to CO emission during the extraction process (8). The biofriendly method i.e. sol-gel synthesis of silica from biomass, which involves converting biomass-derived materials into a carbon-rich precursor, and then mesoporous silica nanoparticles (MSNs) were produced by mixing silica precursor and a surfactant.

Functionalized mesoporous silica refers to silica materials with a highly ordered pore structure at the mesoscale (typically 2-50 nm in diameter) that have been modified or "functionalized" with various organic or inorganic groups on their surface. This modification enhances their properties and makes them suitable for a wide range of applications (9-13).

Sugarcane is the major feedstock for producing sugar and ethanol in the sugarcane processing industry and that yields sugarcane bagasse fly ash (SBFA) as an end product (14). SBFA is considered as a potential feedstock for the production of SiO2 due to the renewable ash generation and its higher silica content (≈70 %) next to paddy husk ash (15). Hence, higher silica content is the prerequisite for the production of MSNs. With this background, the present work is aimed to synthesize biomass-derived MSNs from SBFA using the sol-gel synthesis method.

Drought and heat stress are significant environmental challenges that can severely impact paddy (*Oryza sativa*) growth, development, and productivity. The main strategy to reduce yield losses by heat is canopy cooling. The genes involved in regulating stomatal development and spacing belongs to EPIDERMAL PATTERNING FACTOR/EPF-LIKE families which includes *EPF1, EPF2* and *EPFL9/STOMAGEN*, that encode small secretory peptides. Stomatal development is down regulated by *EPF1* and *EPF2* genes. *EPFL9/STOMAGEN* gene is the positive regulator present in the mesophyll tissues by blocking the action of *EPF1* and *EPF2*. Certainly, overexpression of these genes results in increased/decreased stomatal density (16). Upon gene delivery, these genes are not stable under environmental conditions and create off-target mutations. The present work marks the novel report of conjugating the *EPF1* sgRNA with functionalized mesoporous silica nanoparticles that can be explored for creating targeted mutations to mitigate environmental stress in paddy.

Materials and Methods

Nanoparticle synthesis materials

Sugarcane bagasse fly ash was collected from the sugarcane processing industry located at Perambalur in Tamil Nadu, India. The fly ash was washed with distilled water several times, oven dried, and sieved to get a fine powder using the British Standard Sieve (B.S. S No. 200 (75 µm) mesh size) to yield the uniform-sized ash particles and stored in airtight plastic bags until use. Sulphuric acid (H2SO4), hydrochloric acid (HCl), non-ionic surfactant polyethylene glycol (Mol. Wt. 6000), Toluene anhydrous, 3-aminopropyltriethoxysilane (APTES), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich. Distilled water and Whatman filter paper No. 42 were used throughout the experiments.

Synthesis of Mesoporous Silica Nanoparticles

In the typical Sol-gel synthesis, uniform-sized fly ash particles were subjected to high temperature (900° C) in an inert atmosphere for 10 h under muffle furnace to obtain greyish-white colour ash product. Firstly, the acid treatment was done to remove the impurities such as CaCl2, AlCl2, CuCl2, MnCl2 *etc.* To carry out the acid treatment, the calcined sugarcane bagasse fly ash was treated with 1M HCl and stirred at 600 rpm for 3 h and then the solid residues were oven dried at 70℃. Secondly, the oven dried ash was subjected to 2N NaOH followed by 3 h stirring at 600 rpm and then the pale-yellow colour Na2SiO3 filtrate precursor was yielded using Whatman filter paper No.42. To synthesize silica with mesopore, 2.5 % acidic PEG, which is non-ionic surfactant/ polymer, acted as a template to create a porous material. 2.5 % PEG was dissolved in 0.5 M HCl and then silica-PEG hybrid was prepared by adding 12.5 g acidic PEG to 400 Na2SiO3 precursor under vigorous stirring and flow rate of PEG was maintained at 1 mL min-1. The pH was maintained from 3.5 to 7 to obtain the PEG-silica hybrid gel and then it was kept undisturbed for 16 h. A whitish gel was formed which was broken down with the help of distilled water several times to remove the excess PEG. The template PEG-free mesoporous silica was oven dried at 70℃ for an h and

calcined at 400℃ for 3 h.

Synthesis of Amine functionalized Mesoporous Silica Nanoparticles

In this synthesis method, after surfactant molecules were removed, the silanol group can be functionalized through organosilane, 3-aminopropyltriethoxysilane (APTES). For that, 1 g of MSNs was added to 50 mL of toluene anhydrous, and stirred for 30 min. Then 2 mL of 4.3 mM APTES was prepared and both mixtures were subjected to 50℃ for 24 h. Then the functionalized MSNs was centrifuged at 10000 rpm for 15 min to remove the unreacted amine molecules on the silanol surface. The amine-functionalized MSNs was washed with ethanol twice, air dried, and finally, 900 mg of amine-functionalized MSNs was recovered.

Bioconjugation of sgRNA with amine functionalised MSNs

The short guide RNA of *Os*EPF1 was conjugated with amine-functionalized MSNs. To obtain the optimal binding ratio of the guide RNA to amine- MSNs, 5 µL of various sgRNA were mixed with 0.1% amine-functionalized MSNs at a weight ratio of sgRNA to MSNs (1:10, 1:20, 1:40, 1:60, 1:80, and 1:100) at room temperature and subjected to 250 rpm for 2 h under 4℃. 5 µL of each solution was loaded onto a 1% agarose gel for 60 min at 120 V, where sgRNA alone as the control.

Characterization

Transmission Electron Microscope (TEM)

To visualize the size and morphology of amine-functionalized MSNs, the transmission electron microscope, TEM (FEI Technai spirit) with 120 kV was used. For that, 1mg of amine-functionalized MSNs was dispersed in 1 mL of distilled water and dropped onto the carbon coated copper grid using micropipette, then airdried and placed in the sample holder for imaging.

Fourier Transform Infrared Spectroscopy (FTIR)

The changes in surface functional group as well as chemical bonding of the nanoparticles were determined by Fourier Transform Infrared Spectroscopy using Jasco Model: R-3000-QE. 2 mg finely ground samples were placed on the sample injection port, and the radiation of about 10000–100 cm⁻¹ was passed and part of the radiation absorbed was recorded by the detector in the range of 4000–400 cm⁻¹. The obtained results were the molecular fingerprint of the sample.

Zeta Potential (ζ)

Zeta potential is the measure of the surface charge of the electrical double layer or interfaces in the colloidal dispersion that was measured by using the Nano-Particle Size Analyzer (Model: HORIBA-SZ-100) and in which the zeta potential was measured between -200 mV and +200 mV.

Brunauer–Emmett-Teller (BET) analyser

The nitrogen adsorption-desorption isotherm was recorded using the BET Quantachrome TouchWin™ version 1.22. The samples were subjected to degassing at 300℃ for 3 h and were placed in the sample port for analysis. The adsorbate and adsorbent interaction gave the characteristic pore size, pore volume, and surface area of the synthesized materials.

X-ray Diffraction (XRD)

X-ray diffraction (XRD) analysis is a non-destructive technique used to determine the crystallographic nature when the X-rays interact with the crystal lattice of the material. The diffraction pattern was recorded using Shimadzu, Model: XRD 600 using Cu Kα radiation, 40 kV current with a scan time of 0.5°/min of 50-80℃. The data obtained were plotted using ORIGIN Ver.8.5.

Results and Discussion

In this study, the mesoporous silica nanoparticles were synthesized by the sol-gel technique and functionally modified using 3-aminopropyltriethoxysilane (APTES) for conjugating sgRNA. The size of nanoparticles and morphology were characterized by TEM revealed the average particle size less than 50 nm shown in the Fig. 1 and were

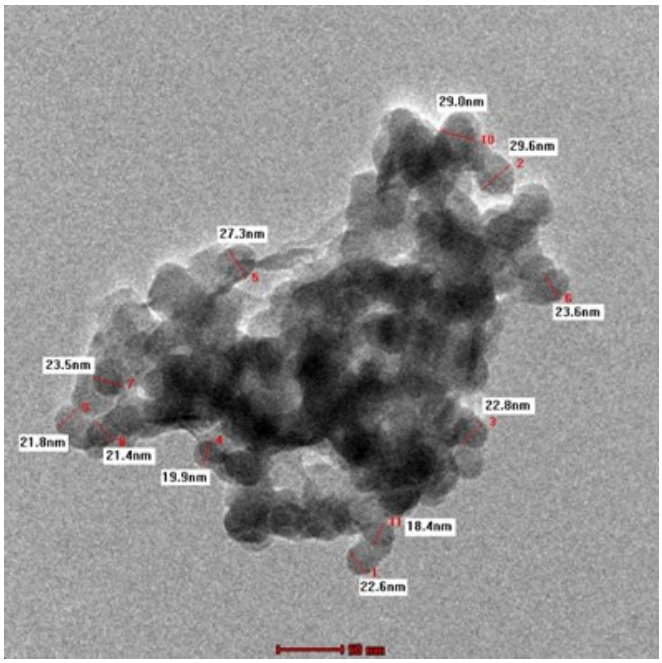

Fig. 1. Surface morphology of Mesoporous Silica (< 50 nm pore size)

spherical in shape with aggregation and the results are in accordance with Sasidhar *et al*., (17). The zeta potential of MSNs showed less than -19.5 mV (Fig. 3), confirming that the particles were anionically charged, mainly due to the silanol group and hydroxyl group, and the results are in line with Wang (18). Furthermore, the functionalized as-prepared MSNs by APTES with NH2 groups resulted in cationic surface charge with a zeta potential value of +26.2 mV (Fig. 4). Therefore, it is confirmed that the 2.3 mM of APTES is required to functionalize the MSNs. The zeta potential results are in line with Ngouangna (19). The FTIR spectra showed the strong absorbance at 3272 cm-1 assigned to the surface hydroxyl groups of mesoporous silica. The peaks at 1650 cm-1 and 1416 cm-1 correspond to the Si-OH bending ions and OH bending ions. The shoulder peak at 1210 cm-1 corresponds to the asymmetric stretching of ≡Si–O–Si≡. The strong characteristic bands can be observed at 1108 cm-1 which attributed to the Si–O–Si stretch of silica is shown in the Fig. 5. The FTIR datasets generated in the current study are in line with the dataset of Alahmadi (20), who reported the similar FT

-IR spectra profile of mesoporous silica nanoparticles. The nitrogen adsorption-desorption isotherm (Fig. 6) showed a typical hysteresis loop (Type IV isotherm). The surface area (400 m2/g), total pore volume (0.34 cm3 /g) and the average pore diameter (10 nm) obtained from the multipoint BET was found to be shown in the Fig. 5 and the results are in line with Bchellaoui *et al.* (21). The chemical and thermal activation increased the surface area and average pore diameter of the silica nanoparticles. The XRD pattern showed the mesoporous silica nanoparticles were amorphous in nature, having the sharp peaks at 23.460, 32.20 and 34.060 (Fig. 2). These results are in accordance with Rida *et al*. (22) and Alves *et al*. (23).

From the gel documentation result (Fig. 7), the binding ratio of short guide RNA (*Os*EPF1) to the synthesized APTES-MSNs was found to be in the increasing order of 1:40, 1:60, 1:80 and 1:100 at room temperature and 250 rpm for 2 h. The obtained results showed that the presence of sgRNA significantly decreases as the ratio of nanoparticle concentration increases. Surprisingly, the optimal binding of

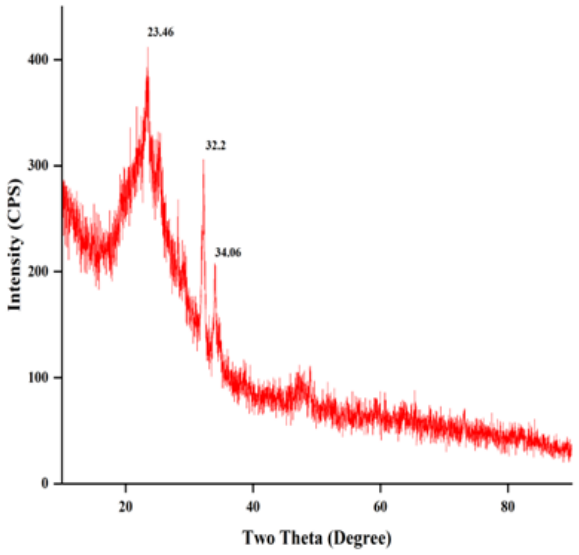

Fig. 2. XRD spectra of MSNs

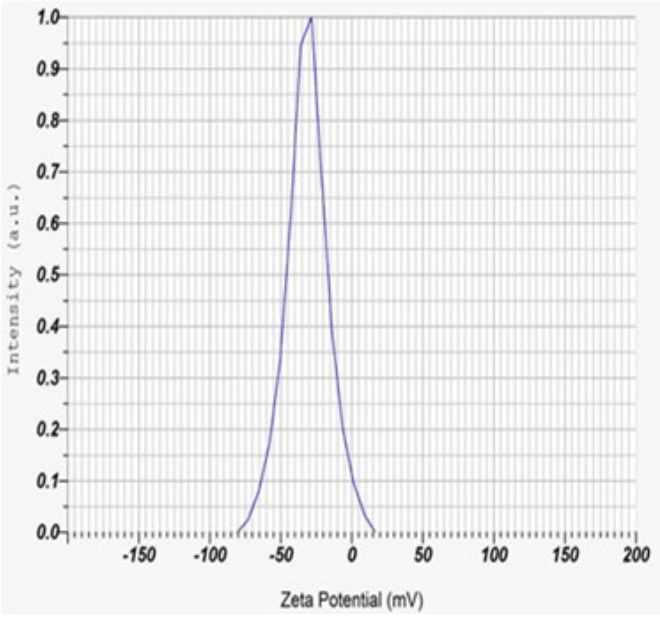

Fig. 3. Zeta potential (MSNs) - 19.5 mV

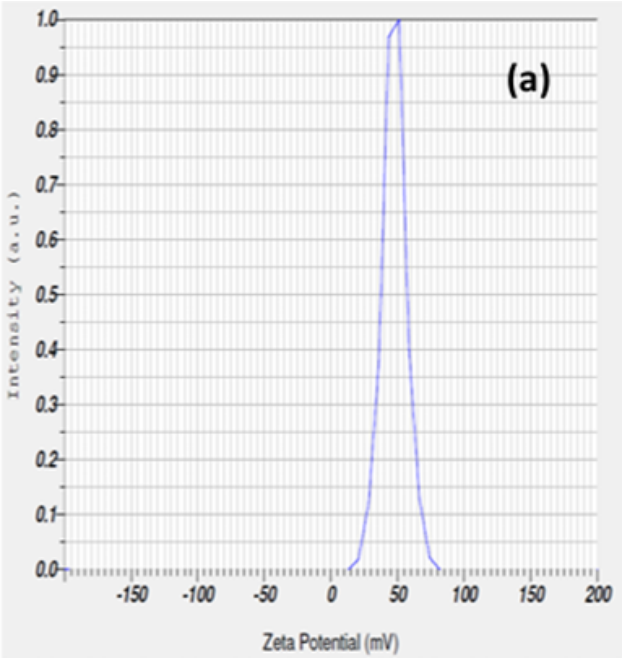

Fig. 4. Zeta potential (Amine-functionalized MSNs) + 26.2 mV

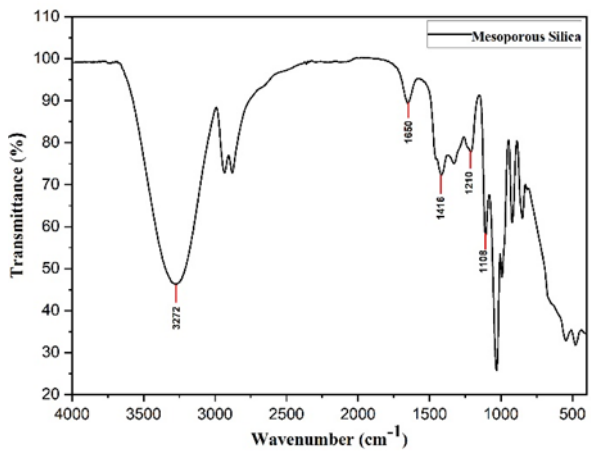

Fig. 5. FTIR Spectra of MSNs

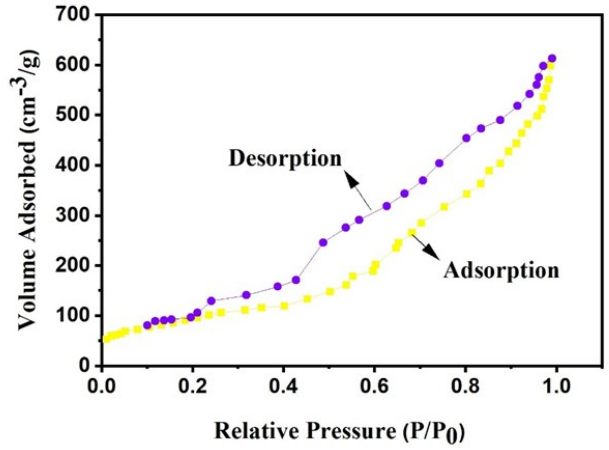

Fig. 6. BET adsorption/desorption isotherms of MSNs

gene material with nanoparticle occurred at the mass ratio of 1:100. The technique followed to conjugate amine-functionalized mesoporous silica with genetic material was reported by Hajiahmadi (24) .

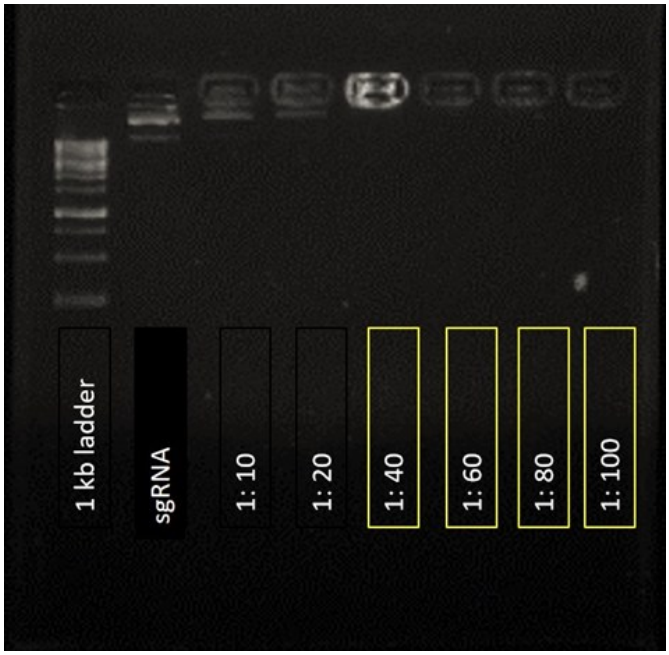

Fig. 7. Bioconjugation of sgRNA with amine functionalised MSNs

Conclusion

The current research successfully conjugated the *Os*EPF1 gene with amine-functionalized mesoporous silica nanoparticles by using the sugarcane bagasse fly ash waste as a precursor. This method can be optimized for conjugating various genes in various plant species as well. This process of synthesis minimizes the dependency on conventional sources of silica like sand. In addition, the use of sugarcane bagasse fly ash waste can lead to the formation of MSNs with unique properties, such as enhanced biocompatibility and tunable surface functionality. Overall, this nanobiotechnological approach offers a sustainable and cost-effective method for conjugating the genetic material with nanoparticles such as mesoporous silica that can be explored for creating targeted mutations to mitigate the adverse effects of climate change on agriculture in future studies and is desirable.

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Authors' contributions

MR and JSS provided the conceptualization, resources and drafted the manuscript. KA carried out the synthesis, characterization and conjugation studies. MP, MS and TK carried out the validation. RR edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors declare that they have no conflict of interests.

Ethical issues: None

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