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Chitosan Nanoparticles for improved plant health

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Abstract
Nanoparticles have emerged as potential tools for improving plant health and its performance during biotic and abiotic stress. However, little to no studies were reported with a focus on application of chitosan loaded nanoparticles to understand the biochemical driven physiological studies in plants. Therefore, in the current review we have focused on synthesis and characterization of chitosan nanoparticles (CSNPs) for improved plant growth and development during unfavorable conditions. Further, we have presented a broad range of nanoparticles that are widely used for plant studies as well. The CSNPs can potentially help in controlling post-harvest storage problems in several crop plants besides there involvement in routine growth and development of plants.

Keywords: Chitosan, Nanoparticles, Synthesis and Characterization of Chitosan Nanoparticles.

1.INTRODUCTION

Owing to its vast array of applications and multidisciplinary nature, building a consensus on a single definition of science of nanotechnology is a tedious task. National Nanotechnology Initiative (NNI) USA**,** defined nanotechnology as "Creation of structures, devices and systems in the range of sub 100 nm by the process of research and development at the atomic, molecular or macromolecular levels".

This emerging area of nanotechnology is now providing scientists with capacity to manipulate atoms for to create stronger, lighter & more efficient material with specifically defined properties [Hunt 2004; Baker and Aston 2005; Bawa et al 2005]. The reduction of size to this level by itself provides certain novel properties in addition to the uniqueness provided by the quantum physics effect [Bawa 2004a; Bawa et al 2005]. Though this may be considered important but experts do feel that restricting the definition to the strict boundaries of size mentioned above may not be well justified as it may exclude many nano scale devices and systems in micro scale which are well considered under nanotechnology by many scientists [Bawa 2004; Bawa 2004b; Baker and Aston 2005]. In sum, nanotechnology is an outcome of amalgamation of physical, chemical, and life sciences. The confusion is heightened by opinion of experts who claim that nanotechnology is not a new science. They buttress their claim by citing examples of nano scale carbon particle which are in application for a about a century as a reinforcement material and by protein vaccine which certainly come under the defined boundaries of NNI definition of nanotechnology. Their claim is strengthened by the fact that many of the biological structures already fall in nano level i.e., peptides are of equal size to that of quantum dots and some viruses are of the scale of nanoparticles used in drug delivery systems.

Thus, we hereby adopt the definition [Bawa and Bawa 2005] which conceives the idea of nanotechnology from its broadness of application: *"The design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometer scale (atomic, molecular, and macromolecular scale) that produces structures, devices, and systems with at least one novel/superior characteristic or property".*

2. Nanoparticles & their general types.

A nanoparticle is a microscopic particle whose size is measured in nanometers (nm). It is defined as a particle with at least one dimension <100nm or nanoparticles are solid colloidal particles ranging in size from 10 nm to 100 nm. They consist of macromolecular materials in which the active principle is dissolved, entrapped or encapsulated, and/or to which the active principle is absorbed or attached [Chen 2004]. Extensive research is currently underway to establish potential role of nanoparticles as particulate carrier in pharmaceutical and medical fields. The most focused application of nanoparticles is:

- Targeted cellular or tissue delivery,
- Prolong drug effect in the target tissue [Vyas et al. 2002],

• To increase stability of therapeutic agent against enzymatic degradation, For crop plants as well as in anti-cancerous activities.

2.1. General Classification of Nanoparticles:

Nanoparticles are classified into eight types based on their source of constituents. They are Quantum dots, Nanocrystalline silicon, Photonic crystal, Liposomal, Gliadin, Polymeric, Solid Lipid Quantum, Metal nanoparticles.

2.1.1. Quantum Dots : These nanoparticles are commonly known as nanocrystals or nanostructures made of semiconductor elements. They range from size 2nm to 10 nm.

2.1.2. Nanocrystalline silicon (Nc-Si): Nc-Si nanoparticles consists of smaller grain of crystalline silicon *i.e.* in nano scale providing unique properties like higher mobility [Shim and Guyot 2004], increased absorption in infra-red region (better function in solar absorption) and higher stability than normal counterpart.

2.1.3. Photonic crystal: Photonic crystals are attractive optical materials for controlling and manipulating the flow of light.

2.1.4. Liposomes: A sphere-shaped vesicle with its membrane composed of bi-layered phospholipids which are efficient in delivering drugs or genetic material into a cell. It can either be made of natural source phospholipids with mixed lipid chains or of pure components.

2.1.5. Gliadin nanoparticles: These are protein nanoparticles rich in neutral and lipophilic residues. The presence of neutral amino acids can promote hydrogen bonding with mucosa, and lipophilic components can interact with biological tissue by hydrophilic interaction. Thus, they are popularly known to be, mucoadhesive. The composition of gladilin explains its high affinity to mucosa [Rao et al. 2004].

2.1.6. Polymeric Nanoparticles: These are the nanoparticles produced by interactions of polymeric compounds with high surface charge which upon treatment with oppositely charged counterpart results in nanoparticle formation. They exhibit higher shelf life and can be surface coated with functional groups for better targeting, hence higher grade than liposomes.

2.1.7. Solid Lipid Nanoparticles (SLN): These are sub-micron level colloidal carriers made of physiological lipids dispersed in water or aqueous surfactant solution [Rao et al. 2004; Muller et al. 2000] and provide best alternate to conventional polymeric nanoparticles. The most advantages involved in them being biodegradability, biocompatibility and non-toxicity.

2.1.8. Metal Nanoparticles: Gold and Silver nanoparticles fall under this category. They are easy to produce by both "*bottom up"* and "*top down"* methods. Gold nanoparticles with DNA attached to surface are examples of some of the materials used in for qualitative and quantitative analysis of samples

3. Synthesis of nanoparticles

Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. The selection of matrix materials is dependent on many factors including: Size of nanoparticles required, Inherent properties of the drug, e.g., aqueous solubility and stability; Surface characteristics such as charge and permeability; Degree of biodegradability, biocompatibility and toxicity; Drug release profile desired; and Antigenicity of the final product.

Nanoparticles are prepared by many methods the most commonly used methods primarily for polymer-based nanoparticles are:

(1) Dispersion of preformed polymers,
(2) Polymerization of monomers and
(3) Ionic gelation or coacervation of hydrophilic polymers.

Besides the above three methods, other methods such as supercritical fluid technology [Rolland et al. 2005] and particle replication in non-wetting templates (PRINT) [Rolland et al. 2005] have also been described in the literature for production of nanoparticles. The latter is claimed to have absolute control of particle size, shape and composition, which could set an example for the future mass production of nanoparticles in industry.

3.1 Dispersion of preformed polymers

Dispersion of preformed polymer preparation methodology is followed routinely when biodegradable nanoparticles are to be prepared. There are two ways in which the method can be applied.

• **Solvent evaporation method:** As the name suggests the method involves dissolution of polymer in an organic solvent. The component to be loaded is also dissolved in the same solvent system. Both the solutions are intermixed under stirring conditions with aqueous solution of a surfactant or emulsifying agent. The organic solvent part is allowed to evaporate. The size of particles is largely dependent on the stirring and stabilizer concentration [Kwon et al. 2001]

• **Spontaneous emulsification or solvent diffusion method:** This is a modified version of solvent evaporation method where water miscible organic solvent system is also used which leads to turbulence while the two solutions are mixed, subsequently leading to particle formation (Kwon et al. 2001).

3.2 Polymerization method

In this method the monomers are polymerized to form nanoparticles. The substance to be loaded is dissolved in the mixture and the particles are finally collected. This technique has been widely used for making poly-butylcyanoacrylate or poly-alkylcyanoacrylate nanoparticles [Zhang et al 2001; Boudad et al. 2001].

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3.3 Coacervation or Ionic gelation method

The method is mostly used to form nanoparticles from biodegradable compounds. The charge on the component systems leads to cross linking the systems leading to nanoparticle formation. Chitosan based nanoparticles prepared by Calvo and co-workers for the first time represented good reference of this method [Calvo et al. 1997 a; Calvo et al.1997b].

4. CHARACTERIZATION OF NANOPARTICLES

Nanoparticle characterization is necessitated for understanding particles, controlling their production to suffice the application for which the particle use is intended. Most of the methods which are used in for characterization are derivative of material sciences. There is an array of equipments which can be used to explore nanoparticles with choice of equipment depending on parameter under study.

Common techniques used include, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), Dynamic Light Scattering (DLS), X-ray Diffraction (XRD), Attenuated Total Reflectance Infra Red Spectroscopy (ATRIR), Fourier transform Raman Spectrometery (FTRS), Nuclear Magnetic Resonance (NMR), Ultraviolet-Visible Spectroscopy (UV-Vis), Dynamic Light Scattering (DLS), N2-Sorpometery, GC-Mass Spectrometery, Rheometric Analysis and Electron paramagnetic resonance spectrometery (ESR).

Transmission Electron Microscopy: To identify the three dimensional phase arrangement, crystal pattern and size distribution of the nanoparticles/materials.
Scanning Electron Microscopy: To identify the external surface morphology and size distribution.
Atomic Force Microscopy: For the visualization of surface morphology of nanoparticles in 2D and 3D as well as in obtaining average grain size.
X-ray Diffractometry: To detect the phase arrangement in the nanoparticles/materials.
UV-visible spectrophotometry: To provide preliminary information about the nanoparticle formation and reaction monitoring.
Attenuated Total Reflectance Infra-Red Spectroscopy: To reveal the functional groups in the nanoparticles after constituent compound interact.
Fourier Transmission Raman spectrometry: To identify the nature of organic functionalities around the nanoparticles.
Zetasizer : For surface charge potential analysis.
Nuclear Magnetic Resonance Spectrometry: To provide information about specific organic environment around the nanoparticles and product identification of the reaction/process
Dynamic Light Scattering machine: To provide the size distribution of the nanoparticles in liquid state.
N_2-sorptometer: To provide details of the external surface area of the nonmaterials.
GC-Mass spectrometer: For analyzing chemical components of nanoparticles formed.
Electron paramagnetic resonance spectrometer: To provide specific information about the magnetic properties of nanoparticles
Rheometric Analysis: This study gives a detailed picture of nanoparticle formation and their assembly process.

5. Applications and potential benefits of nanoparticles

Nanotechnology has led to development of materials with novel properties just because of shrinkage in their size. When these miniature particles are applied in bulk they exhibit potential commercial applicability. Thus, a wide social benefit of these materials is already in use and more can be expected.

5.1. Medicine: Contrast imaging instrumentation and analytical techniques in the field of medicine are now utilizing the nanotechnology research.

5.2. **Diagnostics:** Nano based diagnostic techniques are one more dimension of lab–on–chip technology. The diagnostics tests have now become much faster, much sensitive and flexible due to recent inventions in nanotechnology. Magnetic and gold nanoparticles with DNA attached to surface are examples of some of the materials used in for qualitative and quantitative analysis of samples.

5.3. **Drug delivery:** Nanoparticle based drug delivery offers longer duration of active agent presence in the morbid region as well as reduction of toxicity by increasing specificity

5.4. **Tissue Engineering:** The capacity of nano based materials to act as scaffolds and artificial stimulators has led to "Tissue Engineering", which can help to repair or completely reproduce damaged tissue. This method may be complete replacement of conventional treatments *e.g.,* transplantations of organs or artificial implants.
5.5. Chemistry and Environment: Nanotechnology provides capacity to manufacture nanoparticles with tailored properties like the one with specific ligands or optical properties. Chemical catalysis and filtration techniques are the two prominent examples where nanotechnology is already in use. Chemistry forms the base of all nanotechnology, thus, providing methods for producing tailored material.
5.6. Nanofiltration: Nanoporous membranes are suitable for a mechanical filtration with extremely small pores smaller than 10 nm ("nanofiltration"). Nanofiltration is mainly used for the removal of ions or the separation of different fluids. On a larger scale, the membrane filtration technique is named ultrafiltration, which works in range of 10 and 100 nm. Renal dialysis is one example of such filtration.
5.7. Agriculture: Controlled release of elicitor molecules using Chitosan is currently underway in many laboratories round the world for improving biotic stress resistance in crop plants.

6. CHITOSAN

Chitosan is an N-deacetylated product of chitin. The degree of deacetylation imparts some characteristic properties to chitosan. There is no nomenclature method to name chitosan based on its degree of deacetylation. Due to its properties such as biocompatibility, biodegradability, non- toxicity it is being widely used as natural polymer based functional material.

6.1Production of chitosan:

Chitosan is generally produced from chitin by treatment with alkali which not only leads to deacetylation but also simultaneously leads to removal of some protein components. Total process leads to production of chitosan–glucan complexes complexes. The concentration of alkali dictates how much soluble glycans will be removed with increasing concentration [Madhavan 1992].

6.2 Properties of Chitosan

Chitosan is soluble in mild acid solutions. It has gel forming tendency in N-methylmorpholine N-oxide which was recently been reported [Dutta 1997; Dutta 1999; Ravi Kumar 1999]. There is presence of nitrogen always in the form of primary aliphatic amino group. Because of said group typical reactions such as N-acylation and Schiff reactions are exhibited. Aldimines and kitimines are formed as a result of reaction with aldehydes and ketones. Presence of bulky groups makes hydrogen bonding weaker and hence chitosan swells in water in spite of hydrophobicity of alkyl group.

6.3 Physical and chemical characterization

Chitosan is a heteropolymer and neither random nor block orientation is meant to be implied for chitosan.

6.4 Degree of N-acetylation

Degree of N-acetylation is defined as the ratio of 2-acetamido-2-deoxy-D -glucopyranose to 2-amino-2-deoxy-D -glucopyranose structural units. Chitosan is the universally accepted non-toxic N-deacetylated derivative of chitin, where chitin is N-deacetylated to such an extent that it becomes soluble in dilute aqueous acetic and formic acids. In chitin, the acetylated units prevail (degree of acetylation typically 0.90) whereas in chitosan fully or partially N-deacetylated derivative of chitin with a typical degree of acetylation of less than 0.35 is prevalent.

6.5 Molecular weight

Chitosan molecular weight distributions have been obtained using high performance liquid chromatography (HPLC) [Wu 1988]. The molecular weight (M_w) of chitin and chitosan can be determined by light scattering. Viscometry is a simple and rapid method for the determination of molecular weight; the constants α and K in the Mark–Houwink equation have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution. The intrinsic viscosity is expressed as

$$[\eta]=KM^{\alpha}=1.81\times10^{-3}M^{0.93}$$

The charged nature of chitosan in acid solvents and chitosan's propensity to form aggregation complexes require care when applying these constants. Furthermore, converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration. The weight-average molecular weight of chitin is 1.03×10^6 to 2.5×10^6, but the N-deacetylation reaction reduces this to 1×10^5 to 5×10^5.

6.6 Solvent and solution properties

Chitosan structure exhibits extensive hydrogen bonding resulting in its degradation before melting. The above nature makes it necessary to dissolve chitosan in an appropriate solvent system if its functionality is to be maintained intact. Thus, in sum pH, polymer concentration and temperature are to be studied in extensive form to study functionality.

6.7 Application of Chitosan in Agriculture and other areas:

Chitosan regulates the immune system of the plant and induces the gene expression of pathogen related proteins, thereby improving resistance towards pathogenic attack. Chitosan aids microbes as the carbon source and also assists the root system of plants to absorb vital nutrients from the soil [Bolto et al 2004; Somashekar and Richard 1996]. These responses include lignifications [Felix et al. 1993], ion flux variations, cytoplasmic acidification, membrane depolarization and protein phosphorylation [Felix et al. 1998; Kikuyama et al., 1997; Kuchitsu et al., 1997; Roby et al. 1997], chitinase and glucanase activation [Kaku et al. 1997; Ren et al. 1992], phytoalexin biosynthesis [Yamada et al. 1993; Kuchitsu et al. 1995], generation of reactive oxygen species [Nojiri et al., 1995], biosynthesis of jasmonic acid [Minami et al. 1996], and the expression of unique early responsive and defense-related genes [Cornath et al. 1989; Nishizawa et al. 1999; Takai et al 2001]. In addition, chitosan was reported to induce callose formation [Kohle et al 2010; Simmons and Ryan 1984], proteinase inhibitors [Hadwiger and Beckman 1980], and phytoalexin biosynthesis in many dicot species. Besides these applications in crop protection, chitosan has got wide array of utility in the fields of photography, cosmetics, ophthalmology, paper finishing and drug delivery systems.

7. POLYSACCHARIDE AND CHITOSAN BASED NANOPARTICLES

Polysaccharides are formed as a result of polymerization fraction of monomer units. Depending on their chemical functional groups, polysaccharides exhibit a net negative or positive charge. Thus they have been classified as positively charged (e.g. chitosan) or negatively charged (e.g. heparin, alginate). Due to the presence of varying functional groups, chemical modification of polysaccharides, mainly chitosan provides scope for chemical modifications. The natural forms of polysaccharides are safe, biodegradable and have bio-adhesion properties and hence, can be used for preparation of drug delivery systems. Chitosan based nanoparticles have proved to be good drug delivery systems for epithelia and mucosa, forming bio-adhesion to these tissues [Illum 2000]. Chitosan nanoparticles system can increase the residence time leading to higher grade control on drug release concentration thereby increasing the bioavailability. Current research in polysaccharide/chitosan-based nanoparticles includes efficient loading of polypeptides, proteins, vaccines, nucleic acids, genes. The potential is being exploited for medical, biological, pharmaceutical and agricultural fields [Lee et al. 2000].
Currently, the research on nanoparticle load delivery system focuses on:

a) Selection and combination of carrier materials to obtain suitable load release speed.
b) Surface modification of nanoparticles to improve their targeting ability.
c) Optimization of the preparation of nanoparticles to increase their load delivery capability.
d) Investigation of in vivo dynamic process to disclose the interaction of nanoparticles with cellular receptors.

Polysaccharide based nanoparticle preparation is not a tedious process, however, one has to refine the existing nanoparticle formation protocols to get uniform and well dispersed nanoparticles. Covalent crosslinking, ionic crosslinking and polyelectrolyte complexation are the important methods by which these nanoparticle preparations take place.

7.1 Covalently crosslinked polysaccharide / chitosan nanoparticles

This method facilitates covalent bonding between chitosan and a crosslinker molecule. Glutraldehyde is a potential crosslinker which has been used for the process [Zhi et al. 2005 and Liu et al. 2007], however this crosslinker is toxic, hence application affects cell viability. Other potential crosslinkers include carbidiimide, succinic acid, malic acid etc.

7.2 Ionically cross linked chitosan nanoparticles

Compared with covalent cross-linking, ionic cross-linking has more advantages: mild preparation conditions and simple procedures. For charged polysaccharides like polyaninon chitosan, low molecular weight polycations could act as ionic crosslinkers. To date, the most widely used polyanion crosslinker is tripolyphosphate (TPP) which is non-toxic and has multivalent anions. It can form a gel by ionic interaction between positively charged amino groups of chitosan and negatively charged counterions of TPP.

7.3 Polyelectrolyte complexation (PEC)

Polyelectrolyte polysaccharides can form PEC with oppositely charged polymers by intermolecular electrostatic interaction.

Chitosan Nanoparticles have a great role in improving plant defense and abiotic stress resistance as well. As the chitosan is economically viable and also interferes with plant growth and development, focusing on chitosan loaded nanoparticles may better help the scientists for improving the crop productivity.

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