Synthesis of surface functionalized silica nanoparticles and their use as entomotoxic nanocides

Nitai Debnath a,b,⁎, Shouvik Mitra a, Sumistha Das a, Arunava Goswami a

a Biological Sciences Division, Indian Statistical Institute, 203 B.T. Road, Kolkata–700 108, West Bengal, India
b West Bengal University of Technology, BF–142, Sector–I, Salt Lake, Kolkata–700 064, West Bengal, India

1. Introduction

Silica nanoparticle (SNP) has gained much attention in scientific research because of its easy preparation and its wide range of industrial as well as biological application [1]. The variety of chemical and physical modification possible with silica increases its versatility, and its biocompatibility makes it suitable for biological application. Starting from its use in electronics to DNA delivery [2], SNP has huge potential in agriculture and veterinary sector [3] as well. It can be used as an entomotoxic agent [4], can control grasserie disease of silk worm (a viral disease caused by Bombyx mori nuclear polyhedrosis virus) and can also be applied as an anti-malarial drug in poultry farm [5].

Silica, which is generally inert in nature, becomes biologically potent if its surface is suitably modified. Hydrophobic SNP can absorb parasite induced excess lipid from body circulation and ultimately inhibits viral or parasitic growth.

In recent decades, agrochemical researchers have re-appraised inert dust based pest management strategies because of concerns associated with conventional commercial pesticides like growing resistance, hazards to human health and environmental pollution. The European Union has already embraced naturally occurring Diatomaceous Earth (DE) based pesticides. But insecticidal efficacy of this amorphous silica based insecticide depends on temperature, relative humidity [6–8] and even on the geographical location from which it was excavated [9]. DE is not very effective in a highly humid, tropical region like India. It is known that DE becomes more effective if it contains uniformly sized amorphous silica [10]. We hypothesized that amorphous SNPs having narrow size distribution will show insecticidal property and these will be effective in all climatic conditions.

Among various modes of synthesis, SNPs synthesized from silicon alkoxide as the starting material via sol–gel method, is most popular. It involves simultaneous hydrolysis and condensation of silicon alkoxide groups and silane coupling agents. Functionalization of these SNPs in situ in aqueous solution with silane can be done by covalent bond formation between the surface silanol groups and silane coupling agents.

In this paper, our main objective was to synthesize monodisperse, spherical, thiol and HMDS capped SNP, which can be used in various biological domains, especially in agricultural sector. The core silica particle in nano form was synthesized from Tetraethyl orthosilicate (TEOS) by slightly modifying Stober process [11]. Surface modification of these SNPs was done by two ways — (i) by capping them with 3-Mercaptopropyl triethoxysilane (MPTS) in neutral aqueous medium and (ii) by capping SNPs with Hexamethyldisilazane (HMDS). The insecticidal property of these SNPs were tested against Spodoptera litura (commonly called tropical army worm), a polyphagous insect pest. It attacks cotton, jute, corn, tea, tobacco as well as many vegetables such as eggplants, bean, pepper and a number of Cruciferous and...
Cucurbitacea plants. Our work describes a simple technique to fabricate the SNP with thiol group or hydrophobic methyl moiety and their use as an insecticidal agent.

2. Material and methods

2.1. Chemicals

Tetraethyl orthosilicate (TEOS), Ethanol, Hydrochloric acid (35%) were obtained from Merck, Germany; Hexamethyldisilazane (HMDS), 3-Mercaptopropyltriethoxysilane (3-MPTS) were purchased from Sigma Aldrich. All the chemicals were of analytical grade and were used without further purification. Water used throughout the experiment was of Milli-Q grade with conductivity less than 0.1 μS cm⁻¹.

2.2. Insects

*S. litura* was cultured on leaves of the castor oil plant (*Ricinus communis* L.) in Bidhan Chandra Krishi Viswavidyalaya, an agricultural university in West Bengal, India. The second instar larval stage of the insect was used in the insecticidal bioassay.

2.3. Synthesis of surface functionalized SNP

The core SNP, on which surface capping was applied was synthesized by slightly modifying the Stober process [11]. Thiol capped and HMDS capped SNP was synthesized by using a mixture of ethanol and water taken in a 3-neck round bottom flask equipped with reflux condenser and isopiestic funnel. The mixture was stirred for about 10 minutes. Tetraethyl orthosilicate (TEOS) was added to this mixture in such a way that the final molar ratio of TEOS: ethanol: water was 0.012:4:3. The mixture was kept under reflux condition at a temperature of 75 °C and stirred vigorously for about 30 min. A solution of 3-MPTS in absolute ethanol was added drop wise to this reaction mixture with the help of an isopiestic funnel and the reaction was continued for about 45 minutes. Finally the mixture was centrifuged at 12,000 rpm for 10 min and washed several times with ethanol to remove excess of MPTS. The resultant thiol capped SNP was completely dried at a temperature of 80 °C.

HMDS capped SNP was synthesized under acidic medium with the core SNP functionalized by HMDS. Here, mixture of ethanol and water was taken in a 3-neck round bottom flask equipped with a reflux condenser and two isopiestic funnels containing aqueous 0.075(M) HCl and alcoholic solution of HMDS. TEOS was added to the aqueous mixture of alcohol in such a way that the molar ratio of TEOS: ethanol: water was 0.012:4:3. Initially half of the HCl was added to the reaction mixture with constant stirring and refluxing for about 30 min and the remaining aqueous HCl was added along with the alcoholic solution of HMDS with constant stirring. The reflux was continued for about 1 h. Finally the reaction mixture was centrifuged at 12,000 rpm for 10 min and washed with ethanol several times to remove HMDS. The SNP was dried at a temperature of 80 °C.

2.4. Characterization

Surface morphology of the two SNPs was determined by SUPRA 40 FE-SEM (Carl Zeiss) operated at acceleration voltage of 5.0 kV. Particle size was obtained from high resolution transmission electron micrograph (JEOL 2010). For HR-TEM analysis, a drop of well dispersed alcoholic sample solution was put into copper coated TEM grid and was air dried before imaging. FTIR spectra were recorded in Perkin–Elmer Spectrum 100. For FTIR experiments the samples were ground with KBr (spectroscopic grade) and pressed to pellets. Powder sample XRD (Bruker AXS analytical instruments) was carried out by applying a primary beam monochromator to select the Kα1 component of the employed copper radiation of wavelength 1.54056 Å. The surface morphology of both SNPs was obtained from AFM study (Vecco) in tapping mode. Stability of the capping material was obtained from TG-DTA analysis (Perkin–Elmer Diamond TG/DTA) with a heating rate of 10 °C/min under nitrogen atmosphere.

2.5. Bioassay

To test the pesticidal efficacy of the surface functionalized SNPs, the nanoparticles were dusted uniformly on the bottom surface of plastic containers at three dose rates 0.125 mg cm⁻², 0.25 mg cm⁻² and 0.5 mg cm⁻². No SNP was dusted in the control set. The containers were covered with muslin cloth to allow aeration. For each dose there were five replicates. 10 second instar larva of *S. litura* were introduced in each box after dusting. All bioassays were performed at 30 °C ± 1 °C and 75 ± 5% Relative Humidity (R.H.). Insect mortality was checked.

![Fig. 1. FE SEM image of (a) HMDS caped SNP and (b) thiol capped SNP.](image-url)
3. Results and discussion

3.1. Characterization of surface functionalized SNPs

FE SEM images show that both the SNPs are spherical in nature (Fig. 1). HR-TEM image shows that HMDS functionalized SNP (thiol capped SNP) has the size range of 15 nm to 20 nm (Fig. 2a), whereas MPTS functionalized SNP has the size range of 29 nm to 37 nm (Fig. 2b) TEM micrograph also reveals that both the particles are spherical in shape and distinct uniformity is maintained throughout and yet they do not agglomerate into clusters. Powder XRD pattern is obtained from Bragg’s Law $\lambda = 2d \sin \theta$ using CuKα radiation. Both SNPs i.e. thiol capped and HMDS capped SNP show a single broad peak indicating their amorphous nature (Fig. 3). Therefore non-crystalline nature of the Stober silica is maintained even after functionalization with MPTS or HMDS. This makes the SNPs suitable for biological application. United States Department of Agriculture (USDA) has already approved non-crystalline silica as safe [12].

The surface capping of the SNPs is confirmed by FTIR analysis. SNP synthesized by simple Stober method shows four characteristic peaks

![Fig. 2. TEM image of (a) HMDS capped SNP and (b) thiol capped SNP.](image)

![Fig. 3. X-ray diffraction pattern of (a) HMDS capped SNP and (b) thiol capped SNP.](image)

![Fig. 4. IR spectra of (a) SNP without any surface functionalization, (b) SNP functionalized with HMDS and (c) SNP functionalized with MPTS.](image)
(Fig. 4a). Peak obtained at 3452 cm\(^{-1}\) appears for O–H stretching of the surface silanol groups. Characteristic 1638 cm\(^{-1}\) peak is for the C=O of the carboxylic acid group, 1099 cm\(^{-1}\) is attributed towards Si–O stretching of the SNP and another characteristic peak for SNP appears at 798 cm\(^{-1}\). A small but distinct peak at 1248 cm\(^{-1}\) signifies C–O stretching. In case of HMDS capped SNP (Fig. 4b) two peaks for C–H stretching are observed at 2963 cm\(^{-1}\) and 2905 cm\(^{-1}\) due to asymmetric stretching and symmetric stretching arising from the methyl fragment. The other characteristic peak of Si–O stretching appeared at 1089 cm\(^{-1}\) and characteristic peak of silica was observed at 799 cm\(^{-1}\). For thiol capped SNP (Fig. 4c), 3447 cm\(^{-1}\) peak is for O–H stretching, C–H asymmetric stretching frequency is observed at 2929 cm\(^{-1}\) and C–H symmetric stretching frequency appears at around 2800 cm\(^{-1}\). At the same time very weak stretching at around 2600 cm\(^{-1}\) is for S–H stretching frequency of and the characteristic peak of the Si–O stretching of silica is obtained at around 1077 cm\(^{-1}\) and lower stretching frequency around 800 cm\(^{-1}\) is attributed towards characteristic Si–O stretching. In both SNPs the peak at 1630–1635 cm\(^{-1}\) is for the C=O stretching of CO\(_2\)H group and their corresponding C–O peak appears ~1250 cm\(^{-1}\). The intensity of the O–H stretching frequency is decreased in both cases due to corresponding co-condensation reaction.

The surface topology, obtained from AFM image, shows that both the SNPs are more or less spherical, though there are quite agglomerated in nature. Fig. 5a and b shows that HMDS and thiol capped SNPs have size distribution of nearly 23 nm and 33 nm respectively. HMDS and thiol capped SNPs have surface roughness of 1.25 nm and 1.2 nm respectively by sampling of 65536 number of grains.

The stability of the surface capping agent was studied by TG-DTA analysis. (Fig. 6) shows the percentage of weight loss with the variation of temperature. Normal silica is very stable throughout the span of experimental temperature except in the initial temperature range. Major weight loss started from 50 °C and continued up to 130 °C; 4.8% weight loss during this temperature range is attributed towards the loss of water molecules, adhered to the surface. HMDS functionalized SNP underwent slow decrease in weight throughout the temperature range. It is quite stable upto 350 °C. Whereas MPTS functionalized SNP shows initial weight loss of ~0.2% due to the loss of surface adhered water molecules. Above 230 °C gradual reduction of weight is due to thermolysis of 3-MPTS capping, which has boiling point of 215 °C.

3.2. Bioassay

Fig. 7 shows that at 0.125 mg cm\(^{-2}\) HMDS and thiol capped SNP could kill 58% and 64% of the second instar S. litura larvae respectively. At 0.25 mg cm\(^{-2}\) all insect larvae died in MPTS functionalized SNP treatment, whereas HMDS functionalized SNP caused 84% insect mortality at this dose. There were no survivors for treatment with both SNPs at 0.5 mg cm\(^{-2}\).

Both of these SNPs are equally effective and they show dose dependant insecticidal efficacy. It was observed that the dead bodies of the insects became extremely dehydrated. This was possibly due to the fact that the nanocides caused damage in their cuticular water barrier as a result of abrasion or to some extent because of adsorption of lipids by SNPs present in cuticle. The insects began to lose water from their body and died because of desiccation [13,14]. Absorption property of HMDS and thiol capped SNPs.
have been carried out using standard dye solutions (shown in supplementary information). Fig. S1a and S1b confirmed absorption property of the synthesized SNPs; this absorption property together with abrasion due to surface roughness contributed to its insecticidal efficacy.

Though, there has been an overwhelming academic as well as application oriented interest in nanomaterials over the last decades, application of nanotechnology in agriculture is at a nascent stage. Liposomes are being used for encapsulation of agrochemicals [15]. This nanocapsulation will certainly improve the delivery system and will reduce the deleterious side effects [16].

Nanotechnology has a huge potential to develop alternative pest control strategy and lower risk insecticidal molecules. As silica is rather inert, SNPs can be better alternative to the popular insecticides which are hazardous to human health and because of huge environmental concerns associated with them.

4. Conclusion

Stober [11] pioneered the synthesis of SNP via sol-gel process. Since then a number of synthetic strategies have been employed by various researchers which include different surface functionalizations. SNP synthesized by Stober sol-gel process contains surface silanol groups which can be utilized for various surface fabrication. In this paper we described in situ functionalization of SNPs by co-condensation reaction of silanol groups and silane coupling agents, which resulted in thiol capped and methyl moiety capped SNPs. The beauty of these SNPs is that these can be used as a novel insecticide in agriculture sector. As amorphous silica is relatively biosafe, SNP based nanocides can be an alternative to the conventional hazardous insecticides. Moreover, insects are very unlikely to build up resistance against these physically active nanocides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.powtec.2012.01.009.

References

Novel applications of solid and liquid formulations of nanoparticles against insect pests and pathogens

Arunava Goswami *, Indrani Roy, Sutanuka Sengupta, Nitai Debnath

Biological Sciences Division, Indian Statistical Institute, 203 B.T. Road, Kolkata-700 108, West Bengal, India

Abstract

Nanoparticles are of special interest because of their markedly different physico-chemical properties than their bulk counterparts. This review covers the use of these novel agents to combat real life agricultural and medical issues important to Indian subcontinent. Nanoparticles of oxides like SiO₂ produced and characterized in our laboratory were tested against insect pests and pathogens. Nanosilica against insect pests shows nearly 100% mortality. Survivability of grasserie affected silkworm increased from 0% to 30% with hydrophobic nano-aluminosilicate. Treatment of multi-drug resistant tuberculosis with nanosilver and trypanosome infected mice with Dsethasvan showed significant protection. Sporulation and growth of fungi were inhibited by nanosulfur.

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1. Introduction

Nanoparticles (NPs) have been used for a very long time, probably the earliest use being in glazes for early dynasty Chinese porcelain. A Roman cup, called the Lycurgus cup, used nanosized gold clusters to create different colours depending on whether it was illuminated from the front or the back. The cause of this effect was not, of course, known to those who exploited it. NPs have been used as colloids of gold, silver and copper to embellish calligraphy and in stained glass windows in medieval European churches. Carbon black is the most famous example of a nanoparticulate material that has been produced in quantity for decades. In ancient Indian medical practice therapeutic effects of gold and silver were known and put to use. NPs have been used as colloids of gold, silver and copper to embellish calligraphy and in stained glass windows in medieval European churches. Carbon black is the most famous example of a nanoparticulate material that has been produced in quantity for decades. In ancient Indian medical practice therapeutic effects of gold and silver were known and put to use.

2. Experimental details

All the NPs mentioned in this article were either a gift from Prof. Christian Ulrichs, Humboldt University, Berlin or produced in our lab following standard protocols with some modifications. These were characterized by ultraviolet-visible spectroscopy (UV–VIS), dynamic light scattering (DLS) transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

2.1. Development of entomotoxic solid NPs and their value addition

The first studies were aimed at the generation and testing of lipophilic entomotoxic silica nanoparticle (SNP) in tropical climates and value addition for urban and intensive agriculture, urban horticulture animal and poultry industries.

Surface functionalized spherical SNPs (Fig. 1A) of different size range were synthesized in the laboratory by sol–gel by slightly modifying the Stober process [8]. The other surface functionalized NPs tested for their entomotoxicity were: aluminium oxide (ANP) (Fig. 1B), zinc oxide (ZNP) (Fig. 1C) and titanium dioxide (TNP) (Fig. 1D). We collaborated with M. K. Implex, Canada to generate our products on a large scale.

2.1.1. Application of solid NPs against rice weevil Sitophilus oryzae: Bioassay I

The bioassay on Sitophilus oryzae (rice weevil) was performed in plastic screw capped boxes. 20 g of rice was placed in each box. Rice in...
each box was treated individually with the NPs (different surface functionalized SNP, ZNP, TNP and ANP) at three dose rates—0.5, 1 and 2 g NP kg$^{-1}$ rice. Then the boxes were shaken vigorously to achieve equal distribution of NPs on rice. There were five replicates for each treatment and control. Then 20 adults of *S. oryzae* were introduced into each box. All bioassays were performed at 30 °C ± 1 °C, 75 ± 5% relative humidity. Insect mortality was checked after 1, 2, 4, 7 and 14 days.

2.1.2. Application of alumino silicate NP against silkworm grasserie disease: Bioassay II

The target organism was the silkworm (*Bombyx mori*) affected by grasserie disease. Grasserie is caused by the baculovirus *BmNPV* (*B. mori* nuclear polyhedrosis virus). It is highly infectious and is 100% lethal. 5th instar *B. mori* larvae were inoculated by injecting 5 μl polyhedra in the hemocoel at the third abdominal spiracle [9]. Ethanolic suspension of hydrophobic alumino silicate (AL60102) NP was conjugated with heat-inactivated virus and subsequently injected into diseased larvae. These NPs were gifts from Prof. Christian Ulrichs, Humboldt University, Berlin, Germany. The chemical formula of AL60102 is Al$_2$O$_3$(SiO$_2$)$_3$(H$_2$O) and this is amorphous. These biocompatible alumino silicate NPs have average diameter of 40–60 nm. Silkworm larvae were reared on fresh mulberry leaves for the entire experimental period.

2.1.3. Application of nanoporous microsilica against avian malaria parasite: Bioassay III

The second pathogen, targeted by our lab was the avian parasite, *Plasmodium gallinaceum*. Thirty-day-old, age-matched Ven Cobb breed broilers weighing approximately 250–300 g chickens were used throughout the study. Chicken were housed individually in standard cages and were provided with standard poultry food. All experimental protocols were approved by the animal ethics committee. Frozen stocks of *P. gallinaceum* were purchased from the parasite bank of the National Institute of Malaria Research, New Delhi, India. Equal volume of chicken blood inoculums (0.2 ml) containing less than 0.01% parasitemia were used for artificially infecting chicken via intra peritoneal and intra venous injection. The parasitemia were observed using blood smear examinations. At day 5, when the parasitemia was found to be 4.5 ± 0.2%, amorphous microsilica with nanopores (AMS) dispersed in absolute alcohol were fed to the chicken twice a day. Each dose contained 0.0125 mg of AMS dispersed in 0.5 ml alcohol. Serum cholesterol levels were measured at M/S Pathowind Laboratories, Calcutta, India.

2.1.4. Solid NPs against human pathogen: Bioassay IV

As a natural corollary to the above experiments the effects of NPs on a few human pathogens were studied. Three isolates of *Klebsiella* sp. K1, K2 and K3 causing urinary tract infection (UTI), respiratory tract infection (RTI) and one isolate of *Staphylococcus aureus*, causal agent for pus pyrogenic infections were subjected to treatment with
zinc oxide and titanium dioxide NPs. These were administered in dose range 1, 2, 5, 10, 20, 40 and 80 μg/ml of Muller–Hinton broth, the medium used for pathogen culture.

Moreover, FS, an AMS, AL, an AMS and Dethvasan, a mixture of AMS and amorphous nanosilica (AMS) were applied against Trypanosoma evansi, causal agent of sleeping sickness [10]. The source material of FS and Dethvasan is diatomaceous earth and volcanic soil respectively.

Adult Swiss albino mice of both sexes weighing 20–25 g were used for experimental purposes. Before the commencement of the experiments, the animals were screened for the presence of blood parasites using standard techniques. The mice were divided into five groups (A, B, C, D and E) of 6 mice each. The groups were treated as follows; A] uninfected untreated control; B] infected untreated control, only alcohol was administered; C] treated infected (FS) and D] treated infected (AL). The infected mice were injected with alcoholic suspension of FS, AL and “Dethvasan”. Lipid parameters were estimated with commercially available lipid estimation kits from the mice serum. All experimental procedures were approved by the animal ethics committee.

2.2. Novel application of NP liquid formulation against pathogens

Liquid formulation of nanosilver and monoclinc nanosulfur was applied successfully against one human and fungal pathogen respectively.

2.2.1. Application of nanosulfur against human pathogen (tuberculosis): Bioassay V

We have attempted to devise a novel nanomedicine—bovine serum albumin (BSA) capped nanosilver against multi drug resistant (MDR) tuberculosis (TB).

Five different strains of Mycobacterium were subjected to treatment with BSA capped nanosilver. Of these, one strain was H37Rv (non-tubercular), three were known and characterized TB causing strains and the fifth strain was Mycobacterium xenopi, a Mycobacterium other than TB (MOTT). H37Rv and MOTT served as controls. All strains were inoculated in two batches of 5 ml Middlebrook 7H9 broth. 8, 20, and 40 μg per 5 ml of culture broth (i.e. 1.6, 4 and 8 μg/ml respectively) of nanoparticles were added immediately to the 1st batch. In the second batch, same graded concentrations of nanoparticles were added to the 20 day old broth culture to demonstrate the subsequent growth inhibition or lysis of bacteria by nanoparticles and were monitored for another 4 weeks.

2.2.2. Application of liquid formulation of surface modified monoclinic nanosulfur against fungal pathogen: Bioassay VI

Our latest effort has been to target another common pathogen, Aspergillus niger. Elemental sulfur (S0) is long known as a fungicide and a phytosanlayin. Enhanced fungi-toxicity of sulfur has been envisioned in its nano-dimension against the fungal pathogen A. niger. Sulfur NPs were synthesized using alkaline polysulfides as active sulfur source and weak organic acids as precipitating agents. Average particles size obtained by this method was around 20–50 nm.

Strains of Aspergillus sp. were isolated from rotten potato from commercial market. Fungal isolates were procured through the method after [11] with 1% sodium hypochlorite treatment. Fungi-toxicity of sulfur both in its elemental and nano-form was tested through Poisoned Food Technique after [12]; where 125 parts per million (ppm), 500 ppm, 1000 ppm and 2000 ppm of both the forms were mixed with potato dextrose agar. Growth and sporulation of the fungi were examined after 48 h of incubation at 28 °C. Zone diameter growth was used as the standard parameter for comparing effectivity of nanosulfur as compared with elemental sulfur.

3. Results

3.1. Bioassay I

On day 1, hydrophilic SNP was most effective at 1 g kg−1. At 2 g kg−1 hydrophilic SNP and ANP, when dosage rate was 2 g kg−1. After 7 days of exposure, 95% and 86% mortality was obtained with SNP and ANP, when dosage rate was 2 g kg−1. After 7 days of exposure, 95% and 86% mortality were obtained with hydrophilic and hydrophobic SNP at 1 g kg−1 respectively. Nearly 70% of the insects were killed when the rice was treated with lipophilic SNP at 1 g kg−1. ANP could kill almost all the insects with 0.1 g kg−1 dose (Table 1).

3.2. Bioassay II

Significant reduction of polyhedra bodies in hemolymph was observed in the larvae treated with BmNPV polyhedra–AL60102 complex. 60 ± 3.9% larvae remained alive even after 72 h and well formed hemocytes were observed with few polyhedral bodies present in the hemolymph. Decrease in viral load is inversely related to the survival rate of larvae treated with BmNPV–AL60102 complex. It was found on treatment, that survivability was raised from 0% to 30%. The treated larvae successfully eclosed as moths, which copulated and the females laid viable eggs.

3.3. Bioassay III

The infected chicken showed hepatomegaly, and regression of liver size to near control level was observed after AMS treatment. Similarly, regression of spleenomegaly in AMS-treated malaria-

Table 1

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Table 2

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<th>AMS-treated malaria infected chicken serum (mg/dl; mean ± SE)</th>
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<td>130 ± 4.46</td>
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<td>78 ± 3.01</td>
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<td>80 ± 1.86</td>
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<td>Serum LDL cholesterol</td>
<td>43 ± 2.93</td>
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<td>45 ± 2.08</td>
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<td>Serum VLDL cholesterol</td>
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<td>Serum triglycerides</td>
<td>31 ± 2.47</td>
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infected chicken was observed. The course of the malaria infection is marked by significant increase in serum components specially, serum cholesterol and serum triglycerides. But on administering AMS to diseased chicken it was seen that, the levels of these two serum lipids as well as other lipid constituents came back to normal physiological levels. AMS-treated chicken were alive up to 10 days, after which they were killed for pathological and biochemical studies. On day 7, in control chicken, the parasitemia were found to be 5.5 ± 1.1 % (n = 15). 100% of the untreated malaria-infected chicken (n = 15) died within 7–10 days of inoculation. Total parasite burden was reduced by 78 ± 2% (n = 15). This indicates that due to the presence of the AMS in the chicken body, malarial parasites did not multiply properly. Biochemical studies (Table 2) shows the changes in the level of different kinds of serum cholesterol as well as the total cholesterol content in the control, malaria-infected chicken, and infected chicken treated with 0.025 mg AMS (two doses of 0.0125 mg at 0800 hours in the morning and 2000 hours at night).

3.4. Bioassay IV

It was found that ZnO NPs inhibited growth at doses 10 μg and higher in K1 and K2 strains of *Klebsellia*. TiO2 NPs also inhibited growth in K1 and K2 strains of *Klebsellia* but at dose rate 20 μg onwards. The growth of third *Klebsellia* strain and *S. aureus* culture however could not be inhibited by either NP.

After administration of Dsethvasan in the stipulated dose mentioned earlier the mice could survive for 192 ± 24 h (period of observation: 216 h) which is correlated with the fact that parasite burden was reduced in the slides counts after fixing with methanol and staining by Giemsa stain while, FS treated mice survived for 120 ± 24 h whereas AL treated mice and control infected mice died within 72 ± 24 h of inoculation. Trypanosome infected mice serum shows a significant decrease in the total serum cholesterol and in the serum triglycerides. After feeding Dsethvasan and FS there was a marked increase in some serum cholesterol components (HDLD and LDL) which was more pronounced in case of Dsethvasan (Table 3).

3.5. Bioassay V

In the first batch, *M. xenopi* shows complete growth inhibition in all the concentrations of nanosilver. No strain of *M. tuberculosis* showed any growth inhibition at concentrations of 1.6 μg/ml. At 4 μg/ml concentration, growth is partially inhibited in different strains of *M. tuberculosis* whereas in (8 μg/ml) complete growth inhibition occurred for all four strains (Table 4). In the next batch, effect of nanosilver particles is observed after 20 days of addition of NPs. At 1.6 μg/ml concentration, there was almost no lysis except in *M. xenopi* (showing a scanty growth even in the positive control) at 4 μg/ml concentration; lysis was observed in the isolates from clinical specimens but not in the culture containing isolates from the pleural fluid and the control strain. At the highest concentration, complete lysis was observed in all plates (Table 5). The strain isolated from the pleural fluid showed in vivo resistance to both rifampicin (2 μg/ml) and isonicotinyl hydrazine (5 μg/ml) and by definition of pharmacology, it can be marked as a MDR strain. But even this strain is sensitive to 8 μg/ml concentration of BSA capped nano-Ag.

3.6. Bioassay VI

Nanosulfur controls not only vegetative growth of *A. niger*, but also its sporulation. Fig. 2 shows that nanosulfur, synthesized by two different methods could effectively inhibit fungal growth as well as sporulation.

4. Conclusions

All our experiments have largely validated our premise that NPs of various kinds can be put to use as novel agents of defense against old and common problems that plague mankind.

Stored grain pest control is the biggest challenge faced by scientists working on food security problems. We believe that our work will go a long way in providing a solution that is viable and eco-

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Serum lipid profile of trypanosome infected and treated mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol types</td>
<td>Control mice Serum (mg/dl)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>289 ± 6.23</td>
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<tr>
<td>HDL</td>
<td>46 ± 5.93</td>
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<tr>
<td>LDL</td>
<td>194 ± 4.67</td>
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<tr>
<td>VLDL</td>
<td>49 ± 2.09</td>
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<tr>
<td>Triglycerides</td>
<td>247 ± 4.08</td>
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</table>

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Growth data when BSA capped nano-Ag was added in 20 days old TB culture.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Positive control</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>(++)</td>
</tr>
<tr>
<td>from sputum</td>
<td>(++)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Scanty</td>
</tr>
<tr>
<td>from peritoneal fluid</td>
<td>(+)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>(++)</td>
</tr>
<tr>
<td>from pleural fluid</td>
<td>No growth</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>Scanty</td>
</tr>
</tbody>
</table>
Amorphous nanosilica has been effectively shown in our lab against two stored grain pests, *S. oryzae*, *Tribolium castaneum* and two field pests *Lipaphis pseudobrassicae* and *Spodoptera litura* as a potent and safe pesticide. The mechanism of action is physico-chemical in nature [13]. Amorphous nanosilica has also been declared safe by the US Food and Drug administration.

One exciting finding from these experiments is that no insect infestation is found in the SNP treated stored rice even after 2 months.

**Fig. 3.** Atomic force microscopy and confocal microscopy images show physical alteration of the viral polyhedron by AL60102.
of treatment. So SNP can also be used as an excellent seed protecting agent.

For the first time the deadly grasserie disease caused by BmNPV, has been cured, albeit by only 30 ± 5%. It is clear that AL60102 alone cannot ensure this survival but a combination of AL60102 and polyhedral complex does so. Atomic force microscopy and confocal microscopic results show that AL60102 binds to the viral protein and alters the topological structure (Fig. 3). This might exhibit novel potent epitope(s) which could stimulate the innate immune reaction [14]. Viral protein analysis showed that the major target for AL60102 is a 35 kDa protein of BmNPV. Grasserie disease is most often 100% lethal and is ruinous to sericulturists. It is difficult to detect at an early stage. Our novel approach at developing a nanosilica-inactivated virus conjugate is a promising outcome for tackling this disease.

The results from our avian malaria experiment are most encouraging after the administration of AMS, the levels of these two serum lipids as well as of other lipid constituents came back to the normal physiological level. Chicken farmers usually market 50- to 60-day-old broiler chicken as well as of other lipid constituents. After the administration of AMS, the levels of these two serum lipids as well as of other lipid constituents came back to the normal physiological level. Chicken farmers usually market 50- to 60-day-old broiler chicken.

For the trypanosome experiments, lipid analysis indicates that mechanism of action of Dsethvasan is not lipid pathway dependent. The effect of Dsethvasan on HDL may be an indirect one. Trypanosomai live in the serum and therefore are not easily accessible to larger size hydrophobic drugs and that is why results are less pronounced with FS alone. To the best of our knowledge a combination of AMS and ANS has so far not been tried against any disease. Here, we have investigated the use of three different types of ANS, AMS and a combination of ANS and AMS with different characteristic features as a potential therapeutic drug against trypanosomiasis. We have used here three classes of NPs AL (40 nm to 60 nm), FS (164 nm to 1106 nm) and Dsethvasan having bimodal distribution of particles (78 nm to 142 nm and 200 nm to 800 nm respectively). Amongst the three classes of particle used, Dsethvasan, a mixture of two sub classes of particles is more effective in increasing the longevity of mice artificially infected with trypanosomes whereas FS having a larger sub fraction of particles is only partially effective and AL containing only the smaller fraction of particles is not effective.

TB is a scourge in the developing world. In India about 2 million patients develop active disease and up to half a million die. Moreover emergence of MDR strains and their sinister association with HIV/AIDS has posed a serious threat to the TB control program worldwide [15]. MDR is associated with high mortality rate of 50–80% and spans a relatively short time (4–16 weeks) from diagnosis to death. Effective chemotherapy requires 6 to 18 months of treatment depending on the nature and site of the disease. Failure of remission by first line of drugs in 3–4 months requires introduction of a second line of drugs. Anti tubercular drugs have serious adverse drug reactions (ADR) starting from intolerance to hepatitis, neuropathy, optic nerve atrophy etc. They should be used with extreme caution in pregnancy and lactation due to narrow margin of safety. Moreover, continuous monitoring of liver function test, neurological deficiencies etc. are required. High resolution (HR) SEM studies revealed that BSA capped nano-Ag wrinkles the cell surface and affects the integrity of the surface of the bacterial cell wall. Eventually, the cytoplasmic material was extruded from the cell leading to the collapse of the cell. Therefore, BSA capped nano-Ag kills Mycobacterium in a completely different fashion from that of antibiotics commonly used for treating TB. We may conclude that these nanoparticles damage the cell wall and also on gaining entry into the cell tend to disrupt physiological processes via pathways ill understood at present. At present, we are investigating the cellular mechanisms.

We observed that nanosulfur inhibits sporulation as well as fungal growth. Also nanosulfur proved to be more efficacious over its elemental form in controlling fungal growth. At each dose rate nanosulfur treated samples recorded significantly smaller zone diameters than elemental sulfur. The most dramatic reduction was observed for dose of 2000 ppm.

References

Toxicological & Environmental Chemistry

Toxicological evaluation of entomotoxic silica nanoparticle

Nitai Debnath a b, Sumistha Das a, Prasun Patra a, Shouvik Mitra a & Arunava Goswami a

a Biological Sciences Division, Indian Statistical Institute, 203 B. T. Road, Kolkata - 700108, West Bengal, India
b Department of Biotechnology, West Bengal University of Technology, BF-142, Sector-I, Salt Lake, Kolkata - 700064, West Bengal, India

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Toxicological evaluation of entomotoxic silica nanoparticle

Nitai Debnathab*, Sumistha Das³, Prasun Patra³, Shouvik Mitra³ and Arunava Goswamia

aBiological Sciences Division, Indian Statistical Institute, 203 B. T. Road, Kolkata – 700108, West Bengal, India; bDepartment of Biotechnology, West Bengal University of Technology, BF-142, Sector-I, Salt Lake, Kolkata – 700064, West Bengal, India

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Pest management researchers currently reappraise the use of inert dust-based insecticides because of the growing problem of environmental pollution and increasing insect resistance associated with conventional insecticides. Diatomaceous earth, which is amorphous micron-sized silica derived from fossilized phytoplankton, has become popular as an alternative insecticidal agent in European countries. In this investigation the insecticidal efficacy of amorphous lipophilic silica nanoparticle was examined on red flour beetle (Tribolium castaneum), a stored grain insect pest. The biosafety of this silica nanoparticle formulation was studied in MRC-5 cell line with water-soluble tetrazolium and lactate dehydrogenase activity assays. Acute oral toxicity of these nanocides was studied in mice model following OECD guidelines for testing of chemicals as well as the effects of particle exposure on mouse blood parameters, serum biochemical levels, and histopathological changes in various organs.

Keywords: silica nanoparticle; entomotoxicity; biotoxicity

Introduction

Production of inexpensive and abundant food supply for ever-growing human population is a great challenge which is further complicated by concerns about risks of environmental pollution and human health associated with conventional insecticides. Among the recent technological advances, nanotechnology shows considerable promise to combat this challenge, although its use in agricultural sector is in a nascent stage. Among all the synthesized nanomaterials, silica nanoparticles (SNPs) occupy a prominent position in scientific research because of their easy preparation (Stober and Fink 1968) and wide range of use not only in various industrial applications, such as catalysis, electronic and thin film substrates, electronic and thermal insulators (Herbert 1994), but also in diagnostics, imaging, and drug delivery (Bottini et al. 2007; Jia et al. 2008; Wang et al. 2009). Our group already demonstrated that SNP can also be used as an alternative to the conventional insecticides against a number of stored grain and field insect pests (Debnath et al. 2009, 2010; Goswami et al. 2010).

Since nano-sized particles possess various novel physicochemical properties, it is important to know the adverse effects exerted. In the last few years, several epidemiological studies demonstrated a correlation between ambient ultrafine particles associated...
with human respiratory and cardiovascular diseases (Pettinen et al. 2010; Von klot et al. 2010). It was found that nano forms of carbon titanium oxide may produce more inflammation than their bulk counterpart (Rahman et al. 2002; Lam et al. 2004). Despite a wide range of use of SNP, there is a lack of data regarding human health and environmental hazard associated with this nano material. Although some researchers examined in vitro toxicity of SNP (Xie et al. 2010; Liu et al. 2011), detailed in vivo toxicity studies of these nanoparticles (NPs) remain scarce.

The aim of this investigation was to evaluate the insecticidal efficacy of surface-functionalized SNP against red flour beetle Tribolium castaneum (Coleoptera: Tenebrionidae), a stored grain pest which attacks flour, cereals, beans, spices, pasta, and nuts (Weston and Rattlingourd 2000) and to assess the toxicity of SNP on MRC-5 cells (secondary human fibroblast cells) and murine model system.

Materials and methods

Lipophilic SNP

Amorphous lipophilic SNP, synthesized by the vapor phase method (Swihart 2003), was purchased from M. K. Implex, Canada. Transmission electron microscopy (TEM) revealed that these NPs had a size range of 15–20 nm (Figure 1).

Insecticidal assay of lipophilic SNP

Aqueous suspension of lipophilic SNP at five dosages (0.1, 0.25, 0.5, 1, or 1.5 mg cm$^{-2}$) was thoroughly sprayed on the inner surface of plastic Petri dish (Tarsons, India) and allowed to be air-dried to form a thin film. Controls were sprayed only with distilled water. Twenty unsexed T. castaneum were introduced to each Petri dish after 24 h starvation and dishes were covered with lids. All dishes were kept at 25°C ± 2°C and 55% ± 5% relative humidity in continuous darkness in an insect growth chamber. After a day of exposure to

Figure 1. Transmission electron lipophilic silica nanoparticle.
lipophilic SNP, initial mortality was recorded. The live insects were transferred to glass vials (Borosil, India) containing 50 mg wheat flour for 7 days in the growth chamber. Insect mortality data was further marked after 2, 4, and 7 days. All bioassays were performed in five replicates.

**In vitro cellular toxicity assay of lipophilic SNP on MRC-5 cells**

Cellular toxicity of this SNP was tested on MRC-5 cells with the help of water-soluble tetrazolium (WST) and lactate dehydrogenase (LDH) activity assays. Secondary human fibroblast cells (MRC-5, purchased from American Type Culture Collection) were maintained in continuous culture at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol L⁻¹ glutamine, 1 mmol L⁻¹ sodium pyruvate, 100 U non-essential amino acid, 100 IU mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin. For evaluating the toxicity of lipophilic SNP, 2.0 × 10⁴ cells were placed in each well of a 96-well plate. 20 μL suspension of SNP in pH 7 phosphate buffer saline (PBS) was added to the cells for incubation. The final concentration of SNP was 25, 51, 128, 320, 800, 2000, and 5000 ppm. In negative control wells no treatment was given, only PBS was mixed with cell culture medium. The positive control cells were treated with EDTA, which killed all cells. After 72 h of NP incubation, WST-1 and LDH assays were carried out.

**Cell viability assay**

Viability of MRC-5 cells after lipophilic SNP treatment was assessed in triplicate by water soluble tetrazolium-1 (WST-1) assay. In this study 10 μL reconstituted WST-1 mixture (WST-1 assay kit: BioVision – K302-500) was added to each well of a 96-well plate. Cells were incubated for 4 h at 37°C in a CO₂ incubator. The absorbance of the treated and untreated cells was read at 450 nm. Data was expressed as the percentage of cells alive in control and treated wells.

**Cytotoxicity assay**

The extent of cytotoxicity of SNP on MRC-5 cells was determined in triplicate in a 96-well plate by the measurement of LDH (lactate dehydrogenase) released from control and damaged cells into the medium after the cells were incubated at room temperature for 30 min (LDH assay kit: Cayman chemical – 1008882). The absorbance was measured at 490 nm. Data were expressed as the percentage of cytotoxicity in control and treated wells.

**Acute oral toxicity of lipophilic SNP in mice**

The acute oral toxicity of surface-functionalized SNP at different doses was studied in mice model following the OECD guidelines for the testing of chemicals (OECD 2001). Young nulliparous, non-pregnant female mice and young male mice (average body weight: 20 g) were kept in an animal house with controlled temperature (23°C ± 2°C), humidity (60% ± 10%) at a 12 h light/dark cycle. The mice were fed with standard rodent diet and filtered water. After 7 days of acclimatization, the mice were randomly assigned to control and treatment groups. There were five mice of each gender in each group. Lipophilic SNP was suspended in 1 mL absolute alcohol at three doses 500, 1000, or 2000 ppm. This
suspension (0.5 mL) was fed orally to mice followed by feeding of this same volume after 12 h (i.e., each mouse was fed with 0.5, 1, or 2 g kg\textsuperscript{-1} test substance). Each mouse in control was fed with 1 mL absolute alcohol in two doses of 0.5 mL each within an interval of 12 h.

The animals were kept under close observation for 14 days. Skin and fur changes, eye secretion, and behavior patterns of the mice were observed. Special attention was paid to the clinical signs of toxicity, including tremors, convulsions, salivation, nausea, vomiting, diarrhea, and lethargy. At the end of 14 days, mice were sacrificed. Blood and serum from control and treated mice were analyzed for TC (total count), DC (differential count), PLT (platelet count), LDH, creatinine, alkaline phosphatase (ALP), total protein (TP), cholesterol, triglyceride (TG), uric acid, blood urea nitrogen (BUN), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and phosphorous. Heart, lungs, liver, kidneys, spleen, uteri, and testes were removed and fixed in a 10% formalin solution containing neutral PBS. Thereafter the organs were embedded in paraffin, stained with eosin-hematoxylin, and examined under light microscope. The animals were weighed before and after the completion of the experiment.

Results and discussion

Insecticidal assay

Figure 2 shows that lipophilic SNP did not produce mortality in \textit{T. castaneum} on day 1. On day 2 this nanocide produced 24% insect mortality at 1.5 mg cm\textsuperscript{-2} dosage. Application of this SNP at 1 mg and 1.5 mg cm\textsuperscript{-2} killed 31% and 53% insects, respectively, after 4 days. After 7 days of exposure, 57% and 73% insects died at 0.5 mg and 1 mg cm\textsuperscript{-2} dosages, respectively; whereas 96% insect mortality was found when lipophilic SNP was applied at 1.5 mg cm\textsuperscript{-2}.

In vitro cellular toxicity assay

In WST-1 assay the cell survival rates were determined against the negative control (Figure 3a). Cell survival rate decreased with the increasing concentration of SNP. At 25 ppm
almost 95% of cells were alive, whereas at 128 ppm the cell survival percent was reduced to nearly 71%. Sixty-three percent, 53%, and 47% cell survivability was observed at 320, 800, and 2000 ppm, respectively.

Similar trend of SNP-induced toxicity profile was also obtained from the LDH activity assay. In the untreated cells, there was a negligible amount of LDH activity present in the culture medium because most of the cells were intact. In EDTA-treated cells, the presence of LDH was highest because almost all the cells died due to EDTA treatment and hence the LDH present in the culture medium was taken as 100%. At 25 ppm, lipophilic SNP produced 21.97% cytotoxicity (Figure 3b). Application of SNP at 128, 320, and 800 ppm resulted in nearly 34%, 35%, and 37% toxicity, respectively. CC50 (50% cellular

![Figure 3. (a) Survivability of MRC-5 cells (±SE) after 72 h incubation with lipophilic silica nanoparticle using WST-1 assay. (b) Percentage of cytotoxicity of MRC-5 cells (±SE) after 72 h incubation with lipophilic SNP using the LDH assay.](image)

Table 1. Serum biochemistry and hematology analysis of control and SNP-treated mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>0.5 g kg⁻¹</th>
<th>1 g kg⁻¹</th>
<th>2 g kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g dL⁻¹)</td>
<td>12.2 ± 0.2</td>
<td>10.2 ± 0.3</td>
<td>11 ± 2</td>
<td>11.2 ± 0.2</td>
</tr>
<tr>
<td>TC</td>
<td></td>
<td>4.1 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>4.1 ± 0.4</td>
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<tr>
<td>WBC (×10² mm⁻³)</td>
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<td>56 ± 1</td>
<td>53 ± 1</td>
<td>52 ± 3</td>
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<tr>
<td>DC (%)</td>
<td></td>
<td>42 ± 3</td>
<td>39 ± 1</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td>53 ± 1</td>
<td>57 ± 1</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td>3 ± 1</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Eosinophils</td>
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<td>0</td>
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<td>Basophils</td>
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<td>0</td>
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<td>0</td>
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<td>Platelets (lakh mm⁻³)</td>
<td>1.54 ± 0.02</td>
<td>1.52 ± 0.04</td>
<td>1.54 ± 0.03</td>
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<tr>
<td>LDH (IU L⁻¹)</td>
<td>202 ± 3</td>
<td>204 ± 3</td>
<td>209 ± 5</td>
<td>214 ± 3*</td>
</tr>
<tr>
<td>Creatinine (mg dL⁻¹)</td>
<td>0.71 ± 0.04</td>
<td>0.71 ± 0.03</td>
<td>0.7 ± 0.02</td>
<td>0.74 ± 0.01</td>
</tr>
<tr>
<td>Alkaline phosphate (U L⁻¹)</td>
<td>62 ± 3</td>
<td>64 ± 5</td>
<td>59 ± 4</td>
<td>61 ± 4</td>
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<tr>
<td>Total protein (gm dL⁻¹)</td>
<td>6.1 ± 0.4</td>
<td>6.1 ± 0.3</td>
<td>6.5 ± 0.4</td>
<td>6.8 ± 0.4</td>
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<tr>
<td>Cholesterol (mg dL⁻¹)</td>
<td>136 ± 3</td>
<td>154 ± 4*</td>
<td>156 ± 4*</td>
<td>164 ± 7*</td>
</tr>
<tr>
<td>Triglyceride (mg dL⁻¹)</td>
<td>70 ± 5</td>
<td>72 ± 2</td>
<td>73 ± 2</td>
<td>75 ± 5</td>
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<tr>
<td>Uric acid (mg dL⁻¹)</td>
<td>2.9 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>BUN (mg dL⁻¹)</td>
<td>10 ± 1</td>
<td>11 ± 1</td>
<td>12 ± 2</td>
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<tr>
<td>SGOT (Unit L⁻¹)</td>
<td>12 ± 2</td>
<td>13 ± 1</td>
<td>14 ± 2</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>SGPT (Unit L⁻¹)</td>
<td>11 ± 2</td>
<td>10 ± 1</td>
<td>12 ± 2</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Phosphorous (mg dL⁻¹)</td>
<td>2.8 ± 0.3</td>
<td>3 ± 0.3</td>
<td>3.1 ± 0.3</td>
<td>2.9 ± 0.2</td>
</tr>
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Notes: *Significant difference vs. control, p < 0.05.
cytotoxicity) of this surface-functionalized nanocide was not observed even at 2000 ppm dosage.

Although silica is reportedly inert in nature, the cause of cell death with increasing doses of SNP may be due to the adherence of SNP to the cells in the culture medium and interference with the membrane function or metabolism.

Figure 4. Light microscopic images of eosin–hematoxylin-stained organ sections of control and lipophilic SNP-treated mice: (a) control brain, (b) treated brain, (c) control lungs, (d) treated lungs, (e) control heart, (f) treated heart, (g) control liver, (h) treated liver, (i) control kidney, (j) treated kidney, (k) control spleen, and (l) treated spleen.
Acute oral toxicity of SNP in mice model

There were no significant difference in food consumption and water intake between treated and control groups. There was also no significant dose-related change in the body weight. Blood profile showed that hemoglobin, TC, DC, and PLT remained almost unchanged in control and treated groups (Table 1). Normally in case of liver damage, the levels of ALP, SGOT, and SGPT are increased. There appeared to be a decrease in LDH in the treated mice compared to controls, suggesting that lipophilic SNP did not produce any major damage in the liver. Generally, kidney malfunction is associated with the elevated level of creatinine, BUN, and uric acid (Clarkson et al. 2008). In the treated mice the level of these serum parameters remained unaltered. SNP-treated mice showed a significant increase in cholesterol levels, but TG levels remained unchanged in the treated mice. For serum biochemistry and hematological analysis, there were no marked dose-response alterations in SNP-treated mice.

Pathological observation showed that there was no lesion or damage in any organ, including the lung, liver, kidneys, and spleen. Figures 4 shows the histopathological profile of brain (Figure 4a–b), lung (Figure 4c–d), heart (Figure 4e–f), liver (Figure 4g–h), kidneys (Figure 4i–j), and spleen (Figure 4k–l) exposed to lipophilic SNP on day 14 after administration. Although there are earlier reports of pulmonary and cardiovascular damage produced by NP, silica in nanoform did not induce any major damage in these organs.

Conclusions

Surface-functionalized amorphous SNP has the potential as an alternative insecticidal agent. The in vitro cellular toxicity in human fibroblast cell line and acute oral toxicity study in mice revealed that similar to the amorphous silica particle, its nanosized form is also relatively non-toxic. This study may open up new pathways for nanomaterial-based insecticide for the agrochemical industry.

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References


Novel Entomotoxic Nanocides for Agro-chemical Industry

Nitai Debnath1,2, Sumistha Das1, Arunava Goswami1
1 Biological Sciences Division, Indian Statistical Institute, 203 B. T. Road, Kolkata 700 108, West Bengal, India
2 West Bengal University of Technology, BF-142, Sector-1, Salt Lake, Kolkata 700 064, West Bengal, India
E-mail: nitai.debnath@gmail.com, sumistha.das@gmail.com, agoswami@isical.ac.in

Abstract — In recent years, pest management scientists are reappraising the use of inert dusts as alternative insecticides because of several growing environmental problems of the conventional insecticides like cumulative environmental pollution, increasing insect resistance to them etc. The naturally occurring amorphous micron sized silica (Diatomaceous Earth), derived from fossil phytoplankton has gained enormous popularity as a physically active insecticide in the temperate countries. Here we investigated and compared the insecticidal efficacy of surface functionalized amorphous silica nanoparticle (SNP) with micron sized silica “FossilShield90.0s” on tropical army worm Spodoptera litura. S. litura is a polyphagous pest which attacks a number of crops and vegetables. All micron and nano sized silica were applied on the second instar larvae of S. litura at four dose rates with proper control. It was found that all SNPs were much more effective than “FossilShield90.0s”. Especially, hydrophilic SNP could kill all the larvae within 24 hours of the treatment. In vitro toxicity of SNPs was evaluated on MRC-5 cells with the help of water soluble tetrazolium (WST-1) and lactate dehydrogenase (LDH) assays. Unlike conventional insecticides, SNP kills the insects by causing damage to their cuticular water barrier. Insects lose water through their damaged cuticle and die because of desiccation. As this nanocide is physically active, insects are unlikely to develop resistance against it. United States Department of Agriculture has approved amorphous silica as bio safe.

Index Terms - Silica nanoparticle, Spodoptera litura, Nanocide, Cytotoxicity.

I. INTRODUCTION

According to many anthropologists, the great expansion of agricultural yield is the singular achievement of modern civilization. This was achieved by increasing reliance on mechanical labor and by the use of petrochemical based fertilizers and pesticides to enhance production. But from thermodynamic point of view modern agriculture is the least productive form of agriculture in history. It uses far more energy inputs to power the machinery and provide the synthetic fertilizers and pesticides per unit of every output than any previous period [1, 2].

In spite of this huge energy investment, food security still remains the biggest concern of the mankind. The competition from the insect pests is the major cornerstone in meeting food demands of a rising human population. Particularly, in tropics and sub-tropics the climate provides a highly favorable environment for a wide range of insects. Here, massive efforts are required to suppress population densities of the different field and store grain pests to achieve an adequate supply of food.

Thus, an urgent need is being felt for more scientific and targeted management of the agriculture sector, especially in the use of pesticides. In recent years, pest management scientists are reappraising the use of inert dusts as alternative insecticides because of consumer awareness of environmental pollution and the growing problem of insect resistance to conventional insecticides. The naturally occurring amorphous micron sized silica (Diatomaceous Earth or DE), derived from fossil phytoplankton has gained enormous popularity as a physically active insecticide. But DE is not very effective in highly humid tropical climate [3]. We hypothesized that nanosized amorphous silica would also have insecticidal property and would be needed in lesser quantity in comparison with conventional insecticides because of their huge surface to volume ratio. We have already shown that SNPs can control a number of insect pests [4-7]. In this study we investigated the entomotoxic potential of surface functionalized amorphous silica nanoparticle (SNP) on tropical army worm (Spodoptera litura). S. litura is a polyphagous pest and attacks cotton, jute, corn, tea, tobacco as well as many vegetables such as eggplants, bean and a number of Cruciferous and Cucurbitaceae plants. S. litura has developed resistance against numerous pesticides [8].

Moreover, in vitro toxicity of these nanocides was tested on MRC-5 cells (secondary human fibroblast cells) with the help of water soluble tetrazolium (WST-1) and lactate dehydrogenase (LDH) assays.

II. MATERIALS AND METHODS

A. Nano and micron sized silica

Three types of custom made, surface functionalized amorphous SNPs were purchased from M K Implex, Canada. These were hydrophilic, lipophilic and hydrophobic SNP prepared by the vapor phase method [9] and had size range of 15 – 20 nm (Fig. 1A-C). Micron sized naturally occurring, fossil phytoplankton derived silica “FossilShield90.0s” (FS90.0s) was obtained from Prof. Christian Ulrichs of Urban Horticultural Section at Humboldt University, Berlin. The size range of FS90.0s was 5 – 30 um (Fig. 1D).
B. Insects

*S. litura* was cultured on leaves of the castor oil plant (*Ricinus communis* L.) in Bidhan Chandra Krishi Viswavidyalaya, an agricultural university in West Bengal, India. The second instar larval stage of the insect was used in the insecticidal bioassay.

C. Insecticidal assay of micron and nano sized silica

To test the pesticidal efficacy of the surface functionalized SNPs and micron sized silica, all the SNPs and FS90.0s were dusted uniformly on the bottom surface of plastic containers at four dose rates 0.125 mg cm\(^{-2}\), 0.25 mg cm\(^{-2}\), 0.5 mg cm\(^{-2}\) and 1 mg cm\(^{-2}\). No particle was dusted in the control set. The containers were covered with muslin cloth to allow aeration and the larvae were fed with equal amount of *Ricinus* leaves. For each dose there were five replicates. 10 second instar larvae of *S. litura* were introduced in each box after dusting. All bioassays were performed at 30°C ± 1°C and 75 ± 5% Relative Humidity (R.H.). Insect mortality was checked after 24 hours.

The data were analyzed by using a two-way ANOVA with R 2.8.1 software where insect mortality was the main variable and treatment, dose were the main effects.

D. Incubation of cells with SNPs

The human fibroblast cells (MRC-5, purchased from American Type culture Collection) were maintained in continuous culture at 37ºC with 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 100 U non-essential amino acid, 100 IU/ml penicillin, 100 ug/ml streptomycin. For evaluating toxicity of SNPs 2.0 X 10\(^4\) cells were placed in each well of a 96-well plate. 20 ul suspension of SNPs in pH 7.0 phosphate buffer saline (PBS) was added to the cells for incubation. The final concentration of SNPs was 25, 50 and 100 ppm. In negative control wells no treatment was given, only PBS was mixed with cell culture medium. The positive control cells were treated with EDTA, which would kill all the cells. After 72 hours of nanoparticle incubation WST-1 and LDH assays were carried out.

E. WST-1 and LDH assay

Viability of MRC-5 cells after SNP treatment was assessed in triplicate by WST-1 assay with proper controls. In this study 10 ul of reconstituted WST-1 mixture (WST-1 assay kit: BioVision - K302-500) was added to each well of a 96-well plate. The cells were incubated for 4 hours at 37°C in a CO\(_2\) incubator. Before reading the absorbance, the plates were kept on an orbital shaker for one minute for gentle, homogeneous mixing of the color. The absorbance of the treated and untreated cells was measured using a microtiter plate reader at 450 nm.

The extent of cytotoxicity of SNPs on MRC-5 cells was estimated in triplicate in 96-well plate by measurement of LDH released from damaged cells into the medium (LDH assay kit: Cayman chemical - 1008882). In brief, cell culture plates were centrifuged at 400 X g for 5 minutes. 100 ul of...
supernatant from each well of treated and control cells were transferred to corresponding wells in a new 96-well plate. 100 ul of reconstituted reaction mixture was added to each well using multi channel pipetman. The plate was incubated at room temperature with gentle shaking on an orbital shaker for 30 minutes. The absorbance was taken at 490 nm in a microtiter plate reader.

In WST and LDH assay data were expressed as percentage of cells alive and percentage of cytotoxicity respectively in control and treated wells.

III. RESULTS AND DISCUSSION

A. Insecticidal assay of micron and nano sized silica

Fig. 2 shows all the SNPs had considerable insecticidal effect in comparison with micron sized FS90.0s silica. Especially hydrophilic SNP could kill more than 90% larvae at 0.125 mg cm\(^{-2}\) dosage and at 0.25 mg cm\(^{-2}\) dose or above all the larvae became dead within 24 hours of the treatment. Within this time period, lipophilic and hydrophobic SNP caused 78% insect mortality at 0.125 mg cm\(^{-2}\) dosage. Application of lipophilic SNP at 0.25 mg cm\(^{-2}\) could kill 90% larvae. At this dose hydrophobic SNP killed 86% S. litura. 90% or more insect mortality was obtained when dose rate was 0.5 or 1 mg cm\(^{-2}\) in case of lipophilic and hydrophobic SNP.

In case of micron sized silica, only 23% larvae became dead after 24 hours when FS90.0s was applied at 0.125 mg cm\(^{-2}\). It caused 35% and 60% mortality at .25 and 0.5 mg cm\(^{-2}\) dose respectively. At the highest dose (1 mg cm\(^{-2}\)) 75% larvae died due to FS90.0s treatment.

B. WST and LDH assay

In WST-1 assay tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is cleaved to formazan by mitochondrial dehydrogenase enzyme from live cells and its absorption can be measured spectrophotometrically.

Fig. 2 Mean mortality (± S.E.) of 2\(^{nd}\) instar Spodoptera litura larvae after being exposed to silica nanoparticle (SNP) and micron sized silica (FS90.0s) for 24 hours. ANOVA: treatment F=118.682, P<0.001, dosage F=420.136, P<0.001, treatment X dosage F=12.874, P<0.001.

Hence, the increase in formazan dye is directly correlated to the number of metabolically active living cells. The cell survival rates were determined against the negative control (Fig. 3). Though all types of surface functionalized SNPs were negligibly toxic to the MRC-5 cells, the cell survival rate decreased with increasing concentration of SNPs. At 25 ppm almost 95% of the cells were alive in case of hydrophilic and lipophilic SNP, whereas 91% cells survived when hydrophobic SNP was applied at this dosage. However, at 100 ppm the cell survival percentage was reduced to nearly 75% in case of all three SNP treatments.

The result of WST-1 assay can also be corroborated from LDH assay. LDH is a soluble enzyme located in the cytoplasm of the cells. The enzyme is released into the surrounding culture medium upon cell damage or lysis. LDH activity in the culture medium can therefore be used as an indicator of cell membrane integrity, and thus a measurement of cytotoxicity.

In the untreated cells, there was negligible amount of LDH present in the culture medium because most of the cells were intact. In the EDTA treated cells presence of LDH was highest because almost all the cells became dead due to EDTA treatment and hence, the LDH present in culture medium was considered to be because of 100% cytotoxicity. At 25 ppm hydrophilic, lipophilic and hydrophobic SNP caused 18.14%, 21.97% and 12.64% cytotoxicity respectively (Fig. 4). Application of SNPs at 50 ppm resulted in nearly 25% toxicity. Whereas, at the highest dose hydrophobic and lipophilic SNP seemed to be slightly more toxic (nearly 35%) than hydrophilic SNP (26%).

Though silica is reportedly inert in nature, in cell culture SNPs might attach to the cell surface and interfere with membrane function or metabolism.
Fig. 4 Percentage of cytotoxicity of MRC-5 cells after 72 hour incubation with silica nanoparticle (SNP) using LDH assay. The percentage of cytotoxicity (± S.E.) was also relative data as compared with positive control wells.

C. Advantage of using SNPs over conventional insecticides

Ebeling [10] proposed that insecticidal efficacy of silica becomes enhanced if the particles are finely divided. It was very much evident from our insecticidal assay, where SNPs were much more effective than micron sized FS90.0s because of their highly increased exposed surfaces which could interact with the insect cuticle. FS90.0s and SNPs caused damage to the external cuticle of insect body which forms a water barrier both by sorption and abrasion. Due to much smaller size, SNPs became impregnated into the cuticular wall more effectively and broke its integrity. The insects began to lose water as their water barrier was damaged and die due to desiccation. This was why the bodies of dead S. litura larvae became extremely dehydrated. As these nanocides are physically active, insects are very unlikely to become genetically selected or physically resistant to them.

It is known that inhalation of crystalline silica causes silicosis [11]. XRD analysis of FS90.0s SNPs confirmed that both of these formulations were amorphous in nature (data not shown). United States Department of Agriculture (USDA) has approved amorphous silica as bio safe [12]. In vitro toxicity study showed that SNPs had little cytotoxicity on human fibroblast cells at higher concentration. But it was negligible if we considered the toxicity of the conventional commercial pesticides [13]. As silica is non reactive, it will not pollute soil or contaminate ground water, rather it will enhance structural rigidity and strength of plant [14]. This may be one of the possible reasons for which there was an age old tradition of using silica dust as protective agent for stored seeds by different ethnic races all over the world.

IV. CONCLUSION

In this study we have shown that SNPs can be used as an alternative insecticide. These nanocides are much more eco-friendly than the conventional insecticides. However, it will be premature to comment on the toxicity of nanomaterials in living system until elaborate toxicity studies are conducted. It should be applied in controlled environment until detailed toxicity study of NPs is performed.

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REFERENCES

Patent Facilitation Form

Proforma for filing of patents in Nanobiotechnology

Part - A

1. Title of Invention: Nitentox: Entomotoxic hydrophilic silica nanoparticle

2. Name of the Inventors:
   i. Dr. Arunava Goswami, AERU
      Biological Sciences Division,
      Indian Statistical Institute, Kolkata 700108.
   ii. Nitai Debnath, AERU
       Biological Sciences Division,
       Indian Statistical Institute, Kolkata 700108.
   iii. Dipankar Seth, AERU
        Biological Sciences Division,
        Indian Statistical Institute, Kolkata 700108.
   iv. Sumistha Das, AERU
       Biological Sciences Division,
       Indian Statistical Institute, Kolkata 700108.
   v. Somesh Chandra Bhattacharya,
      Agricultural and Ecological Research Unit,
      Indian Statistical Institute, Giridih, Jharkhand.

3. Address: Biological Sciences Division, Indian Statistical Institute, 203 B. T. Road, Kolkata, India. Telephone: ++919433479088 Fax: 033-2577-3049 E-mail: agoswami@isical.ac.in / srabanisopanaranava@gmail.com

4. Name of the applicant: Dr. Arunava Goswami

5. Whether the invention is made out of DBT-funded project (Yes/No) Yes
   If Yes,
   b.) Subject area: Nanobiotechnology
   If No,
   a.) When and Where the invention was developed
   ..........................................................................................................................
   ..........................................................................................................................
   b.) Sponsoring organization/ agency ..............................................................................

6. Description of the invention (indicating prior art and the highlights of the invention).

   • 15 nm, 30 nm and 60 nm size, amorphous spherical shaped nanosilica were synthesized by sol-gel method from aqueous alcohol solution of silicon alkoxide by slightly modifying Stober process as well as by altering the molar concentration of the reactants. Nanosilica thus produced was lyophilized and resultant nanopowders were
Patent Facilitation Form

Proforma for filing of patents in Nanobiotechnology

Part - A

1. Title of Invention: **Sumentox: Entomotoxic hydrophobic silica nanoparticle.**

2. Name of the Inventors:

   i. Dr. Arunava Goswami, AERU
      Biological Sciences Division,
      Indian Statistical Institute, Kolkata 700108.

   ii. Nitai Debnath, AERU
       Biological Sciences Division,
       Indian Statistical Institute, Kolkata 700108.

   iii. Dipankar Seth, AERU
        Biological Sciences Division,
        Indian Statistical Institute, Kolkata 700108.

   iv. Sumistha Das, AERU
       Biological Sciences Division,
       Indian Statistical Institute, Kolkata 700108.

   v. Somesh Chandra Bhattacharya,
      Agricultural and Ecological Research Unit,
      Indian Statistical Institute, Giridih, Jharkhand.

3. Address: Biological Sciences Division, Indian Statistical Institute, 203 B. T. Road, Kolkata, India.
   Telephone: ++919433479088 Fax: 033-2577-3049 E-mail: agoswami@isical.ac.in / srabanisopanarunava@gmail.com

4. Name of the applicant: **Dr. Arunava Goswami**

5. Whether the invention is made out of DBT-funded project (Yes/No) **Yes**
   If Yes,
   b.) Subject area: **Nanobiotechnology**
      If No,
   a.) When and Where the invention was developed
      ………………………………………………………………………………………………………
      ………………………………………………………………………………………………………
   b.) Sponsoring organization/ agency ……………………………………………………………

6. Description of the invention (indicating prior art and the highlights of the invention).

   - Hydrophobic nanosilica powder was synthesized by vapor phase method and the average particle size is 15nm (Figure 1A & 1B). Nanosilica thus produced was lyophilized and the hydrophobic silica nanopowder was tested for its entomotoxic
Patent Facilitation Form

Proforma for filing of patents in Nanobiotechnology

Part - A
1. Title of Invention: **Dipentox: Entomotoxic lipophilic silica nanoparticle.**

2. Name of the Inventors:
   
   i. Dr. Arunava Goswami, AERU
   Biological Sciences Division,
   Indian Statistical Institute, Kolkata 700108.
   
   ii. Nitai Debnath, AERU
   Biological Sciences Division,
   Indian Statistical Institute, Kolkata 700108.
   
   iii. Dipankar Seth, AERU
   Biological Sciences Division,
   Indian Statistical Institute, Kolkata 700108.
   
   iv. Sumistha Das, AERU
   Biological Sciences Division,
   Indian Statistical Institute, Kolkata 700108.
   
   v. Somesh Chandra Bhattacharya,
   Agricultural and Ecological Research Unit,
   Indian Statistical Institute, Giridih, Jharkhand.

2. Address: Biological Sciences Division, Indian Statistical Institute, 203 B. T. Road,
Kolkata, India.
   Telephone: ++919433479088 Fax: 033-2577-3049 E-mail: agoswami@isical.ac.in /
srabanisopanarunava@gmail.com

3. Name of the applicant: **Dr. Arunava Goswami**

4. Whether the invention is made out of DBT-funded project (Yes/No)  **Yes**
   If Yes,
   b.) Subject area : **Nanobiotechnology**
   If No,
   a.) When and Where the invention was developed
   ………………………………………………………………………………………………………………………………
   ………………………………………………………………………………………………………………………………
   b.) Sponsoring organization/ agency ……………………………………………………………………………

5. Description of the invention (indicating prior art and the highlights of the invention).
   
   • Lipophilic nanosilica powder was synthesized by vapor phase method and the average
   particle size is 15nm (Figure 1A & 1B). Nanosilica thus produced was lyophilized and
   resultant nanopowders showed high level of pesticide activity against store grain