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Chlorpyrifos-loaded Silver/Polyethylene Glycol/Chitosan Nanocomposite: Improved Termiticidal Activity against *Microcerotermes diversus*. (Isoptera: Termitidae)

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Abstract

Pesticides play an important role in increasing food production by pest control. Most pesticides are less soluble in the aqueous media and need organic solvents for desirable efficacy, causing environmental pollutions. Nanopesticides overcome the problems associated with native pesticides through reduction in the quantity of pesticide and controlling the risks for human and environmental health. In this research, chlorpyrifos (CPS) was loaded in Ag/PEG/Cs nanoparticles (Ag/PEG/Cs/CPS NPs) and then evaluated as a nanopesticide against *Microcerotermes diversus* Silvestri (Isoptera: Termitidae). The target nanopesticide was characterized by XRD, UV-VIS, FT-IR, and TEM. The results revealed that the NPs were spherical with the medium-sized of 11 nm. The biological activity of the NPs was also estimated *in vitro* against *Microcerotermes diversus*. The results confirmed the effectiveness of the nanopesticide at concentrations of 0.05 µl/l (5 ppm) after 48 h against termites and also at a lower concentration of 0.005 µl/l (50 ppm) after 72 h. The nanopesticid could be considered as a promising alternative to the conventional harmful pesticides for controlling termites.

Keywords: Chlorpyrifos; Nanopesticide; *Microcerotermes diversus*; Nanosilver.

Introduction

Insects damage food production and induce serious problems toward the agricultural economy. The use of high amounts of pesticides for controlling pests causes many environmental pollutions. Indeed, the lack of full control of pesticides is reportedly responsible for pesticide resistance to chemical pesticides [1]. Recently, the impressive progress in the nanotechnology has encouraged researchers to develop nanopesticides to remove all problems associated with pesticide resistance and improve the efficacy of the low-dose pesticide. In this regard, various nanomaterials have been exclusively developed for the pesticide delivery.[1] Nanotechnology has endowed the target pesticide with new specific properties including improved low-dose effects of pesticides in the aquatic media, controlled release, appropriate stability of pesticides [2, 3].

Nowadays, many pesticides have been produced as nanopesticide by different routes and for specific

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targets. Various nanoformulation of pesticides have been so far introduced as nanopesticides such as nanoemulsion of beta-cypermethrin [4], emanated-benzoate (Fan et al., 2010) [5], nanopolymer formulations of tebuconazole [6], porous hollow silica NPs of avermectin [7], layered double hydroxides and clay NPs of atrazine [8]. Also, there are many studies reporting priority of the nanopesticide formulations compared with their commercial analogues against target organisms with less toxicity against non-target organisms [9]. Most of the nanopesticide are composed of degradable and non-toxic polymers for sustained release. For example, Gonzalez et al. developed a nanocomposite based on chitosan (Cs) and silver NPs to encapsulate gentamicin. The results showed that the nanoformulation had much better activity than free gentamicin confirmed by *in vitro* analyses [10]. In other studies [11, 12], comparison between nanosilver synthesized using *Heliotropium Indicum* plant leaves and crude aqueous extract of the leaf showed more biological activity of nanopesticides against three mosquito species than extracted plant leaves. The synthesized silver NPs had potential larvicidal activity against *Culex quinquefasciatus*, *Anopheles subpictus*, and *Pediculus humanus*. Also, nanotubes containing pesticides filled with aluminosilicate could damage certain physiological functions due to the effectively attachment to the surfaces of the plant, hairs of the insect, and finally body penetration [13].

Several polymers such as chitosan (Cs) and polyethylene glycol (PEG) have been used for construction of nanopesticides. Cs has attracted more attention due to its unique characteristics. The amino groups of Cs act as an excellent hydrogen-bonding group and facilitate the formation of the network. The interaction between silver and Cs was explained by Budnyak [14] and Cheng et al. [15] Kima et al. [16] synthesized retinol-encapsulated Cs NPs through electrostatic interaction between amine and hydroxyl functional groups of Cs and hydroxyl groups of retinol with a spherical shape and size average of 50–200 nm. Navaladian et al. [17] used thermal decomposition technique for preparation of PEG/PVP coated silver NPs with the size below 10 nm. In this method, polyvinyl alcohol (PVA) was used as a reducing and coating reagent, and had a crucial role to control the size of the NPs.

Termites are abundantly found in all world places and certain areas and cause severe damages. They can destruct the agricultural plants, wood structure, and building by feeding on cellulosic materials. In the south of Iran, *Microcerotermes diversus* Silvestri (Isoptera: Termitidae) is economically the most destructive termite species for buildings.

Chlorpyrifos (O, O-diethyl O-3, 5, 6 -trichloropyridin-2-yl phosphorothioate, CPS) is an organophosphate insecticide that is used for control of agricultural, domestic, and industrial insect pests. It is highly toxic to *Formosan subterranean* and *Formosanus Shiraki* in the dosage of 8 litters per acre [18].

This study explains synthesis of the aqueous stable silver NPs coated with Cs and PEG loaded with chlorpyrifos insecticide (CPS). The NPs were prepared by mixing the same ratio of neutral polymers Cs/PEG in deionized water. These two polymers contain hydroxyls and amine groups chelating with silver ions and forming hydrogen bonds with the surface of silver NPs. The electrostatic steric repulsion between Cs coated NPs can efficiently solve the target nanosystem and prevent the aggregation resulting in the improved solubility and efficacy of CPS in the aqueous solution [19]. Silver nitrate as silver source was added into the solution of mixed polymers under nitrogen atmosphere to produce Ag/Cs/PEG NPs. Then, CPS was added to absorb in the nanosystem to give Ag/Cs/PEG/CPS NPs (Fig. 1). This nanopesticide was evaluated by the contact treatments in the *in vitro* test on termites. The results of the biological study showed this compound would be useful to develop a practical control method for termites which is a severe pest of wooden, structures, and crops [20].

Materials and Methods

Chemicals

$AgNO_3$ (98%) and PEG were from Merck and High-purity deionized water was obtained from milli-Q. Chlorpyrifos (CPS) as powder was taken from Aldrich-Sigma as technical grade of 98%, Cs was purchased from Aldrich-Sigma. Other chemicals were analytical grade supplied from Aldrich-Sigma, Merck, and used as received.

Synthesis

For the synthesis of Ag/Cs/PEG/CPS NPs, Cs (20 mg) in 1.0 wt% of AcOH (10 ml) and PEG-6000 (20 mg) were separately dissolved in 10 ml deionized water. These two solutions were mixed in 100 mL conical flask and stirred at room temperature for 15 minutes. Then, 40 mg of silver nitrate was added under the nitrogen atmosphere to prevent oxidation during Ag NPs formation. The amount of 80 mg $NaBH_4$ was added in the solution and followed by the addition of CPS (100 mg) in acetone (1 ml). The obtained mixture was stirred for 4 h to load pesticide into the Ag nanocomposite. The colour of the solution converted from pale brown to dark brown, indicating the formation of Ag NPs. Finally, the solution was centrifuged and washed four times with deionized water, dried at 40 ºC in dark under nitrogen atmosphere. The precipitate was triplicate centrifuged and washed with distilled water and EtOAc (to remove unabsorbed CPS). The black-grey precipitate was dissolved in 20 mL of H_2O. Four concentrates solution of the target nanopesticide was prepared for further studies (100, 50, 10, and 5 ppm). CPS as technical grade (98%, 100 ppm) was prepared, and 100 µl of each sample was applied on filter paper for the biological test. The obtained black-grey precipitate (Ag/Cs/PEG/CPS) was also characterized.

Nanoparticles characterization

The synthesis of silver NPs (the reduction process of Ag^+ to Ag^0 NPs) was confirmed by visual observation of color change of the solution to dark grey upon reaction of silver ions with reducing agent followed treating with polymers/pesticide. Morphology of the Ag/Cs/PEG/CPS NPs (Fig. 1) was characterized by depositing the suspension (2 µl) onto Formvar-coated copper grids and excess water was removed by filter paper. The obtained samples were left to dry under ambient air and then visualized via TEM (Zeiss EM10C-100 VK). The size of the NPs was analyzed using software (version 3.2, Soft Imaging System GmbH) equipped with the TEM.

UV-vis spectrophotometer (Perkin Elmer, lambada 25) was used at different concentrations FT-IR spectra of the Ag/Cs/PEG/CPS NPs were determined by using a Shimadzu IR prestige-21. Size distributions of Ag/Cs/PEG/CPS NPs were measured by DLS using Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., UK). The synthesized silver NPs were analyzed by X-ray diffractometer (XRD, STOE-STADV) and equipped with copper X-ray source.

The entrapment efficiency of Ag/Cs/PEGNPs

To determine entrapment efficiency, the prepared NPs (80 mg) were mixed with CPS (100 mg). The mixture was centrifuged at 15,000 rpm for 15 min. The entrapped CPS was determined by measuring the remaining CPS in the supernatant at $\lambda = 290$ nm by the UV-Visible spectrophotometer and standard curve equation [20].

Release studies

To study the release behavior of the target system, Ag/Cs/PEG/CPS NPs (30 mg) were dispersed in a vial containing 15 ml of phosphate buffer (pH= 6.5) at room temperature [21, 22]. At specified time points, 3 ml of medium was taken to estimate the amount of chlorpyrifos released from the target system by UV-Vis spectrometer.

***In vitro* study on the effect of Ag/Cs/PEG/CPS NPs against Termites.**

Termites were collected from a garden of Khuzestan province (*Microcerotermes diversus Silverti*). The Termites were put in Petri dishes containing moisture filter paper, and placed in a dark room. Each treatment was repeated four times. Three samples including water, CPS technical grade, and Ag/Cs/CPS in four different concentrations (100, 50, 10, and 5 ppm or µl/l), were used in the contact treatment (n=4). Distilled water was used as the control. For the contact treatment, ten groups of termites were placed in Petri dishes (92 mm × 16 mm). 100 µl of each sample was applied on filter paper (90 mm, Whatman) and then termites were then transferred to the Petri dishes. The Petri dishes were placed into the incubator at 28 ± 1 °C with high humidity, and total darkness (except during observation).

Statistical analysis

The average termite mortality was calculated by Probit Analysis. Other statistics at 95% confidence limits of upper confidence limit and lower confidence limit, and Chi-square values were calculated using the Statistical sas. Results with $p < 0.05$ were considered to be statistically significant.

Results and Discussion

Due to the well-known properties of nanoproducts, nanotechnology has intensively penetrated in many branches of science such as biomedical, electronic [23], catalytic [24], and optical applications [25]. Because of the numerous advantages such as biocides activity for preventing and treating diseases, the use of nanosilver (Ag NPs) has attracted more attention among researchers. In the present study, a new nanopesticide containing Ag NPs stabilized with PEG and Cs were used for CPS loading (Fig. 1).

Typically, the synthesis was performed by a chemical reduction of silver nitrate Ag^+ to Ag^0 in the presence of PEG and Cs by sodium borohydride leading to the high yield of the product [26]. The applied polymer could effectively chelated with silver ions and control the size of the target NPs. The reaction was performed fast at room temperature (Fig. 1).

Huang et al. [27] synthesized different Ag-Cs nanocomposites in aqueous solution by the reduction of different salts of silver by $NaBH_4$ [28-30]. The nanocomposite was applied for gentamicin loading.

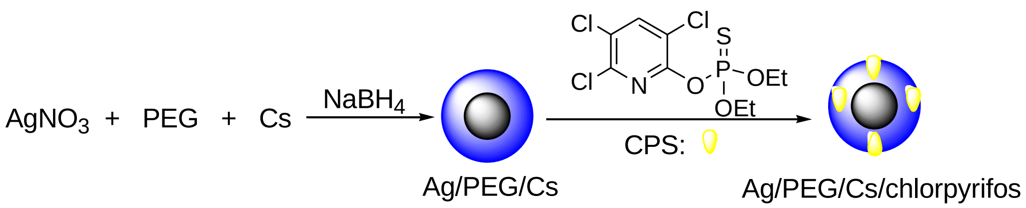

Figure 1. The schematic representation of Ag/Cs/PEG/CPS NPs synthesized from $AgNO_3$ by reduction method.

The entrapment efficiency of the NPs was calculated 85% by dividing the initial amount of CPS in the supernatant to the initial amount of CPS taken for loading [20]. The progress of the reaction and formation of silver NPs can be easily monitored by the colour changing of silver ions, after metal reduction from grey to dark suspension. The formation of the NPs was confirmed by the TEM, DLS, UV-visible, and IR and XRD analyses (Figs. 2-4).

The suspended solution of Ag/Cs/PEG/CPS NPs was stable after the reaction, without any precipitate or aggregate of the formulation. In a similar study, the silver-impregnated Cs films were prepared from silver nitrate and Cs was applied as a stabilizer and ascorbic acid as a reducing agent via thermal reaction [26, 32]. The prevalent stabilizers such as Cs and PEG are employed to control dispersion of the NPs. Notably, Cs has gained much attention for the synthesis of the stabilized NPs due to its biocompatible and biodegradable properties. This compound contains an amino functional group in β-(1, 4) polymer chain of D-glucose produced from the deacetylation of natural chitin [27, 33]. Controlling the particle growth, and preventing the oxidation of NPs can be performed by using Cs and PEG polymers [34, 35].

The TEM image of Ag/Cs/PEG/CPS NPs showed

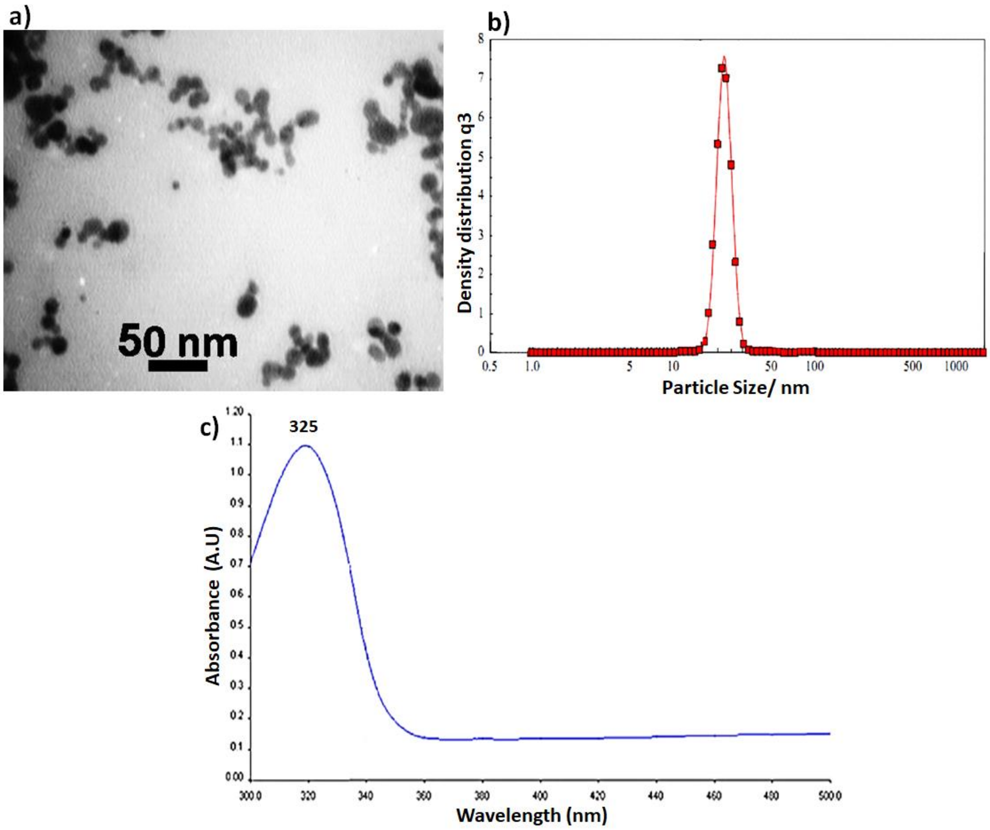

Figure 2. a) Representative TEM image and b) DLS and c) UV-visible spectra of Ag/Cs/PLG/CPS NPs .

that the NPs were spherical having the average size of 25-35 nm. DLS analyses also confirmed formation of NPs with average size of 30 nm (Fig. 2).

Also, the UV-visible analysis was performed to confirm formation of the silver NPs (Fig. 2). The spectrum indicated a broad peak at about 325 nm to related to the excitation of surface plasmon resonance of elemental Ag confirming successfully formation of Ag NPs [37]. Also, CPS absorption band was disappeared at 290 nm confirming successfully absorption of CPS into the matrix of the NPS. The result was in agreement with previously published work by Shameli et al., reporting preparation of spherical Ag NPs have in the absorption bands at around 400 nm in the UV-visible spectrum [38].

FT-IR spectrum of the NPs showed a broad peak at 3432 cm^{-1} related to stretching vibration of O-H bonds overlapped with stretching vibration of N-H bonds, peaks at around 2900 cm^{-1} related to the stretching vibration of aliphatic C-H bonds and a week band at 1663 cm^{-1} which could be related to C=O bonds (Fig. 3). The band appeared at 1340 cm^{-1} could be due to the stretching vibration of C-N bonds. The peaks appeared at 1411 and 834 cm^{-1} could be ascribed to the CPS loaded on the silver surface of the NPs [39].

The X-ray diffraction (XRD) pattern of the target NPs is shown in Fig. 4. The average crystalline size of Ag/Cs/PEG/CPS NPs was calculated by the Scherrer equation.

$$d = \frac{K\lambda}{\beta \cos\theta}$$

Where K=1, λ (X-ray wavelength = 0.15), β is the line broadening at half the maximum intensity in radians and is Bragg`s angle [40]**.** The average size of the NPs was obtained 11 nm by Sherrer's equation. The diffraction peaks were appeared at 2θ = 10.89º, 19.08º, 27.53º, and 38.05º, confirmed successfully formation of the NPs [40]. Pure PEG [41] showed strong reflections at 2θ of 19.23° and 23.34° and the weak reflections at 13.61° and 27.32°; while pure Cs [42] showed two peaks at 2θ of 19.08° (Fig. 4). The powder X-ray diffraction peaks at 2θ of 38.24°, 44.43°, 64.62°, and 77.50° could be attributed to the 111, 200, 220, and 311 crystallographic planes of the face-centered cubic silver crystals, respectively [34], (JCPDS Card No. 04-0783). Other peaks could be related to the presence of the polymer on the structure of the silver NPs.

The release profile of Ag/Cs/PEG/CPS NPs during 15 days was determined by UV-Visible spectrophotometer in aqueous media (Fig. 5). CPS concentration in the solution was measured based on the absorption peak of CPS at 290 nm. It was found that the release rate of insecticide increased over time. There was an initial fast release on the third day but followed the steady release having advantageous of readily controlling the existence of insect and subsequently kill new incoming insects in the termite colony. This was in

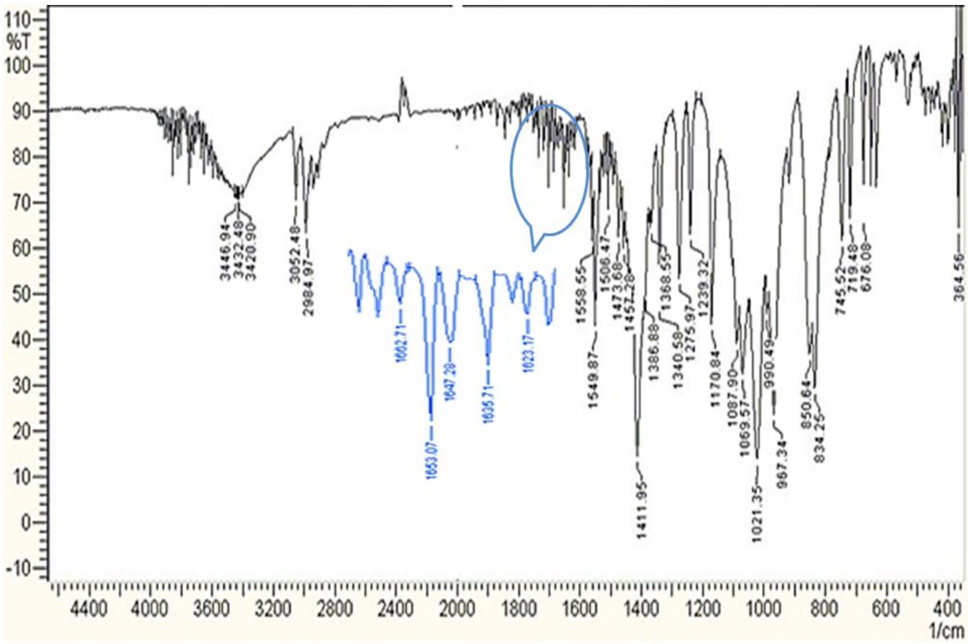

Figure 3. FT-IR spectrum of Ag/Cs/PLG/CPSNPs, the inset is related to the expanded part in the region of 1690-1615 cm^{-1}

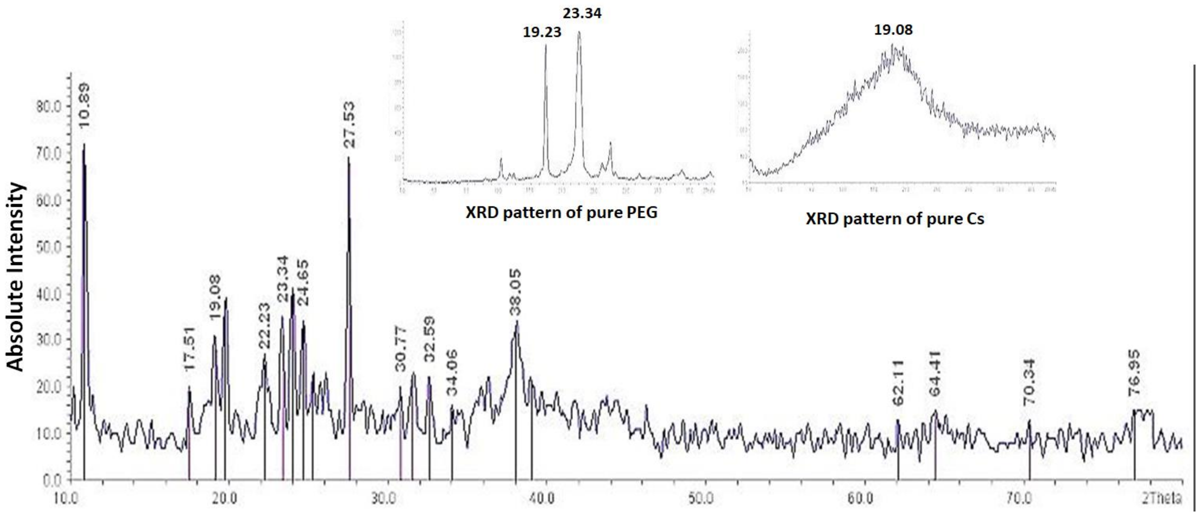

Figure 4. The powder XRD pattern of Ag/Cs/PEG/CPS NP. The inset images are related to the pure PEG and pure Cs.

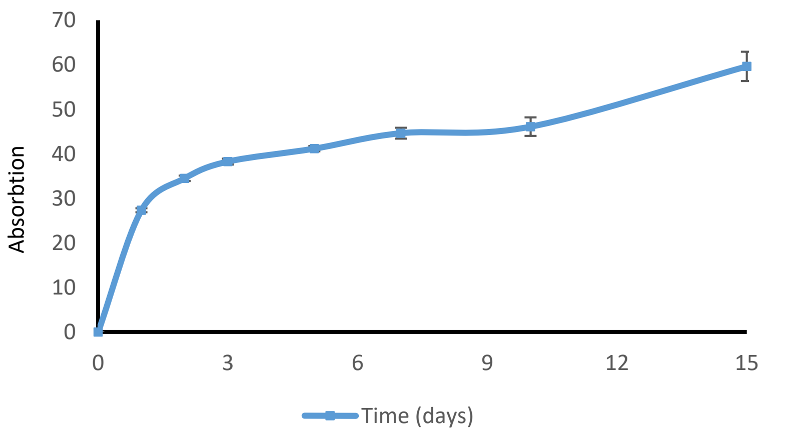

Figure 5. Release profile of Ag/Cs/PEG/CPS NPs during 15 days with standard error bars, 95% confidence, (n = 4).

agreement with a study reporting the improved of herbicidal activity of silver nanoparaquat due to the ability for slow release [43]. Ihegwuagu, et al. [44] suggested that the presence of nanosilver in the matrix of Cs creates an intricate polymer structure by intercalation with the effect that the diffusion/migration path length within the matrix becomes less tortuous, causing the release of the active ingredient easier and faster.

Table 1 shows mortality percentage of each treatment after 12, 24, 48, and 72 hours. The results revealed that after 12 hours, there was no significant difference between 100, 50, and 100 ppm. At 5 ppm, there was no mortality. After 24 hours, commercial CPS (100 ppm) induced 100% mortality, but in Ag/Cs/PEG/CPS NPs treatment, there was some live termite. There was no significant difference between free CPS (100 ppm) and nanopesticide (100 ppm), but after 72 hours all treatments have been shown 100% mortality. The results showed that adding 5 ppm of nonopesticide after 72 hours was equal to the efficiency of 100 ppm of CPS but in a very lower dose. However, after 72 hours of treatment, sustained insecticidal effect caused by Ag/Cs/PEG/CPS NPs nanoparticles led to the effective result. Compared with commercial CPS, the nanoformulation would be highly desirable, as it increased the likelihood of horizontal transfer of pesticide. Hence, it provides the control improvement of vast populations of termite colonies [45].

As shown in Table 2, several nanoformulation of pesticides and commercial ones applied on termites. Most of the nanopesticides had slow release manner

Table 1. Insecticidal activity of Ag/Cs/PEG/CPS NPs against adult termite at different time after training

Concentration	12h	24h	48-h Mortality (%)±SE	72h
100 ppm	22.5^{ab}	72.5^{B}	97.5 ± 0.43^{ab}	100^{A}
50 ppm	20.0^{ab}	72.5^{B}	85.0 ± 0.50^{b}	100^{A}
10 ppm	12.5^{bc}	40.0^{C}	50.0 ± 0.70^{c}	100^{A}
5 ppm	0.0^{c}	32.5^{C}	37.5 ± 0.82^{c}	100^{A}
100 ppm	30.0^{a}	100^{A}	100^{a}	100^{A}
control	0^{c}	0.0^{D}	0.0 ± 0.0^{d}	0^{B}

The same letters in each column mean there is no significant difference between treatments. Probit Analysis for calculating, and other statistics at 95 % confidence and $p < 0.05$.

Table 2. The Efficacy of nanoformulations compared to more conventional formulations

Ag/Cs/PEG/CPS NPs	100% mortality of termite after 72 h having slow release comared with free CPS.	
Nanofipronil (Nanosilica formulation)	100% mortality after 3 days, allowing better elimination of colony.	Ref- 45
Essential oil of *O. japanica*	90% mortality of termite after 3 days of treatment	Ref-46
Eucalyptus-Cs nanoemulsion	İncreased contact toxicity and 75% mortality was obtained after 72 h. The nanoemulsion improved essential oils performance. 8% mortality was achieved by chlorpyrifos and Eucalyptus globulus pesticide.	Ref-47
Metalic NPs of ZnO with size range of 30-70 nm containing neem extract as biopesticides	The efficacy of commertial pesticides: Thiodan (24%), CPS (53%), Tenkil (29 %) in soil.	Ref 48
NPs prepared from silver (Ag), boron (B), copper (Cu), zinc (Zn), zinc oxide (ZnO), zinc borate ($B_2O_6Zn_3$), and titanium dioxide (TiO_2)	Wood treatments with the NPs may play an important role in the next generation of wood protection systems against termites.	Ref-49

than commercial ones, such as nanosilica fipronil [45], which presented 100% mortality in termite after a longer time than Termidor as a commercial pesticide. In our study, Ag/Cs/PEG/CPS NPs showed 100% mortality after 72 h and a more slow release than CPS. In another study, the plant extract of Eucalyptus (8%) loaded in Cs NPs were evaluated biologically and the result showed 75% mortality after 72 h. In many studies, some popular pesticides such as third, stencil, and Essential oil of *Orixa japonica* as a biopesticide exhibited an efficient germicidal activity in the soil [46, 47]. Also, several nanometals were successfully synthesized and showed high insecticidal activity on *Microcerotermes diversus* Silvestri.

In other studies, various nanopesticides were prepared which showed high mortality percentage. Silver NPs synthesized by *Helitropium Indicum* plant leaves showed appropriate activity against late third instar larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* [11]. The synthesized Ag NPs from *H. Indicum* was highly toxic than crude leaf aqueous extract in three important vector mosquito species. The study performed by Ihegwuagu [44] also showed that presence of nanosilver in the starch matrix causes faster diffusion and release of the active ingredient. The pesticide delivery system presented in this work can be developed for other pesticide delivery systems.

Conclusion

The nanopesticide was prepared by a facile, inexpensive, and reproducible method in aqueous media and showed improved insecticide efficiency than free CPS. The biological study revealed that Ag/Cs/PEG/CPS NPs could be highly effective in lower dose and had promising feature to be applied for further studies.

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