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Research Article

Effect of Carbon Nanomaterial – Graphene and Reduced Graphene Oxide on the Silkworm Biological and Commercial Characters

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ABSTRACT

Silkworm is one of the economically beneficial insects and a potent in vivo animal model to test the toxicity of nanoparticles. Intensive efforts are being made to manifest the silk fibroin properties using nanoparticles for biomedical applications, which is also an emerging area of commercial importance in agri/seri-biotechnology. Graphene, for its superior mechanical properties, is widely applied as reinforcement in preparing high-performance materials. It has shown massive potential in energy, electronics, medicine and more. The reduced graphene oxide is a derivative of Graphene Oxide (GO), devoid of attached functionality with more potent electrical conductivity, biocompatibility, bioactivity and found applications in developing antibacterial surfaces and drug delivery. These two materials affect the biological and commercial traits of the silkworm, B. mori (PM × CSR2), in the oral route of administration, along with the mulberry leaf, which has a dose-dependent effect. In both nanoparticle-treated batches of the two different concentrations (0.1% & 0.5%), 0.1% showed as an optimum concentration that has potential for enhancing the cocoon and silk filament properties significantly compared with 0.5%. An increase in the total protein content of haemolymph tissue may also increase the total silk protein synthesis due to 0.1% graphene. Moreover, no significant change was noticed in the total protein content and profile of the haemolymph tissue in Reduced Graphene Oxide (rGO) treated silkworm batches. Overall observation showed no traceable toxic effect in the silkworm, B. mori, to rGO-based mulberry leaf diet, which clearly indicates the beneficial effect of rGO that can be exploited for the improvement of silk traits.

Keywords: *Bombyxmori, Graphene, Graphene oxide, Invertebrate model system.*

INTRODUCTION

The silkworm, *Bombyx mori* L., is a domesticated, monophagous lepidopteran insect that feeds on the foliage of the mulberry plant to biosynthesize economically important structural protein in the form of silk fibre. The different strategies have been followed to increase the cocoon productivity; enrichment of mulberry leaves is one among them.

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Fortification of mulberry leaves with supplementary compounds such as vitamins (Etebari et al., 2004; & Rahmathulla et al., 2009), hormones (Trivedy et al., 1993; & Saha & Khan, 1997), amino acids (Saha et al., 1994; & Saha & Khan, 1997), minerals (Islam & Khan, 1993; & Khan & Saha, 1995) and micro nutrients (Vishwanath & Krishnamurthy, 1983) to increase the larval growth and development. The application of nanoparticles in the improvement of biological traits has become an active area of research over the past few decades. The increased surface area and altered surface chemistry of nanoparticles make them a potential material that can effectively interact with any biological systems. Silver nanoparticles have shown significant improvement in cocoon shell and pupal weight after being treated along with mulberry leaves (Prabu et al., 2011).

Among many nanoparticles, Graphene is a two-dimensional (2D) material made up of a thin single layer of carbon atoms bonded together in a repeating flat honeycomb pattern of hexagons. Graphene is widely regarded as one of the strongest materials with combining lightweight properties, often conductive and transparent in nature. Due to its extraordinary characteristic features, Graphene has a wide application in several industries such as electronics, medicine, aviation and much more. However, since Graphene is expensive and relatively difficult to synthesize, great efforts have been put forth to develop cost-effective alternatives in the form of graphene derivatives or related materials. In this context, recent developments have shown the possible modifications in Graphene through chemical reaction that can enhance the biocompatibility of Graphene. One such derivative of Graphene is graphene oxide, which is synthesized from the chemical reduction reaction. Due to the presence of oxygen atoms instead of reactive carbon atoms, graphene oxide (GO) is considered less dangerous and a better alternative to native graphene material. Further, GO has triggered considerable interest in developing a variety of new types of composites owing to its huge potential in various fields, including biomedicine (Geim & Novoselov, 2007). The reduced graphene oxide is a derivative of GO, which is devoid of attached functionality, having more potent electrical conductivity. The material exhibits superior electrical conductivity and improved biocompatibility, making it suitable for applications such as antibacterial surfaces, drug delivery and also the biological methods employed in the synthesis of nanoparticles have made a significant impact rather than the chemical synthesis methods.

On the other hand, the silk fibroin, a protein derived from cocoons, has been identified for its excellent biocompatibility and tunable degradability. In general, the *Bombyx mori* silk fibre (SF) is a semi-crystalline biopolymer with 80–85% of glycine, alanine and serine. SF has for a long time been applied in textile, biotechnological and biomedical fields due to its high strength, rupture elongation, environmental stability and biocompatible properties (Marsh et al., 1955; Vepari & Kaplan, 2007). These properties make silk fibroin one of the most widely used biomaterials in both academic and industrial fields. In general, to achieve the enhanced mechanical or functional properties of SF (Trabbic & Yager, 1998), some nano or functional materials are added to mix with the silk protein through an artificial spinning process. The use of carbon nanotube (CNT) to reinforce SF has been tried in an artificial spinning process, and the presented regenerated SF. It has been found to have enhanced mechanical and electrical properties (Asakura et al., 2010; Terry et al., 2004).

Graphene, known for its superior mechanical properties, is widely applied as reinforcement in preparing high-performance materials (Kim et al., 2014). Graphene, considered as a "miracle material" has shown massive potential in energy, electronics, medicine and more. Silkworms, the larvae of silk moths, spin the silk threads made of sericin and fibroin, which are produced in their silk glands. The carbon-enhanced silk was twice as tough as regular silk and could withstand at least 50% higher stress before breaking and this silk filament has the ability to withstand when it is heated up to 1050°C (Wang et al., 2016). When silkworms are fed a graphene-supplemented diet, they can synthesize high-quality silk with several beneficial properties that can increase its applications in the production of durable protective fabrics, biodegradable medical implants and eco-friendly wearable electronics (Wang et al., 2016). Nanoparticle supplementation through diet has also been shown to improve uptake efficiency in livestock, which may directly correlate with enhanced productivity. On this rationale, the present research has been initiated to explore the effect of Graphene and rGO supplemented mulberry leaf diet on the properties of cocoon, silk and also substantially the in-vivo reinforcement to enhance silk mechanical properties.

MATERIALS AND METHODS

Materials:

Experimental animal

Disease free layings of crossbreed PM × CSR2 were procured from the National Silkworm Seed Organization (NSSO), Mysuru.

Nanoparticles

The Graphene was procured from the United Nanotech Innovation Pvt. Ltd., Bangalore. The reduced graphene oxide (rGO) is a derivative of Graphene oxide (GO), which was extracted from leaves of *Justicia wynaadensis* and the Centre for Material Science and Technology, Vigyan Bhavan, University of Mysore, Mysuru provided it.

Methods:

Maintenance of silkworm and experimental methods

Disease free layings were incubated at 25°C temperature with relative humidity of 80-85% in the rearing house. Upon hatching, larvae were brushed along with chopped fresh mulberry leaves. After 3rd moult, 100 larvae in each replication and three replications for each treatment were maintained and reared. After larvae attained maturity, they were mounted on bamboo mountages for spinning separately. Appropriate care was taken during rearing and mounting the worms for spinning as per the standard rearing protocol (Dandin & Giridhar, 2014).

Preparation of graphene oxide

Graphene oxide (GO) was prepared by modified Hummer's method (Kopelevich et al., 2007). 5 g of graphite was mixed with 2.5 g of sodium nitrate and 115 ml of cold sulphuric acid at a temperature below 20℃, and maintained the same temperature to control the exothermic reaction upon adding Potassium permanganate (KMnO4). KMnO4 was added slowly to the reaction mixture and magnetically stirred at 35℃ for 7 hours, and the reaction temperature was reduced using an ice bath whilst adding water to terminate the reaction. 25 ml of 30% H_2O_2 was added, and there was a visual change in colour to yellowish brown. Dilute Hydrochloric acid was added to remove metal impurities, and the solution was washed repeatedly to neutral pH and further subjected to vacuum evaporation, dried and stored in an air-tight container.

Preparation of reduced graphene oxide (rGO) using *Justicia wynaadensis* extract

The reflux method was used to reduce graphene oxide using *J. wynaadensis* extract (Cong et al., 2010). 250 mg of rGO was dispersed in 500 ml of water and sonicated for 30 min to obtain a dispersed Graphene Oxide solution. 0.5 g of dried *J. wynaadensis* was mixed in 5 ml of ultrapure water by vortexing, and the Graphene Oxide dispersion mixed with an aqueous solution of *J. wynaadensis* was refluxed at 100℃ for 10 hours.

Application of Graphene and reduced graphene oxide to mulberry leaves and feeding to silkworm larvae

Graphene and rGO of 0.1% and 0.5% were prepared on the ventral surface of the mulberry leaf and dried in the shade. Silkworm larvae at their 4^{th} and 5^{th} instar were fed with these leaves once a day in the evening. The control batch T0 larvae were fed with normal leaves, and batch T1 larvae were fed with distilled water-smeared leaves. The batch T2 larvae were fed with 0.1% graphene and rGO smeared leaves, and batch T3 larvae were fed with 0.5% graphene and rGO smeared leaves. In each treatment, three replications were maintained.

Analysis of Biological and Commercial Traits

The impact of Graphene and rGO was measured by determining the larval mortality rate of the fifth instar. Furthermore, the larvae that survived and successfully spun the cocoons were taken into account to measure their health status (Hussain et al., 2011). For this purpose, approximately 6 larvae, cocoon and pupae were randomly selected and their average weights were recorded. The post cocoon characters like filament length, filament weight, denier and renditta were also calculated using the standard formula (Ganga & Chetty, 1991). All data derived from three replications were statistically analyzed through SPSS.

Protein estimation by Lowry's (1951) method

Quantitative estimation of protein in the samples derived from Graphene and rGO nanomaterial fed and control larvae of crossbreed PM × CSR2 were estimated using a Biophotometer (Eppendorf). 10 µl of collected protein samples (haemolymph) was taken from each treatment separately into a test tube, and the volume was made up to 1ml by adding 990 µl of distilled water. The optical density (OD) was taken at 660 nm using a biophotometer (Lowry et al., 1951).

X-Ray Diffraction (XRD) analysis of nanomaterial reduced graphene oxide

X-ray diffraction analysis is a unique characterisation technique to know the crystallinity of a material and measure the average spacing between layers of atoms. XRD is primarily used for the characterization of nanomaterials. The analysis was performed using Rigaku SmartLab-II, CuKα radiation at the Centre of Materials Science and Technology, Mysuru.

Dynamic Light Scattering (DLS) technology

Particle size analysis of selected nanomaterials (Graphene and rGO) was determined using the Microtrac Zeta analyzer. About 10 mg of the sample (Graphene and rGO) was taken in a clean and dry Eppendorf tube to which 2 ml of ultrapure water was added, and was sonicated for about 30 min using an ultrasonicator to obtain a complete dispersion of the nanomaterial. This material was used for DLS analysis (particle size determination), considering ultrapure water as a reference. The analysis was performed at the Centre of Materials Science and Technology, Mysuru.

Data analysis

All the data derived from three replications of each group were subjected to the statistical analysis with ANOVA using SPSS software Version 20.

RESULT AND DISCUSSION

X-Ray Diffraction (XRD) analysis of Graphene and reduced graphene oxide nanomaterial.

Powder XRD is a characterization technique to analyze the crystalline structure of a material that measures the average spacing between layers of atoms. Powder XRD pattern of Graphene with a sharp diffraction peak at $2\theta = 26.3^{o}$ (with 002 diffraction plane) indicates the presence of crystalline segments present in pure graphene sheets (Figure 1). The XRD analysis of GO revealed a sharp diffraction peak (2θ) at 10.69^{o} with an interlayer distance of 0.826 nm (Figure 2). This indicates the presence of highly crystalline and ordered atomic layers present in the GO freshly synthesized. Whereas the rGO showed a broad peak (2θ) at 25^{o} (Figure 3). This suggests the presence of close d-spacing in rGO, and the disappearance of the diffraction peak (2θ) at 9.75° further indicates that the oxygen-containing group of GO has been efficiently removed during the reduction process.

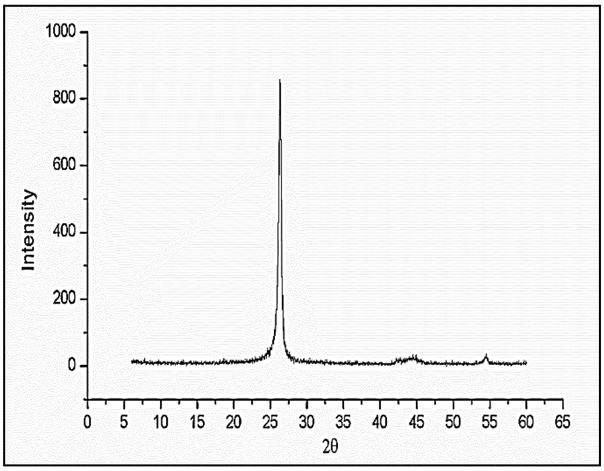


Figure 1. Powder XRD pattern of commercial graphene nanomaterial

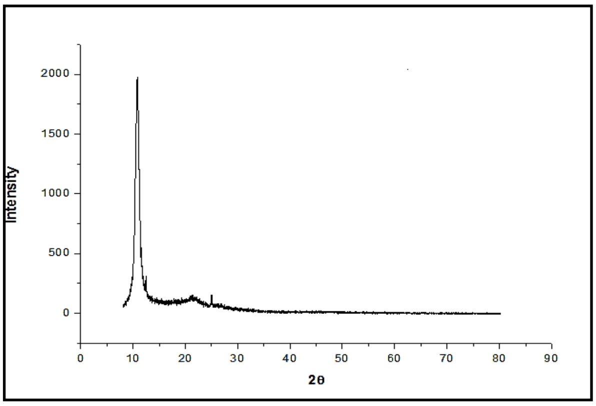


Figure 2. XRD pattern of synthesized GO with sharp diffraction peak at 2θ=10.69^0

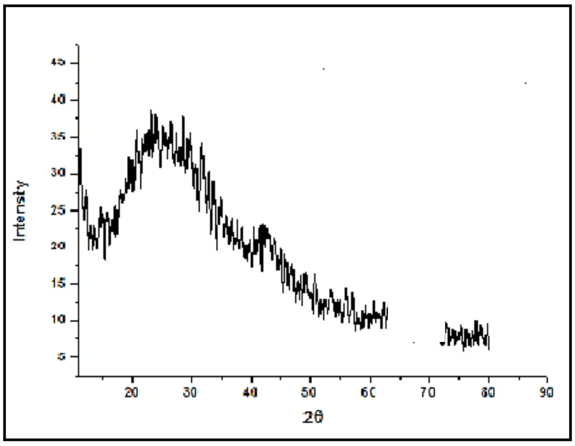
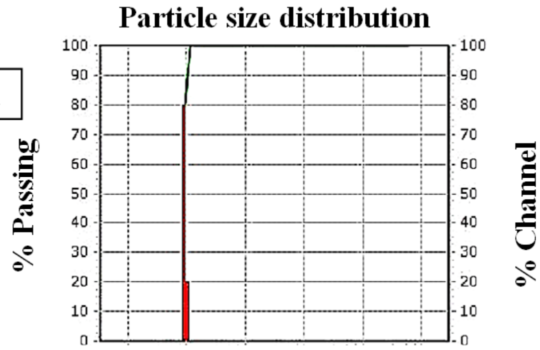


Figure 3. XRD pattern of synthesized rGO with sharp diffraction peak at 2θ=25^o

Particle size analysis of graphene nanomaterial

The Size of the graphene nano material was characterized using the Dynamic light scattering (DLS) technique through the principle of the light scattering effect. The DLS analysis of Graphene showed a single peak that ranges between approximately 1 nm. This monomodal distribution indicates the homogenous nature of uniformly distributed similar sized particles (Figure 4A & 4B). Further the average size of the particles present in the suspension was found to be 1.1 nm. This analysis confirmed the uniform distribution of graphene particles of the same size.

4A



Diffraction angle 2θ Size (nanometer)

4B

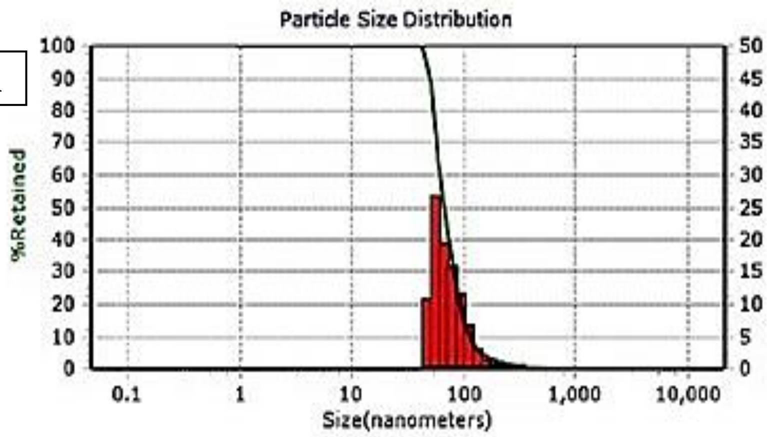
Peaks Summary		
Diameter (nm)	Volume (%)	Width (nm)
0.98	100	1.1

Figure 4A. Particle size analysis of the Graphene as measured by the DLS technique
Figure 4B. The overall summary of the analysis, indicating the size of the particles, is shown in the form of table

Particle size analysis of rGO nanomaterial

The particle size of the rGO analyzed using the DLS technique. Based on the size of the particle, the intensity of the scattered light varies significantly. rGO showed a single peak that ranges between approximately 50-100 nm. This monomodal distribution indicates the homogenous nature of uniformly distributed, similarly sized particles (Figure 5A & 5B). Furthermore, the average size of the particles present in the suspension was found to be 48.9nm. This analysis confirmed the presence of uniformly distributed, same-sized rGO particles that were taken for our experimental studies.

5A



5B

Peaks Summary		
Diameter (nm)	Volume (%)	Width (nm)
77	100	48.9

Figure 5A. Analysis of reduced graphene oxide particle size by the DLS technique
Figure 5B. The overall summary of the analysis indicating the size of the particles

Toxicity Assessment

Larval growth

The growth rate of IV instar silkworm larvae was studied under the influence of Graphene and rGO nanomaterial administration. Slight changes in the larval growth were noticed during 1st day of IV instar in treated batches like graphene, rGO and control batches. The highest weight, 0.13g, was recorded in both the concentration of Graphene (0.1 and 0.5%) and rGO (0.1 and 0.5%) treated batches. Meanwhile, in the control batches, the larvae's weight is about 0.11g. The weight of IV instar 3rd day larvae of 0.1 and 0.5% Graphene treated batches was 0.60 and 0.57g, respectively. Whereas 0.60 and 0.50g weights were recorded in 0.1 and 0.5% of the rGO-treated batches. The mean larval weight of control and distilled water-treated batches was 0.60 and 0.63g, respectively.

The influences of Graphene and rGO on the fifth instar larvae of crossbreed PM × CSR2 were measured on the 3rd and 6th day. The mean weight of the larvae was found to be decreased in the Graphene-supplemented batches on the 3rd day of treatment. The larval weight recorded to 1.96 and 1.75g when treated with 0.1 and 0.5% concentration. Whereas, a decrease in the rGO treatment on the 3rd day has resulted in an increase in larval weight, which was recorded to 1.72 and 1.76g in 0.1 and 0.5% concentration treated batches. The highest weight of 2.0g was recorded in the control groups of V instar larvae on the third day of treatment. Whereas on the 6th day, the actual effects were seen through increased larval weight of 4.07 and 3.91g were recorded in the silkworm batches treated with 0.1 and 0.5% graphene supplementation and almost similar weights were recorded in the larval batches treated with 0.1% (4.05g) and 0.5% (3.91g) of rGO. The larval weight was recorded to be 3.88g and 3.84g in the control and distilled water-treated batches, respectively. There was no adverse effect induced by graphene and rGO supplementation in the silkworm larvae, which was evident from the morphological appearance of the silkworms in all the treated groups.

Table 1: Effect of Graphene and rGO nanomaterial on the larval growth, of the silkworm *B. mori* cross breed PM × CSR2

Treatments		Larval weight (g)			
		IV instar		V instar	
		1st Day	3rd Day	3rd Day	6th Day
Control		0.113±0.11	0.60 ±0.02	2.0 ±0.09	3.88 ±0.19
D. Water		0.115±0.02 (0.00)	0.63±0.40 (+4.44)	1.72 ±0.14 (-16.64)	3.84 ±0.14 (-1.12)
Graphene	0.1%	0.127±0.006 (12.09)	0.600±0.030 (0.00)	1.963±0.074 (-4.85)	4.073±0.162 (4.89)
	0.5%	0.133±0.012 (17.99)	0.573±0.045 (-4.44)	1.747±0.075 (-15.35)	3.910±0.020 (+0.69)
rGO	0.1%	0.137 ±0.00 (+20.94)	0.60 ±0.04 0.00)	1.72 ±0.06 (-16.48)	4.050 ±0.11 (+4.21)
	0.5%	0.13 0±0.03 (+15.04)	0.5 ±0.02 (-15.56)	1.76 ±0.05 (-14.70)	3.910±0.74 (+0.60)

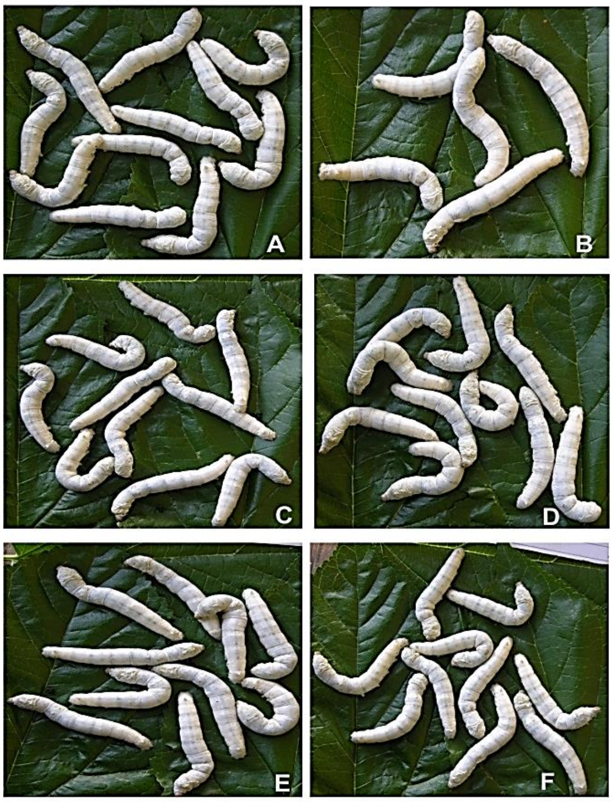


Figure 6. Healthy silkworms treated with Graphene and rGO nanomaterial orally
A - Control; B-Distilled water; C- Graphene 0.1%; D- Graphene 0.5%; E- rGO 0.1%; F - rGO 0.5%

Effect of Graphene and rGO nanomaterial on the cocoon and silk traits of the silkworm *B. mori* cross breed PM × CSR2.

Cocoon weight

The influence of Graphene and rGO on cocoon weight was analyzed by measuring the weight of the cocoon in all the groups. The weight of the cocoon spun by PM × CSR2 silkworm larvae was found to be significantly increased when compared to control batches due to the influence of Graphene and rGO nanomaterial at different concentrations (0.1 and 0.5%). The highest cocoon weight of 2.18g was recorded in 0.1% Graphene-treated batches, followed by 2.08g in the 0.1% rGO-supplemented mulberry leaf diet. The batch with 0.5% treatment of Graphene and rGO was reduced to 1.96 and 1.98g, respectively. Whereas the control and distilled water-fed batches showed no change in their cocoon weight (1.96 g). (Figure 7, Table 2).

Shell Weight

The shell weight determines the effect of treated carbon nanomaterials on silkworm biosynthesis capacity. The influence of Graphene and rGO over the shell weight was analyzed by measuring the weight of the cocoon shell from all treated groups. The average weight of the shell fed with 0.1% of Graphene and rGO supplemented mulberry leaf diet was found to be 0.40 and 0.34g, respectively. Whereas the average shell weight in 0.5% treated batches was observed to be 0.37 and 0.33g. The weight of the shell from the control and distilled water-fed batches was shown to be 0.33 g, respectively (Table 2).

Shell percentage

Shell percentage indicates the total improvement of quantitative silk in the investigated crossbreed PM × CSR2 cocoons. The cocoon shell ratio of 18.21 and 18.36% was recorded in 0.1 and 0.5% graphene nanomaterial treated batches, which are the highest among the treated batches. The Shell ratio of 15.92 and 16.69% was recorded in rGO-treated batches of concentrations 0.1% and 0.5% respectively. Here, the rGO-treated batches showed the lowest shell percentage among the treated groups. Whereas the shell percentage of control and distilled water fed batches were shown to be 16.85 and 16.72% respectively (Table 2).

Pupal weight

The pupal weight often reflects the quality of food intake by the silkworm larvae. The presence of toxicity or residual toxicity in the supplements could directly affect the larval and pupal metamorphosis. The pupal weight of 1.62g and 1.63g was recorded in the control and distilled water batch. However, there was an improvement noticed in the Graphene treated batch i.e. 1.77g & 1.66g with respect to 0.1 & 0.5% concentrations. Whereas in rGO treated batches, the weight of the pupa obtained i.e. 1.74g (0.1%), followed by 1.64g (0.5%), respectively (Table 2).

Filament length

The filament length was potentially increased in the Graphene treated batches. Even at a concentration of 0.1%, the average length of the silk filament was recorded as 1001 m, while in the 0.5% treated batches, the filament length was 932m. A reduction in the length of the silk filament was observed in those batches which were treated with a higher concentration of Graphene. Whereas in rGO-treated batches, the length of the silk filament was found to be 981m and 930m, respectively. Interestingly, although the shell percentage was lower in the rGO-treated batches, the non-breakable filament length increased compared to the control (902m) and distilled water-treated batches (877m) (Table 2).

Filament weight

The weight of the silk filament influenced by the nanomaterials was measured after the raw silk was obtained. It was recorded as 0.31 & 0.29 g in the graphene-treated batch. The weight of 0.303 & 0.288g was recorded in the rGO-treated batch, which shows the improvement over the control (0.251g) and the distilled water (0.288g) treated batch. Notably, the graphene-treated batch exhibited an enhanced filament weight (Table 2).

Denier

Denier is a unit of measurement of the fineness of the silk thread. The denier of the silk filament influenced by the graphene nanomaterial was recorded as 2.80 and 2.79 D in the 0.1 % and 0.5 % concentration-treated batches, respectively. Interestingly, the denier in the rGO-treated batches was 2.78 and 2.79 D, respectively. In contrast, the average denier from the larvae in the control and distilled water-fed batches was 2.57 and 2.67 D, respectively. A similar trend of results was observed among the batches.

Table 2: Effect of Graphene and rGO nanomaterial on the cocoon and silk traits of the silkworm *B. mori* cross breed PM × CSR2.

Treatments		Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell %	Filament length(mt)	Filament weight(g)	Diener (D)	Renditta (g)
Control		1.96±0.114	1.62±0.103	0.33±0.022	16.85±1.21	877±5.317	0.251±0.005	2.57±0.038	8±0.291
D. Water		1.97±0.046 (0.34)	1.63±0.037 (0.58)	0.33±0.007 (-0.40)	16.72±0.161 (-0.80)	902±13.516 (+2.87)	0.268±0.014 (+6.91)	2.67±0.104 (+3.90)	7±0.520 (-5.92)
Graphene	0.1%	2.18±0.09 (+11.05)	1.77±0.072 (+9.23)	0.40±0.023 (+21.3)	18.36±0.565 (+8.93)	1001±4.500 (+14.20)	0.311±0.006 (+23.94)	2.792±0.043 (+8.53)	7±0.208 (-10.29)
	0.5%	2.05±0.04 (+4.76)	1.66±0.028 (+2.32)	0.37±0.003 (+13.45)	18.21±0.357 (+8.04)	932±8.592 (+6.29)	0.290±0.004 (+15.69)	2.80±0.014 (+8.85)	7±0.226 (-9.26)
rGO	0.1%	2.08±0.01 (+6.34)	1.74±0.016 (+7.38)	0.34±0.004 (+3.24)	15.92±0.679 (-5.56)	981±5.953 (+11.89)	0.303±0.002 (+21.01)	2.78±0.006 (+8.16)	7±0.041 (-12.00)
	0.5%	1.98±0.01 (+1.09)	1.64±0.003 (1.38)	0.33±0.009 (+0.30)	16.69±0.368 (-0.98)	930±6.874 (+6.07)	0.288±0.002 (+14.89)	2.79±0.004 (+8.32)	7±0.038 (-11.90)

Figure7. Healthy silkworms treated with Graphene and rGO nanomaterial orally (A-control; B-Distilled water; C- Graphene 0.1%; D- Graphene 0.5%; E- rGO 0.1%; F - rGO0.5%)

Quantitative changes

The total protein content in the haemolymph of 5th instar 5th day larvae was influenced by the graphene nanomaterial in treated batches. The protein content in haemolymph recorded was 283.33 and 225.00 mg/ml. The protein content in the 0.1% graphene-treated batch (283.33 mg/ml) showed an 18.88% improvement compared to the control batch (238.33 mg/ml), indicating the positive effectiveness of graphene material. However, the total protein content in the haemolymph of silkworm larvae (PM × CSR2) was not significantly altered by the rGO nanomaterial-supplemented mulberry leaf diet. The total protein content was found to be highest in the Graphene 0.1% batch, followed by control (238.33 mg/ml), rGO 0.5% (230.55 mg/ml), Graphene 0.5% (225.00 mg/ml), rGO 0.1% (202.78 mg/ml) and distilled water 152.78 mg/ml respectively.

Table 3: Effect of Graphene and rGO nanomaterial on protein concentration (mg/ml) of the silkworm *B. mori* haemolymph of cross breed PM × CSR2.

Treatments		Protein Concentration (mg/ml)
Control		238.33±9.61
D. Water		152.78±4.81 (-35.90)
Graphene	0.1%	283.33±14.43 (18.88)
	0.5%	225.00±36.32 (-5.59)
rGO	0.1%	202.78±4.81 (-14.92)
	0.5%	230.55±4.81 (-3.26)

Tensile strength

In order to investigate the mechanical properties of the obtained silk fibres with different treated batches against the control batch, a test was conducted using a Universal Testing Machine (UTM) at the Department of Polymer Science and Technology, Sri Jayachamarajendra College of Engineering, Mysuru.

The tensile strength is the maximum load that a material can support without fracture when being stretched, divided by/original cross-sectional area of the material. Here, the tensile strength of the silk fibres that were derived from silkworm cocoons fed with Graphene of different concentrations (0.1% and 0.5 %) was recorded as 0.127 and 0.133 GPa. Whereas, the rGO-treated batch showed 0.137 & 0.13 GPa with respect to 0.1 & 0.5% concentrations, respectively. Incorporation of the Graphene and rGO into the larvae significantly strengthened the silk fibre compared to the control (0.113 GPa) and the distilled water (1.127 GPa) treated batch, respectively (Table 4).

Table 4: Effect of Graphene and rGO nanomaterial on the silk tensile strength of the silkworm *B. mori* cross breed PM × CSR2.

Treatments		Tensile strength (GPa)	Elongation at peak load (m)	Yield strength (Gpa)	Elongation at break load (m)
Control		0.113±0.11	0.60 ±0.02	2.0 ±0.09	3.88 ±0.19
Graphene	0.1%	0.127±0.006 (12.09)	0.600±0.030 (0.00)	1.963±0.074 (-4.85)	4.073±0.162 (4.89)
	0.5%	0.133±0.012 (17.99)	0.573±0.045 (-4.44)	1.747±0.075 (-15.35)	3.910±0.020 (+0.69)
rGO	0.1%	0.137 ±0.00 (+20.94)	0.600 ±0.04 0.00)	1.720 ±0.06 (-16.48)	4.05 ±0.11 (+4.21)
	0.5%	0.13 0±0.03 (+15.04)	0.500 ±0.02 (-15.56)	1.760 ±0.05 (-14.70)	3.91 ±0.74 (+0.60)

In the present study, we used graphene and rGO nanomaterial in the silkworm to find out the possibilities to improve the commercial characteristics as well as the enhancement of silk properties. On the other hand we analyzed the effect of commercially available Graphene and biologically synthesized rGO using the silkworm.

The results from our XRD analysis show a crystalline peak for Graphene at a diffraction angle 2θ at 26.3 degrees (Figure 1), which indicates the purest form of Graphene was used for our experimental analysis. Similarly, the physical characterization of Graphene using particle size analysis shows a single characteristic peak that represents a monomodal distribution of ≈1nm graphene particles (Figure 4). Thus, the physico-chemical characterization of the graphene particles used in our experiment was successfully validated and employed further for our biological analysis. Crystalline peak for GO at a diffraction angle 2θ at 2θ=10.69^{o} degrees (Figure 2) as confirmed with the previous reports (Marcano et al., 2010). Whereas the crystalline peak for rGO at a diffraction angle 2θ at 2θ=25° (Figure 3), in contrast to GO, is probably due to restacking of graphene layers by removing the oxygen-containing group of GO (Gao et al., 2009). This indicates that the purest form of rGO used in the present study to examine its impact on the growth and cocoon parameters silkworm *Bombyx mori*. Further, the physical characterization of rGO using particle size analysis shows a single characteristic peak that represents a monomodal distribution of ~49 nm rGO particles (Figure 5). Thus, the physico-chemical characterization of the rGO particles used in our experiment was successfully validated and employed for further biological analysis.

Other than its economic importance, the silkworm is a burgeoning model animal in most scientific research areas. Among many fields, nanoscience researchers are also extensively using silkworms for their many scientific innovations. The method of feeding and rearing silkworm is a new method for the development of in-situ enhanced silk. Nanomaterials possess unique properties, such as surface effect, small size effect and quantum confinement effect, etc., which result in their distinct optical, electric, magnetic, thermal and mechanical properties (Ramalingam et al., 2020). With the development of science and technology, nanomaterials are becoming more and more popular. Among these, there is also a lot of scope to improve silkworm cocoon yield through the nanoparticle feeding method, and also the silkworm serves as a model animal to test nanoparticle toxicity (Pandiarajan & Krishnan, 2017).

The change in larval morphometric properties due to Graphene and rGO-smeared mulberry leaf diets was analyzed during the 4th instar stage of their life cycle. There was no characteristic change in larval weight in all the four experimental groups (Control, Distilled water, Graphene and rGO at 0.1% and 0.5%). The scenario of larval weight improvement was dependent on their treated concentrations, and silkworms performed well in the lower concentration (0.1%) treated batches than in higher concentration (0.5%) treated batches. However, there was a significant difference observed between the 1st, 2nd, 3rd, and 4th day of the 4th and 5th instar stages, respectively. These findings clearly explain how the silkworm is useful as a model animal to test the toxicity of nanoparticles. The digital images of the larvae before spinning (5th instar) showed no changes in their characteristic appearance, even after being fed with 0.1 and 0.5% graphene and rGO-smeared mulberry leaf diets (Figure 6).

The cocoons from 0.1 and 0.5% Graphene treated silkworm larvae showed increased cocoon weight, shell weight, shell percentage, filament length and denier followed by decreased renditta compared to either rGO/ control treated batches. In the Graphene-treated batches, in 0.1% concentration was observed 11.05% improvement in the cocoon weight over the control. In the same concentration of the rGO-treated batch, 6.34% of improvement was noticed. Interestingly, the improvement in shell percentage was 8.93 % and 8.04 % in the 0.1 % and 0.5 % of graphene-treated batches, respectively, whereas in the rGO-treated batches, the shell percentage declined by -5.56 % and -0.98% in 0.1 and 0.5% concentrations. Two main observations were recorded in the present investigation, i.e. Graphene steadily improve the silkworm larval weight, cocoon weight, shell weight and filament length compare to control. But in rGO treated batches, though the shell percent was lower than control but the non-breakable filament length increased significantly with the improvement of 11.89 and 6.07% in 0.1 and 0.5% treated batches. This evidence clearly explains the rGO nanoparticle improves the non-breakable capacity of silk filament. More than its larval weights, graphene quantum dots improve mechanical property of silk when it was injected or fed with mulberry leaves, because it can easily entered into silk by the metabolism of silkworm (Ma *et al*., 2019). An in-situ biomineralization strategy achieved superior mechanical properties in silk fibers, strengthening them compared to normal silk fibers. Our investigation results may also strongly admired, the Graphene and rGO helps to improve the mechanical properties of silk through in situ administration strategies (Guo et al., 2018)

Denier obtained from the cocoon fed with 0.1 & 0.5 % Graphene smeared mulberry leaf diet varied slightly among the batches, followed by 0.1 &0.5% rGO treated batches and the distilled water treated batch. Whereas, there was a significant difference observed against the control batch. This might upshot the effect of carbon nanomaterial (Graphene & rGO) even in the denier character. The observed results were in correlation with renditta, which confirms the improvements of silk filament length with respect to silk filament weight. There was a significant improvement noticed in carbon nanomaterial-fed batches compared to the control batch. Moreover, carbon nanomaterial (Graphene & rGO) with a lower concentration (0.1%) treated batches showed notable improvement over the control batch. The obtained results of the cocoon properties showed the significant improvements in treated batches over the control batch, imparting the positive effect/impact of the carbon nanomaterial (Graphene and rGO).

The change in total protein content present in the haemolymph tissue isolated from the 5th instar 5th day larvae was analyzed through Lowry's (1951) method. The quantitative analysis showed an overall increase in the total protein content present in haemolymph tissue collected from 0.1% Graphene-fed silkworm batches when compared with control, distilled water, and rGO-fed silkworm larvae batches. The quantitative analysis showed a decrease in the total protein content present in haemolymph tissue collected from rGO-fed silkworm batches when compared with the control. Utilization of nanotechnology in sericulture not only improves the survival rate, growth, and development of the silkworm but also the quality of the fibre. This also improves the biological effect by exhibiting the therapeutic properties without toxicity (Fometu et al., 2021). Similarly, silkworm larvae fed with carbon nanomaterial (Graphene & rGO) also improve the total protein content, which might also help in the biomedical field.

In this work, a simple process was developed for obtaining high-strength silk directly from silkworms by feeding the worms with Graphene and rGO. The present investigation resulted in the finding that there is no toxicity of these materials, and also, the mechanical properties were enhanced in lower concentrations (0.1%) of carbon nanomaterial (Graphene % rGO) treated batches.

The effect of Graphene and rGO in terms of mechanical strength to the silk fibre was determined through a tensile strength test. Here, the strength of the fibre increased considerably in carbon nanomaterial (Graphene & rGO) treated batches when compared to the control and distilled treated batches. As carbon nanomaterials have been a major focus of scientific research, feeding or injecting these materials into silkworm larvae through mulberry leaves enhances the mechanical strength of the silk fibre or biosynthetic material, potentially leading to significant advances in future scientific applications (Kelly et al., 2020; Ma et al., 2019).

CONCLUSION

The result of the present investigation revealed that inorganic 2-dimensional carbon nanomaterials, Graphene and reduced graphene oxide, affect the biological and commercial traits of the silkworm *Bombyx mori* (PM × CSR2) in a dose-dependent manner. Interestingly, of the two different concentrations (0.1% and 0.5%), 0.1% showed as an optimum concentration that has potential for enhancing the cocoon and silk filament properties significantly compared with 0.5%. More importantly, it is evident from the present study that no traceable toxic effect in the silkworm *B. mori* due to carbon-based (Graphene and reduced graphene oxide) mulberry diet was observed. This clearly indicates that the beneficial properties of carbon nanomaterials shall be availed by incorporation with the mulberry leaf diet. An increase in the total protein content of haemolymph tissue may also increase the total silk protein synthesis due to a 0.1% concentration diet. Therefore, Graphene and reduced graphene oxide nanomaterials can be supplemented through mulberry leaves to enhance the cocoon and silk properties.

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