EARLIER DENATURATION OF DNA BY USING NOVEL TERNARY HYBRID NANOPARTICLES

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(Received: 10th July 2021; Accepted: 1st November 2021; Published on-line: 4th July 2022)

ABSTRACT: Two novel ternary hybrid nanoparticles (THNp) consisting of graphene oxide (GO) and reduced graphene oxides (rGO) were added to samples of DNA. The effect of the addition of nanoparticles on the thermal denaturation of DNA samples was studied by measuring the absorbance using a temperature-controlled Perkin Elmer UV spectrophotometer. Adding GO-TiO_2-Ag and rGO-TiO_2-Ag nanoparticles lowered the denaturation temperature of template DNA significantly. The nanoparticles affect the denaturation rate. The optimal GO-TiO_2-Ag and rGO-TiO_2-Ag concentrations were found to be 5×10^{-2}, which resulted in 86- and 180-folds augmentation of DNA denaturation (6.5 µg/mL), respectively, while it resulted in 2- and 7-folds augmentation of DNA denaturation (11.5 µg/mL), respectively, at temperature as low as 80 °C. The results indicated that rGO-TiO_2-Ag nanoparticles exhibited significantly higher DNA denaturation enhancement than rGO-TiO_2-Ag nanoparticles, owing to their enhanced thermal conductivity effect. Therefore, these nanoparticles could help to get improved PCR yield, hence enable amplification to be performed for longer cycles by lowering the denaturation temperatures.

ABSTRAK: Dua ternar baru nanopartikel hibrid (THNp) mengandungi oksida grapen (GO) dan oksida grapen yang dikurangkan (rGO) dan dimasukkan ke dalam sampel DNA. Kesan penambahan nanopartikel pada denaturasi termal pada sampel DNA telah dikaji dengan mengukur penyerapan menggunakan kawalan-suhu Perkin Elmer UV spektrofotometer. Penambahan GO-TiO_2-Ag dan rGO-TiO_2-Ag nanopartikel telah mengurangkan suhu denaturasi pada templat DNA dengan nyata. Nanopartikel memberi kesan pada kadar denaturasi. Kepekatan optimal GO-TiO_2-Ag dan rGO-TiO_2-Ag didapati sebanyak $5 \times 10^{-2,}$ menyebabkan penambahan sebanyak 86- dan 180-lipat pada DNA denaturasi (6.5 µg/mL), masing-masing, sementara ia menyebabkan sebanyak 2- dan 7-

lipat penambahan pada DNA denaturasi (11.5 µg/mL), masing-masing, pada suhu serendah 80 °C. Dapatan menunjukkan nanopartikel rGO-TiO_2-Ag mempunyai kenaikan penambahan DNA denaturasi nyata berbanding nanopartikel rGO-TiO_2-Ag, disebabkan kesan kekonduksian penambahan suhu. Oleh itu, nanopartikel ini dapat membantu bagi penambah baikan pengeluaran PCR, membolehkan penguatan dapat dilakukan dalam kitaran lebih lama dengan merendahkan suhu denaturasi.

KEYWORDS: *DNA denaturation; polymerase chain reaction (PCR); nano-PCR; hybrid nanoparticles*

1. INTRODUCTION

PCR is a widely used tool in molecular biotechnology to generate billions of copies of target DNA from a single templet DNA strand. This mechanism is the basis for detecting genetic mutation and disease diagnosis in various medical and OMICS applications. The PCR process involves three major stages: denaturation, annealing, and extension. These steps are performed by rapid heating and cooling of the samples to a specific temperature at the defined time. The denaturation is the first step of PCR which involves the unwinding of double-stranded DNA into two single-stranded DNA by applying heat [1,2]. From a thermodynamic perspective, the intricate arrangement and bonding of two adjacent base pairs in the DNA (A, T, G, and C) is the most critical aspect for the stability of the DNA double helix. Therefore, the energy required to denature the DNA should be equal to or greater than those bonding energies holding the base pairs. In genetics, pyrimidine/purine (YR) and A: T rich regions are less exposed to the stacking energies due to double hydrogen bonding than the G: C rich region with triple hydrogen bonds. Therefore, the TATATA sequence will melt readily once the reaction is heated to the denaturation temperature. Denaturation or melting is modifying the molecular structure of the DNA by breaking the weakening linkages of the DNA. The application of heat to the DNA sample increases the system's kinetic energy and entropy, leading to transitional and rotational movements between the DNA helix causing a collision of the atoms and molecules with one another in the DNA. These collisions reduce the strength of the hydrogen bonds, which eventually break, allowing a double-stranded DNA helix to unwind into two single strands [3].

The initial denaturation step usually occurs at 94 °C to 98 °C for each amplification cycle depending on the optimal temperature for *Taq* DNA polymerase activity and the G-C content of the template DNA used in the reaction. The denaturation temperature in a PCR assay is usually set at 95 °C, regardless of the characteristics of the DNA template. Therefore, the denaturation temperature may only vary the duration of the denaturation step instead of the temperature. Hence, *Taq* DNA polymerase gradually inactivates under these conditions, and its half-life will be reduced from 130 min at 92.5 °C to 40 min 95 °C [4]. As the amplified product serves as a template in subsequent cycles, the Taq DNA polymerase activity might be limited later. Moreover, some templates of double-stranded DNA wind during a typical denaturation stage, while others will not unwind easily (e.g., DNA templates of mammalian promoter GC-rich sequences are complicated to denature initially). Increasing the temperature of the denaturation step by more than 95 °C can assist in enhancing denaturation, which may lead to better yield. However, biomolecules in PCR reactions are stressed when the denaturation temperature is excessively high. Polymerases' half-times will decrease by a factor of 3–9 between 95 °C and 100 °C, depending on enzyme type [5]. Hence, DNA polymerase activity could be improved by the judicious use of lower denaturation temperatures.

Nanomaterial-assisted PCR technology has emerged to solve this problem by incorporating nanomaterials with excellent thermal conductivity into a PCR reaction [6]. This leads to enhanced DNA denaturation and ultimately improves the PCR yield, as many researchers have studied in the past two decades [7]. In addition, some studies have proven the theory that by using nanoparticles as an additive, the DNA denaturation process starts eventually at temperatures due to the excellent heat transfer property of the nanoparticles used in the PCR reaction [8]. For instance, graphene nanoflakes helped better heat dissipation, leading to enhanced DNA denaturation during the first PCR step [9]. Moreover, hexagonal boron nitride nanoparticles enhanced Acanthamoeba DNA yield at a lower denaturation temperature of 91.5 °C [10].

Two novel ternary hybrid nanoparticles (THNp) consisting of graphene oxide (GO) and reduced graphene oxides (rGO) were synthesized and characterized in a recent study [11]. They were coated with two other nanoparticles, silver (Ag) and titanium dioxide (TiO_2). The potential use of GO-TiO_2-Ag and rGO-TiO_2-Ag THNps as PCR enhancer additives is primarily discussed in this study. The primary target is the first step in the PCR reaction (DNA denaturation step). The use of GO-TiO_2-Ag and rGO-TiO_2-Ag nanoparticles is expected to lower the denaturation temperature template DNA; this could help to get improved PCR yield of product, hence enables amplification to be performed for longer cycles. THNp of five different concentrations to two different concentrations of DNA were used. The samples are investigated in a temperature-controlled spectrophotometer to check the absorbance of DNA with THNp at different concentrations.

2. MATERIALS AND METHODOLOGY

2.1 Synthesis of THNp

The hydrothermal method was used to synthesize THNps as described in our previous study [11]. Graphene oxide (GO) was dispersed in deionized water at 1 mg/mL concentration using an ultrasonic stirring treatment for about two hours. A 10 mL by volume of Titanium isopropoxide was mixed with 10 mL of isopropyl alcohol, and then the solution was added dropwise to 50 mL of GO suspension. Next, 10 mL of 0.2 M $AgNO_3$ was added dropwise to the solution. The solution was stirred for two hours to ensure complete mixing and homogeneity. The pH of the solution was adjusted to 1.1, and then it was heated at 160 °C for 24 hours using a stainless steel autoclave lined with Teflon. The product was washed with ethanol and then with water to remove all the remnants and unreacted ions and finally filtered. The resultant residue (GO-TiO_2-Ag nanocomposites) was dried at 80 °C. rGO-TiO_2-Ag THNps was synthesized with the same procedure; however, ammonia and hydrazine were added to GO suspension to remove oxygen molecules from GO sheets and their functional groups. The characterization of THNp is described in our previous study [11].

2.2 Preparation of THNp-based Nanofluids

GO-TiO_2-Ag THNp were weighted using Sartorius Entris® balance and then dispersed in molecular biology-grade sterile/DI water to make the final stock solution concentration of 5 x 10^{-2} wt % (Sample A). Next, the THNps were dispersed in DDH_2O and then sonicated using ultrasound probe sonication for 2 min, followed by water bath sonication for about 4 hours to obtain a homogenous solution of nanofluids without sedimentation. The stock solution was then serially diluted for about four more concentrations, named Concentration B($5x10^{-3}$)wt%, C($5x\ 0^{-4}$)wt%, D($5x10^{-5}$)wt%, and E($5x10^{-6}$)wt%. rGO-TiO_2-Ag nanofluid was prepared in the same way.

2.3 DNA Isolation

A salmon fish tissue sample was used as a template in this study. Genomic DNA was extracted and purified using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Template concentration was ascertained using Thermo Qubit 3.0 Fluorometer, and it was found to be 11.5 ng/µL and 6.5 ng/µL (A and B resp.) and stored at 4 °C for further use.

2.4 Evaluation of DNA Denaturation by UV Spectroscopy

Perkin Elmer UV spectrophotometer was used to measure the absorbance of samples at 200-400 nm. The thermal denaturation experiments were carried out using two DNA concentrations, named A (6.5 ng/µL) and B (11.5 ng/µL) in the presence and absence of the synthesized graphene-based ternary hybrid nanofluids (GO-TiO_2-Ag and rGO-TiO_2-Ag) to determine the effect of nanoparticles on the early DNA denaturation. Five levels of concentrations, A($5x10^{-2}$)wt%, B($5x10^{-3}$)wt%, C($5x10^{-4}$)wt%, D($5x10^{-5}$)wt% and E($5x10^{-6}$)wt% were tested. The measurements were performed by mixing DNA samples with the nanofluid at the ratio of 5:2 and then filled in a clean 5 mm spectrometer quartz Cuvette cell. The absorbance was measured at various temperature ranges starting at 80 °C, with 2 °C increments, till 96 °C using the Peltier system.

3. RESULTS AND DISCUSSION

3.1 Effects of THNps on DNA Denaturation

The novel GO-TiO_2-Ag and rGO-TiO_2-Ag ternary hybrid nanoparticles exhibited a superior enhancement of PCR and led to a 28.5% reduction of total cycles and enhanced the PCR yield 16.89-folds for GO-TiO_2-Ag and 15.75-folds for rGO-TiO_2-Ag, compared to control in our earlier study [refer PCR manuscript]. In this study, the same ternary hybrid nanoparticles were added to two DNA samples (samples A and B), and the absorbance is measured using a spectrophotometer. The absorbance (at 260 nm) of two DNA samples (A:6.5 and B:11.5 ng/µL) with and without the synthesized graphene-based ternary hybrid nanofluids) at five concentration levels at various temperatures were measured as shown in Fig 1. The absorbance of the DNA in the presence of THNp can give us a deeper insight into the behavior of DNA during the denaturation step. GO-TiO_2-Ag and rGO-TiO_2-Ag THNp exhibited higher absorbance values than the control (without nanoparticles) over the measured temperature range (from 80 ºC to 96 ºC) in two different DNA samples A and B with concentration, indicating early denaturation of DNA even at temperatures as low as 80 °C.

Interestingly, the concentration of nanoparticles has an impact on the extent of DNA denaturation. A more significant denaturation was exerted with the higher concentration of these nanoparticles than the lower one at all studied temperatures (Fig. 1). When comparing the DNA samples containing the nanoparticles, rGO-TiO_2-Ag nanoparticles were significantly more effective than GO-TiO_2-Ag THNp at higher concentrations ($5x10^{-2}$) wt%. The absorbance of the DNA samples containing $5x10^{-2}$ wt% GO-TiO_2-Ag nanoparticles was ~86- and ~2-folds higher than the control DNA samples at 80°C, for DNA samples A and B, respectively, while the absorption in the presence of rGO-TiO_2-Ag nanoparticles was ~180- and ~7-folds higher than the control in DNA samples A and B, respectively. Moreover, DNA was denatured earlier in the presence of rGO-TiO_2-Ag nanoparticles at a concentration higher than C ($5x10^{-4}$)wt%. At the same time, it was still earlier denatured in the presence of GO-TiO_2-Ag even at a concentration as low as E ($5x10^{-6}$) wt%. These results

indicate the importance of an optimal nanoparticle concentration for the maximal heat transfer effect.

The earlier denaturation of the DNA in the presence of the THNps could be due to the enhanced thermal conductivity of nanoparticles. The denaturation of double-stranded DNA typically happens if the temperature exceeds 90 °C. As the kinetic energy and entropy of the reaction system increase, the transitional and rotational motions, which causes a collision of the atoms and molecules of the reagents, increase. These collisions reduce the strength of the hydrogen bonds, which eventually breaks, allowing a double-stranded DNA helix to unwind into two single strands [12]. Thus, the presence of nanoparticles enhances the DNA's denaturation due to the more excellent heat dissipation in the reaction mixture, which causes a collision of the atoms and molecules between nanoparticles, and PCR reagents [13]. Thus, these results are proof that DNA could be denatured earlier in the presence of THNps. Furthermore, the percentage of enhancement that we achieved is significantly higher than previously published reports.

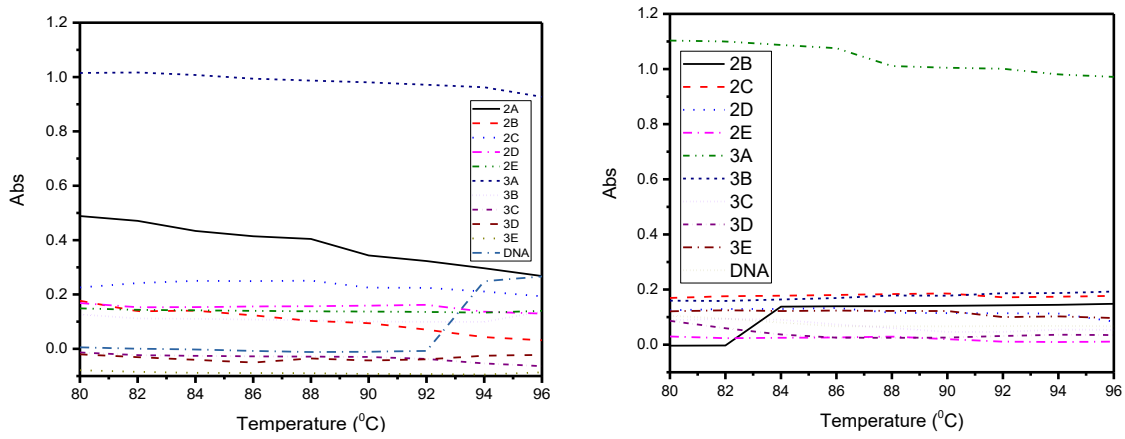

Fig. 1: Near UV absorption spectra (260 nm) of DNA in presence and absence of increasing concentration of GO-TiO_2-Ag and rGO-TiO_2-Ag ($5x10^{-2}$ - $5x10^{-6}$ wt%) to determine the effect of nanoparticles on DNA denaturation. To the left, A (6.5 ng/µL), and to the right, B (11.5 ng/µL) DNA samples.

The absorption spectra for GO-TiO_2-Ag and rGO-TiO_2-Ag over 200 to 400 nm are presented side by side based on the concentration in Figs. 2 and 3. It can be seen from Fig 2, the strong absorption below 210 nm in the DNA spectrum, at all temperatures, resulted from absorptions of phosphate groups and sugar parts. The second maximum DNA absorption position was located at 260 nm due to DNA base absorption. DNA spectra had confirmed that the DNA was denatured at temperature 92-94 °C as all spectra absorption at temperature ≤92 °C were significantly lower than that observed for spectra at temperature ≥ 94 °C. Once the absorbance of UV light in the spectrophotometer has increased until it has completely melted or un-wound to two single strands, the denaturation can be determined. The absorbance will remain constant even if the temperature or heating is further increased. The hypochromic effect refers to the fact that single-strand DNA absorbs 50 percent more UV light than double-strand DNA before reaching the melting point. Renaturation is the reversible process of denaturation, which occurs when the temperature is lowered below the melting point. The renaturation time can be used to calculate the repetitive fractions as well as the base composition [14]. The melting point, or T_m, of different DNA will vary depending on various factors such as the length of the DNA strand, base composition, topological condition of DNA, buffer composition, etc. Compared to a longer strand of DNA, a shorter DNA strand will melt faster and more efficiently [15].

Because many variables influence the melting point of DNA, it is difficult to predict the exact melting temperature of a given DNA sequence. (a)

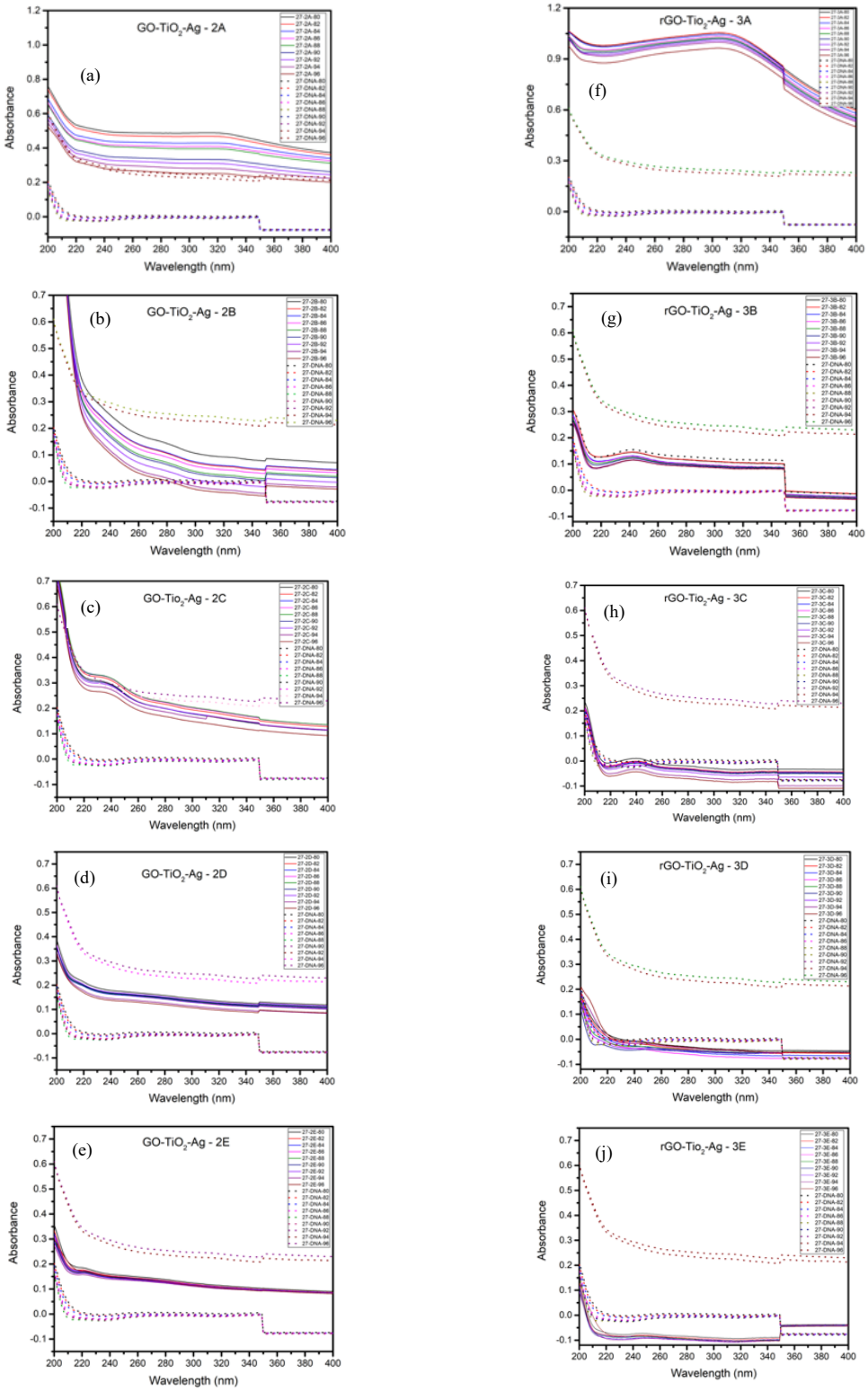

Fig. 2: (a), (b), (c), (d), and (e) are near UV absorption spectra of 6.5 ng/µL DNA in presence and absence of GO-TiO_2-Ag samples of concentrations A($5x10^{-2}$) wt%, B($5x10^{-3}$) wt%, C($5x10^{-4}$) wt%, D($5x10^{-5}$) wt% and E($5x10^{-6}$) wt%, respectively; (f), (g), (h), (i) and (j) are near UV absorption spectra of 6.5 ng/µL DNA in presence and absence of rGO-TiO_2-Ag - samples of concentrations A($5x10^{-2}$) wt%, B($5x10^{-3}$) wt%, C($5x10^{-4}$) wt%, D($5x10^{-5}$) wt% and E($5x10^{-6}$) wt%, respectively.

https://doi.org/10.31436/iiumej.v23i2.2148

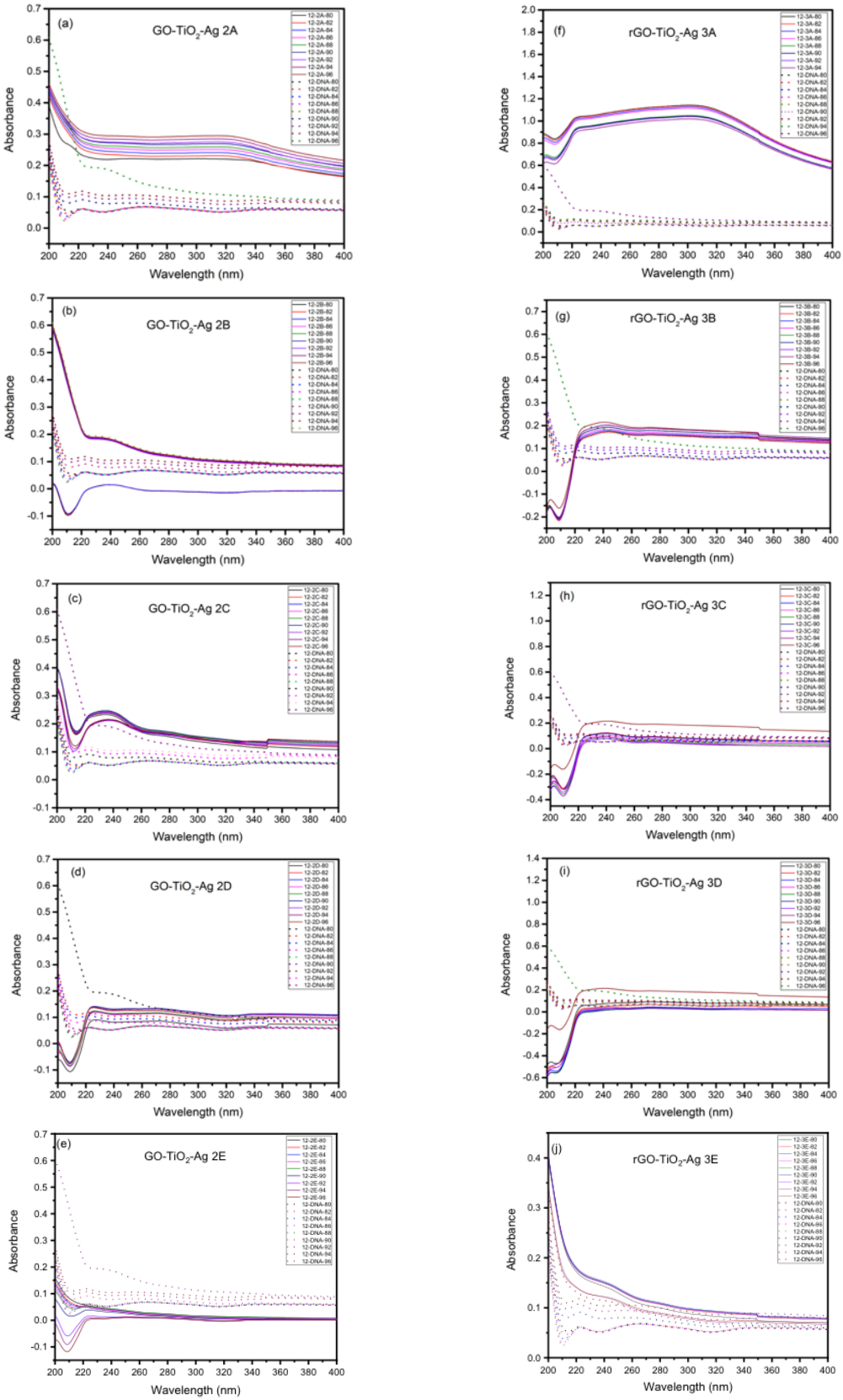

Fig. 3: (a), (b), (c), (d), and (e) are near UV absorption spectra of 11.5 ng/µL DNA in presence and absence of GO-TiO_2-Ag samples of concentrations A($5x10^{-2}$) wt%, B($5x10^{-3}$) wt%, C($5x10^{-4}$) wt%, D($5x10^{-5}$) wt% and E($5x10^{-6}$) wt%, respectively; (f), (g), (h), (i), and (j) are near UV absorption spectra of 11.5 ng/µL DNA in presence and absence of rGO-TiO_2-Ag samples of concentrations A($5x10^{-2}$) wt%, B($5x10^{-3}$) wt%, C($5x10^{-4}$) wt%, D($5x10^{-5}$) wt% and E($5x10^{-6}$) wt%, respectively.

The shift in the DNA absorption peak upon addition of GO-TiO_2-Ag reveals the interaction of the DNA–THNps. The significant absorbance increase observed upon the addition of various concentrations of GO-TiO_2-Ag at temperature ≤ 92 °C indicates that the GO-TiO_2-Ag THNps augmented the denaturation of the double-stranded DNA. However, and at a temperature ≥ 94° C, a higher DNA denaturation was exhibited with the presence of at least $5x10^{-2}$ wt% of GO-TiO_2-Ag THNps. Similar absorbance patterns can be seen for rGO-TiO_2-Ag THNps spectra at all study concentrations except at a concentration of $5x10^{-2}$ wt%; their absorbance was significantly higher than that of GO counterparts.

Additionally, rGO-TiO_2-Ag nanoparticles did not enhance the denaturation at concentration $5x10^{-3}$ wt% to $5x10^{-6}$ wt%, indicating the importance of an optimal nanoparticle concentration for the maximal heat transfer effect. A similar absorption pattern was observed for DNA samples A in the presence and absence of THNps; however, DNA absorption peak shift upon THNps addition was more prominent. The DNA denaturation was augmented at all concentrations of THNps except at $5x10^{-6}$ wt% and $5x10^{-5}$ wt% for GO-TiO_2-Ag and GO-TiO_2-Ag, respectively (Fig. 3). Moreover, there was a positive impact of GO-TiO_2-Ag THNps concentration on the denaturation. For instance, the absorption of the DNA samples containing $5x10^{-2}$ wt% and $5x10^{-5}$ wt% GO-TiO_2-Ag nanoparticles was 4.0-, 1.99-folds, respectively, higher than the control A DNA samples, and 54.64-, 20.57-folds, respectively, higher than the control B DNA samples, at 86 °C. Thus, decreasing the concentration of GO-TiO_2-Ag THNps in DNA samples, from $5x10^{-2}$ wt% to $5x10^{-5}$ wt%, decreased the absorption significantly by 50% and 38% for DNA samples A and B, respectively. On the other hand, reducing the concentration of rGO-TiO_2-Ag THNps in the DNA sample, from $5x10^{-2}$ wt% to $5x10^{-5}$ wt%, decreased the absorption by 3.4% for DNA samples A and B, respectively. Interestingly, at the lowest concentration of the THNp, their absorbance is negative, which indicates that the UV light passing through the DNA samples in the presence of THNp gives out a greater intensity of light. Therefore, it may have important significance.

4. CONCLUSION

The experiment has demonstrated the impact of adding the two novel ternary hybrid nanoparticles on DNA denaturation. The rationale behind the use of graphene based THNps is its unique heat transfer properties. DNA denaturation data showed that the enhancement of the DNA denaturation was nanoparticle concentration-dependent. The higher concentrations exhibit the maximum enhancement of the DNA denaturation owing to the enhanced thermal conductivity effect. rGO based THNp showed better results at higher concentrations compared to GO-based THNp. We propose THNp can be effectively used to achieve early denaturation of DNA samples.

ACKNOWLEDGEMENTS

This research work was financially supported by a grant from the Fundamental Research Grant Scheme (FRGS/1/2018/WAB09/UIAM/02/5), Ministry of Higher Education, Malaysia and a grant from the Knowledge Transfer and Assimilation Grant Scheme 2021 (2-2/25/15/11-21), Universiti Malaysia Terengganu (UMT). We would also like to thank the reviewers for their constructive comments to improve this manuscript.

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