THERMAL ENHANCEMENT OF PCR USING TERNARY HYBRID NANOFLUIDS

BY

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Engineering)

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JULY 2021

ABSTRACT

Polymerase chain reaction (PCR) is a vital tool in molecular diagnostics. Still, it is challenged with a host of poor yield, low sensitivity, specificity, contamination, non-specific target amplification, time-consuming, higher cost, energy-intensive, etc. In this study, the performance of PCR as a thermocycler is enhanced from a mechanical engineering perspective by adding additives leading to the enhancement of thermal conductivity of the reaction. In this study, two types of novel ternary hybrid nanoparticles (THNp) or tri-hybrid nanoparticles (GO-TiO_2-Ag and rGO-TiO_2-Ag) were synthesized consisting of three different nanoparticles graphene oxide, titanium dioxide, and silver decorated on each other. The two THNp were synthesized and then characterized using various techniques. The THNp were dispersed in lab-grade DDH_2O and sonicated substantially to form stable ternary hybrid nanofluids (THNf). Zeta potential of the prepared nanofluids was measured to check their stability, and it was in a range of 25 mV to 35 mV. The nanofluids were then serially diluted to 5 levels.Thermal conductivity measurements were performed, and the measurements showed a significant enhancement of about 66% and 83% for both GO-TiO_2-Ag and rGO-TiO_2-Ag, respectively, with THNp in the base fluids. The nanofluids' dynamic viscosity measurements show that the ternary hybrid nanofluids behave as Newtonian and non-Newtonian, where the viscosity decreases with the increase in temperature. Rheological investigations of both the ternary hybrid nanofluids exhibit Newtonian behavior with the stock solution. At the same time, it behaves as non-Newtonian, shear-thinning, or pseudo-plastic fluid when the concentration is diluted. At higher temperatures and low shear rates, the viscosity decreases significantly, which indicates shear thinning behavior. Concentration played a vital role in the change of viscosity due to the variation of temperature. Agglomeration is believed to be the reason for such behavior.Effects of concentration, temperature, and stresses applied in the non-linear viscoelastic fluid revealed linear viscoelastic (LVE) region through amplitude and frequency sweep tests. PCR experiments were performed on the extracted DNA. The initial PCR amplicons from the agarose gel electrophoresis showed that a higher concentration of nanoparticles is not of much significance to PCR, while lower concentration ($5x10^{-3}$ wt.%) of both GO-TiO_2-Ag and rGO-TiO_2-Ag contribute a significant enhancement of PCR reaction while reducing the number of cycles to about 40% when compared to PCR without nanoparticles (control). Subsequent PCR study showed PCR amplicon yield increased by 16.74-folds and 15.30- folds with the addition of GO-TiO_2-Ag and rGO-TiO_2-Ag, respectively. Band intensity study corroborated the same, indicating that the addition of THNp contributes to the thermal enhancement in the reactions. Sanger sequencing results showed the presence of a conserved region, and no DNA damage was observed with the addition of THNp. Separately, DNA denaturation tests were performed with and without the use of THNf for all prepared concentrations. The results showed significantly higher absorbance of UV light in the samples with THNf, indicating earlier denaturation of DNA strands due to the enhancement of thermal conductivity of the reaction. Numerical simulations using ANSYS thermal transient model were performed for a PCR setup with and without THNp. The temperature contours showed a significant enhancement of the heat transfer in the PCR reaction with THNp as an additive. They reduced the overall time by about 40%, corroborating our experimental results.

خلاصة البحث

) أو THNpفي هذه الدراسة ، تم تصنيع نوعين من الجسيمات النانوية الهجينة الثلاثية الجديدة ( ) مع ثلاثة من الجسيمات $rGO\text{-}TiO_2\text{-}Ag$ و $GO\text{-}TiO_2\text{-}Ag$الجسيمات النانوية ثلاثية الهجين (النانوية المعروفة ذات السمعة الطيبة وهي أكسيد الجرافين وثاني أكسيد التيتانيوم و فضة. ثم تم تمييز الجسيمتين النانويتين الهجينين باستخدام تقنيات توصيف مختلفة. تم تحضير الموائع النانوية المركب وصوتنة. تم إجراء قياسات جهد زيتا للتحقق من الثبات ثم تم تخفيفها THNpباستخدام بشكل متسلسل إلى 5 مستويات. تم إجراء قياسات الموصلية الحرارية على المخزون ، والسوائل . أظهرت قياسات الموصلية الحرارية أن هناك KD2 proالنانوية المخففة تسلسليًا باستخدام جهاز في السوائل. أظهرت القياسات تحسنًا THNpموصلية حرارية معززة للسوائل الأساسية في وجود على التوالي. تُظهر $rGO\text{-}TiO_2\text{-}Ag$ و $GO\text{-}TiO_2\text{-}Ag$كبيرًا بنسبة 66٪ و 83٪ لكل من قياسات اللزوجة الديناميكية للسوائل النانوية أن السوائل النانوية الهجينة الثلاثية تتصرف كسلوكيات نيوتونية وغير نيوتونية حيث تنخفض اللزوجة مع زيادة درجة الحرارة. أظهرت التحقيقات تظهر سلوكًا نيوتونيًا مع محلول GOالريولوجية أن السوائل النانوية الهجينة الثلاثية القائمة على المخزون بينما يتصرف كسلوك ترقق غير نيوتوني أو سائل بلاستيكي زائف عند تخفيف التركيز. في درجات الحرارة المرتفعة ومعدلات القص المنخفضة ، تنخفض اللزوجة بشكل كبير مما يشير إلى سلوك القص الخفيف. لعب التركيز دورًا حيويًا في تغيير اللزوجة في التباين في درجة الحرارة. يعتقد أن التكتل هو سبب هذا السلوك. أظهرت تأثيرات التركيز ودرجة الحرارة والضغوط المطبقة) من خلال LVEعلى اللزوجة المرنة غير الخطية وجود منطقة المرونة اللزجة الخطية (اختبارات اكتساح السعة والتردد. ثم تم استخدام الموائع النانوية لإجراء تفاعل البوليميراز المتسلسل ، وأظهرت أمبليكونات تفاعل البوليميراز المتسلسل من الرحلان الكهربائي لجيل الاغاروز أن التركيز العالي للجسيمات النانوية يثبط تفاعل البوليميراز المتسلسل بينما التركيز المنخفض يساهمان تحسن كبير في $rGO\text{-}TiO_2\text{-}Ag$ و $GO\text{-}TiO_2\text{-}Ag$ بالوزن٪) لكل من $5)x10^{-3}$ تفاعل تفاعل البوليميراز المتسلسل مع تقليل عدد الدورات إلى حوالي 40٪ بالمقارنة مع تفاعل البوليميراز المتسلسل بدون جزيئات نانوية. أكدت دراسة شدة النطاق نفسه. يشير هذا إلى أنه مع في التحسين. بشكل THNP ، يساهم التحسين الحراري في التفاعلات الناتجة عن THNPإضافة منفصل ، تم إجراء اختبارات تمسخ الحمض النووي باستخدام وبدون استخدام السوائل النانوية الهجينة الثلاثية لجميع التراكيز المحضرة. أظهرت النتائج أن هناك امتصاصًا كبيرًا لضوء الأشعة ، مما يشير إلى تمسخ سابق لخيوط الحمض THNpفوق البنفسجية في العينات التي تحتوي على النووي والذي يمكن أن يرجع أساسًا إلى تعزيز التوصيل الحراري للتفاعل. أظهرت نتائج التسلسل مع الأملاح الموجودة في THNp. قد يكون ذلك بسبب تقارب PCRوجود ضوضاء في منتج PCR الحراري العابر لإعداد ANSYSالمنتج. تم إجراء عمليات محاكاة عددية باستخدام نموذج . أظهرت ملامح درجة الحرارة أن هناك تحسنًا كبيرًا في انتقال THNpباستخدام وبدون استخدام مع انخفاض كبير بنسبة 40 ٪ تقريبًا في الوقت الذي THNp مع PCRالحرارة في تفاعل تستغرقه التحسينات الحرارية التي تدعم نتائجنا التجريبية.

APPROVAL PAGE

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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To Almighty Allah for means; And to my beloved family and friends for support.....

ACKNOWLEDGMENTS

In the Name of Allah, the Most Compassionate, the Most Merciful

Allah - beginning with the name of - the Most Gracious, the Most Merciful Most Auspicious is he whose control is the entire kingship, and he can do all things [67:1]. All Praise to Allah, the Lord of the creation, and countless blessings and peace upon our Master Mohammed, the leader of the Prophets (peace be upon Him).

My humble thanks to my supervisor, Professor Dr. Ahmed Faris Ismail, for being a pillar of support during the entire journey of my study. My special heartfelt gratitude to both of my brotherly co-supervisors, Dr. Akbar John and Dr. Abdul Khaliq Rasheed, for always being there for me to not only help in my academics but also for their patience and for knowing me very personally, for being my guiding light during my entire study and before. Words are not enough to thank them for their immense help in my studies and their guidance. My sincere acknowledgment to Prof. Dr. Mohammed Khalid and his team Priyanka and Mahesh Vaka from the Graphene advance research lab at Sunway University for their help with my rheological and denaturation experiments.

My sincere thanks to Anton-Paar, Malaysia, for helping us with the equipment for many of our experiments. Also, my sincere thanks to all my colleagues and friends in the "ECTRMG post-graduate lab" for their cooperation and support. And not to forget Br. Oualid from the preparation lab. My sincere acknowledgment and gratitude to Sis Batoul Allam from Kuliyyah of Pharmacy, Kuantan, for her help with my PCR experiments and also to Dr. Mohammed Abdulmalek Aldheeb for his help in my ANSYS simulations and finally, my heartfelt gratitude and thanks to my good friend Dr. Abdul Aabid from S&M Research Lab., Prince Sultan University, Riyadh, KSA for his immense help in guiding me how to write my thesis and all his immense help to complete this thesis as what it is today.

Special thanks to my Parents for their faith and tremendous patience to complete this study and to my siblings for their constant encouragement and prayers throughout my life.

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LIST OF SYMBOLS

ρ	Density
φ	Volume Fraction
k	Thermal Conductivity
C	Specific Heat Capacity
μ	Viscosity of Hybrid nanofluids
f	Fluid
p	nanoparticles
eff	Hybrid nanofluids
T	Temperature

LIST OF ABBREVIATIONS

3D	Three-dimensional
PCR	Polymerase chain Reaction
RT-PCR	Reverse transcriptase Polymerase chain Reaction
THNp	Ternary Hybrid Nanoparticles
THNf	Ternary Hybrid Nanofluids
GO	Graphene oxide
rGO	Reduced Graphene oxide
TiO_2	Titanium Oxide
Ag	Silver
SEM	Scanning electron microscopy
FTIR	Fourier-transform infrared spectroscopy
XRD	X-ray powder diffraction
DNA	Deoxyribonucleic acid
UV	Ultraviolet
FEA	Finite Element Method
GC	Guanine-Cytosine
ATGC	Adenine (A), Cytosine (C), Guanine (G), And Thymine (T).

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

This introductory chapter covers the research background, problem statement, research philosophy, scope, methodology, and objectives. This presentation includes a brief overview of the topic followed by the research problem and reasoning to the solution based on the philosophy of the study. The scope of the research is briefly clarified along with a specific end goal to answer the problem statement and followed by the research objectives laid down in steps. Then, the critical flow of the current study is explained in the research methodology. Finally, the outline of the thesis is presented.

1.2 BACKGROUND OF THE STUDY

The polymerase chain reaction (PCR) is an *In vitro* reaction technique that enables us to multiply an available DNA of any organism from a few strands to multiple billion copies of the targeted DNA. The PCR was an invention of Kary Mullis in 1984 based on the concept devised by Kjell Kleppe and H. Gobind Khorana by using assays of enzymes to replicate DNA templates with primers (Kleppe et al., 1971). The PCR is a fundamental building block of all genetic testing by multiplying copies of targeted DNA (Dwyer et al., 2002; M. Li, 2005; Siqueira and Rôças, 2003). This fundamental technique is widely used in contemporary molecular biology research and clinical medicine. PCR is an inexpensive, rapid, and simple means of producing large numbers of copies of DNA molecules from a small number of source DNA material (Goodman et al., 1993; Mullis et al., 1986; Ponce and Micol, 1992).

PCR mainly relies on thermal cycling, where the temperature of the DNA and its additive reactants are rapidly changed in many heating and cooling stages to allow different temperature-dependent reactions in each stage (Marie et al., 2003). The reactions are rapidly done in many cycles, where the DNA is multiplied exponentially with each cycle. A small amount of DNA strands from materials like tissues, skin, hair, saliva, or blood peripherals are multiplied exponentially until a desired copy of the DNA is obtained. PCR is a crucial technique used in almost all medical and biotechnological laboratories and research centers for numerous applications like criminal forensics, genotyping, biomedical researches, cloning of DNA, cloning or duplication of genes, monitoring of infectious diseases, pathogen detection (Giovannini and Concilio, 2002), amplification of ancient DNA in archaeology, diagnosis of hereditary diseases, genetic fingerprint and identity analysis as in parentage testing and many more research-based applications. PCR is highly significant to be used when a specific set of DNAs is amplified within minutes in an automated machine called a thermocycler. That is called specificity, wherein only the target set of DNA or genomic material is amplified. The PCR has several challenges which researchers are working hard to address to make it a better technique.

Some of its challenges include the inadequate availability of DNA in the sample, template's high GC content, low specificity in amplification, lower efficiency, the requirement of higher melting temperature in some samples, difficulty in amplifications of some DNA samples due to its secondary structure, the higher formation of impurities or primer- dimer, etc. (Pan. et al., 2012). The typical PCR reaction consists of a three-step recycling process: denaturation, annealing, and extension. The main components of a PCR reaction include PCR buffer solution of about ten times, deoxynucleotide triphosphate (dNTP) mixture, Mg2+, DNA templates, and Taq enzyme.

The PCR relies on thermal cycling in which the content mixture goes through repeated temperature changes called cycles consisting of three temperature steps, as mentioned above. The initialization is done by heating the reaction mixture to a temperature of 94-96 °C for about 1 -10 minutes. The first step of the cycle is denaturation, which consists of heating the reaction mixture to 94–98 °C for about 20–30 seconds. This stage causes the DNA to melt and de-naturate or open strands by breaking the hydrogen bonds between complementary bases, which yields two single-stranded DNA molecules. The next step is annealing, in which the reaction temperature is lowered to about 50–65 °C for about 20–40 seconds. This reduction in temperature allows the primers of each single-stranded DNA template to bind itself to the target region. This is also known as a hybridization stage in which the primers (short DNA fragments) of different strands attach themselves to the denatured strands of DNA. The next stage in the reaction is the elongation or extension in which the temperature is raised to 75–80 °C. This helps synthesize a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture. This cycle is repeated as required to amplify the DNA target to millions of copies as required. The general formula used for a given number of cycles is $2^{n,}$ where n is the number of cycles in the reaction.

PCR is a very widely used method for many applications to simulate *In vivo* DNA replication in many fields of scientific research like DNA cloning for sequencing, gene manipulation, and cloning, construction of DNA-based phylogenies, gene mutagenesis monitoring and diagnosing of hereditary diseases; ancient DNA amplification (Markwell, 2009) analyzing of DNA for profiling to be used in forensic sciences and diagnosis of infectious diseases by detection of pathogens in nucleic acids. However, PCR is still compromised with its low specificity, sensitivity, and false-

negative results, especially in GC-rich fragments (Cao et al., 2009). PCR is prone to have errors due to contamination and primer-dimer formation, where other unwanted DNA is also multiplied by the targeted ones due to the *in vitro* nature of the reaction. Efforts have been made to increase PCR efficiency by various means to improve specificity, sensitivity, reduction of time per cycle, and increase the yield, especially in the GC-rich regions.

PCR is a vital tool with many applications, and it is highly desirable to enhance efficiency using various methods. Some of the conventional methods used are by selecting high-fidelity enzyme such as pfx DNA polymerase, optimizing the PCR reaction conditions, and adding special chemical reagents such as glycerol, etc. (Chisholm et al., 2002; D Cui and al., 2004; Sellner, Coelen, and Mackenzie, 1992; Tombline et al., 1996). Other biochemical analysis methods require a significant number of biological materials, whereas PCR requires very little, and it has the ability of higher sensitivity, detection, and amplification. It is known from the literature that metal ions such as Mg2+ significantly increase the PCR efficiency by maintaining the highest activity of Taq enzyme.

It is imperative to develop newer ways and techniques to enhance the amplification efficiency and specificity (He et al., 2016). Many researchers have found that a few materials such as dimethyl sulfoxide (DMSO), glycerol, formamide, betaine, etc. (Henke, 2008; Varadaraj and Skinner, 1994) enhance the amplification of PCR. Even though these methods can slightly improve the specificity and efficiency of PCR, there is still a considerable void that should be addressed to improve the efficiency of PCR. Few other ways to increase the efficiency of the PCR are to develop better thermocycler machines or by adding additives such as nanoparticles into the PCR reaction known as NanoPCR. The latter is an inexpensive method that has led to

intense investigation due to their fascinating physical and chemical properties (J. Wang et al., 2015). It has attracted considerable attention from researchers in the last decade. Various types of nanoparticles have been tried and used in the PCR to enhance its efficiency and obtained better results.

1.2.1 NanoPCR

In the last decade, nanoparticles have attracted more considerable attention and gradually penetrated various engineering and life sciences fields because of their unique chemical and physical properties, such as large surface area and small size effect, which significantly promoted the development of life science and technology. Their high thermal conductivity and surface-to-volume ratio have led many researchers to adopt them in PCR and named them NanoPCR (Shen et al., 2009). NanoPCR using Gold nanoparticles has proved to increase- the sensitivity of PCR detection 5- to 10-fold in a slower PCR system (i.e., conventional PCR) (S. H. Hwang et al., 2013). Khaliq et al. observed that TiO_2 nanoparticles effectively enhanced the PCR efficiency of low as well as high GC- rich DNA templates, with maximal augmentation up to 6.9- fold at 0.4 nM concentration (Khaliq et al., 2010). Platinum nanoparticles capped with β-cyclodextrins helped improve the overall efficiency of the PCR and increased the sensitivity while reducing the use of reagents (Petralia et al., 2012).

Carbon-coated silica nanocomposites or hybrid nanoparticles were found to be effective PCR enhancers which also induced strong interaction between polymerase and primers (J. Y. Park et al., 2015a). ZnO-TiO_2 hybrid nanoparticles were found to enhance the overall efficiency and specificity of PCR reaction while reducing overall time to about 50% (Fadhil, 2014). Better PCR enhancements can be obtained from other novel nanoparticles, which are yet to be studied. This technology has become a current

research hotspot. The mechanism and application of PCR amplification technology based on various nanoparticles are being explored. Nonspecific amplification and primer mismatch significantly affect PCR amplification. The addition of nanoparticles highly improves the sensitivity, specificity, and yield of PCR due to their excellent surface properties, heat conduction, and specific binding to single-stranded DNA or protein.

1.2.2 Hybrid NanoPCR

Hybrid NanoPCR is a term given to PCR in which a combination of more than one nanoparticle is used. Since the nanoparticles to be added in the PCR reaction are initially mixed with either water or the PCR buffer solution, they are called nanofluids. When one or more nanoparticles are used to prepare the nanofluids to be used as additives to PCR, they are called hybrid NanoPCR. Hybrid NanoPCR is a very new concept. A significant number of studies reported to date has mainly focused on single material nanoparticle as PCR enhancers. At the same time, very few studies have been done on the bi-hybrid or two-material nanoparticles, which can also be referred to as nanocomposites. The aim of synthesizing a multiple-material nanoparticle is to enhance the overall efficiency of PCR and increase the specificity, yield, and efficiency in profiling less abundant, low expressive genes. It also aimed to reduce the time taken per cycle, the overall reaction time of the PCR. At the same time, it should also try to eliminate or at least reduce the formation of primer-dimer or the formation of another unwanted potential by-product of PCR. NanoPCR has the advantage of exhibiting more than one characteristic to improve more aspects in enhancing PCR. The rationale behind the use of hybrid nanoparticles could be because:

- Possibility of modification in the DNA molecular structure and its biochemical and bio-thermal activities due to interactions between the nanoparticles and DNA molecules (M. Li, 2005)
- They exhibit higher heat conduction since nanoparticles have a large surface area (Abdul Khaliq et al., 2010)
- Approximately 20% of atoms of particles measuring less than 20 nm are carried on their surface, facilitating them instantly open for heat transfer (Zhizhou Zhang et al., 2008)
- Enhanced heat transfer due to micro-convection of fluid as a result of the movement of the nanoparticles attributable to the infinitesimal size (Mi et al., 2007)
- Small size and less weight of the particles evade the problem of particles sedimentation (Zhizhou Zhang et al., 2008)
- Percolation structures also help faster transfer heat within the nanoparticles and surrounding fluid (X. Cao et al., 2011).

1.3 STATEMENT OF THE PROBLEM

PCR is one of the fundamental tools in molecular biology. Various nanomaterials have opened up newer opportunities for improving PCR. Several reports have confirmed that an optimal concentration of various nanoparticles acts as enhancers that enhance the yield, specificity, and overall reduction of the PCR reaction time. However, scores of debates prevail concerning the underlying mechanisms. NanoPCR efficiency is affected by parameters such as size, shape, concentration, and type of nanoparticles used, either metallic or non-metallic/carbon-based. All these parameters were also found to have a substantial effect on the thermal conductivity of nanoparticle-