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Research Article
Optimization of Graphene Oxide-based Quencher-free Molecular Beacon for Meat Product Authentication

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Abstract

Background and Objective: Sensitivity is very important in DNA detection. Various attempts have been made to increase detection sensitivity, including increasing the detection capabilities of devices and using DNA probes. This study was aimed to develop a DNA detection method using a quencher-free molecular beacon (QFMB) probe with the help of graphene oxide (GO) as a quencher. **Materials and Methods:** The GO has the specific ability to adsorb DNA in the form of a single strand but not in a double strand. The optimum interaction between the MB probe and the target DNA (pig DNA) could produce a double-stranded DNA (dsDNA) so that it is detached from the GO surface. The dsDNA that escapes from the surface of the GO can be detected using a spectrofluorometric technique at an excitation wavelength of 482 nm and an emission of 519 nm, with an intensity comparable to its concentration. **Results:** The optimum condition that can be used is a GO concentration of 5 μg mL^{-1}, a reaction temperature of 65°C, an incubation time of 6 min, a reaction pH of 7.5 and cation levels of 40 nM. Analysis of the target pork meatball DNA carried out at a concentration interval of 0-500 pg mL^{-1}. **Conclusion:** So it was concluded that the DNA detection system uses a combination of a quencher-free molecular beacon and graphene oxide, providing a good prospect to be developed into a new method in the halal authentication of meat products using the spectrofluorometric method.

Key words: Spectrofluorometric method, meat products, molecular beacon, graphene oxide, halal authentication, pork meatball

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The DNA is a biomolecular compound that carries important genetic information from each individual in both regeneration and physiological functions by synthesizing functional proteins[1]. The DNA detection was made to find its presence in samples or mixed sample matrices and it can also be performed for diagnostic, screening or analytical purposes. Various DNA detection methods have been developed by applying various analytical methods, probes and biosensors with nanomaterial platforms[2]. A detection system aimed at detecting targets in small quantities leads to high sensitivity and selectivity. The polymerase chain reaction (PCR) method is one of the detection methods that have good sensitivity because it goes through the target amplification stage[3,4].

A DNA detection that requires amplification and targets specific species requires specific oligonucleotide primers. A specific primer can be developed with a specific gene from those species using an in silico method[5]. Detection sensitivity can be achieved by using a specific oligonucleotide that could bind to the DNA target called probe. The specific oligo which in some conditions would bind the target can be detected using some methods, depending on the modification of the 5' or 3' end of the oligo-probe[6]. One of the developed probes is the molecular beacon (MB) probe[7]. The MB has been generally modified with a fluorophore and a quencher on the 5' and 3' end, which could activate the probe to the next detection procedure. It is one of some specific probes that could be applied as a DNA detection tool[8].

A nanoparticle platform becomes a common tool in the application of a DNA detection system. The surface of the nanoparticle can be functionalized as the anchor of some molecular probe that could improve signal detection[9]. Graphene oxide (GO) is one of the non-metal nanoparticles known to have some advantages applied in a DNA detection system[10]. The GO is a modified oxidized graphene that can separate a single-stranded and double-stranded DNA (dsDNA) through the π-π stacking interaction. The adsorption ability of the GO surface can also quench the fluorophore attached to the probe. This phenomenon gives us the advantage to design a quencher-free molecular beacon[11]. The GO can improve the selectivity of the detection by increasing the signal-to-background ratio[12].

The MB probe is one of many probes that can be functionalized with graphene oxide for the DNA detection system[13]. Some studies have applied GO and the molecular beacon for DNA detection systems, such as Ca^{2+} in solution detection[14], DNA damage detection[15] and T4 polynucleotide kinase activity[11]. Therefore, this study was aimed to optimize the use of molecular beacon probes with a quencher-free design by utilizing graphene oxide to develop a DNA detection method. The ability of graphene oxide to separate ssDNA and dsDNA was explored in the production of a quencher-free probe for meat product authentication using a DNA detection system.

MATERIALS AND METHODS

Apparatus and reagents: This study was conducted at the Integrated Research and Testing Laboratory of Universitas Gadjah Mada in Yogyakarta, Indonesia, from November, 2017-May, 2018. The fluorescence intensity was measured using Spectrofluorometer Shimadzu RF-6000 (Japan). Scanning was performed in the excitation and emission wavelength between 450 and 600 nm. The sample was prepared using refrigerated centrifugation Sigma Sartorius 3K30 (UK). The pH of the solution was determined using SevenEasy pH meter Mettler Toledo (USA). The sample was incubated in an IF55plus incubator, Memmert (Germany). Graphene oxide and another chemical reagent with a pro analytic grade were purchased from Sigma Aldrich (USA). Isolation of the DNA sample was performed using PureLink™ DNA Mini Kit from Invitrogen (Thermo Fisher Scientific, USA). Oligonucleotide probes and targets were designed using the Beacon Designer 8 software; the probe was modified with the addition of fluorescence 5'-FAM. The sequence obtained was as follows: Quencher free molecular beacon probe of porcine: 5'-FAM-CGCGATCACAAATGTGTGTAACTGATGAGAAGATCGCG-3', complemented target: 5'-TTCTCATCAGTT ACACACATTTGT-3', non-complemented target: 5'-AAGAGTAGTCAATGTGTG TAAA CA-3'.

The DNA samples of pigs, cows, goats and chickens were isolated using the protocol that established the PureLink™ DNA Mini Kit, based on the preparation procedure for the category of mammalian tissue and mouse/rat tail lysate samples. The amount of sample prepared was 25 mg, which was incubated at 55°C for 1-4 h until lysis was complete with an occasional vortex. Purification of the sample was carried out through a series of centrifugation processes utilizing spin columns and buffer C provided by the PureLink™ DNA Mini Kit. The DNA samples obtained were analyzed using a UV/Vis spectrophotometer at wavelengths of 260 and 280 nm. Indirectly used DNA was stored in a refrigerator at 4°C (short-term) or 120°C (long-term). A molecular beacon probe was also incubated at 55°C with the aim of melting the complemented stem parts so that the open sequence would facilitate binding to the DNA target. This was followed by the addition of GO 5 µL and then fluorescence intensity was measured after incubation for 5 min.

DNA target detection: The optimization design using a quencher-free molecular beacon (QFMB) was carried out according to the method of Zhou *et al.*[16] pig, cow, goat and chicken DNAs were used as targets to test the selectivity of the molecular beacon probes. The sensitivity of the test was carried out on the target porcine meat and meatball DNA as samples of food products. A FAM-fluorescence signal measurement uses a spectrofluorometer by scanning within a wavelength range of 400-600 nm to obtain the excitation and emission wavelength. The reaction conditions were optimized using the parameters of temperature, incubation time, pH of the solution and effect of ionic strength.

Data analysis: The experiments were conducted at least in triplicate. The value results were reported as mean±standard deviation (SD) from three independent batches. Analysis of variance (ANOVA), specifically one-way ANOVA was performed to determine the difference among groups and $p<0.05$ was considered significant.

RESULTS

Feasibility of analytical methods and study of GO concentration effects: The feasibility of the analytical method was tested to determine the effect of fluorescence reduction from the molecular beacon probe through graphene oxide (Fig. 1). Figure 1 shows the effect of adding graphene oxide to the intensity of fluorescence probe molecular beacons. The results show that fluorescence intensity was reduced and GO concentration increased at a concentration interval of 1-9 μg mL^{-1} with a linear regression equation of y = -20.088x+90.046, R^2 = 0.9019. This study selected a concentration of 5 μg mL^{-1} for subsequent experiments.

Effect of incubation temperature: To obtain the optimum incubation temperature, the hybridization reaction was tested at a temperature range of 25-85°C. The data in Fig. 2 shows the results of reaction temperature optimization, where at a temperature interval of 25-65°C, fluorescence intensity increases, while at an interval of 65-85°C, it tends to decrease. Therefore, in this study, the temperature of 65°C was chosen as the incubation temperature of the QFMB probe hybridization reaction with the target DNA.

Effect of incubation time: Optimization of incubation time was carried out in 2-10 min intervals. The data in Fig. 3 shows the optimization results of the time, revealing that from 2-4 min, the intensity of fluorine decreased and then increased in the 6th min and dropped back at 8-10 min. Therefore, in this study, the incubation time of the QFMB probe reaction with the target DNA was used for 6 min.

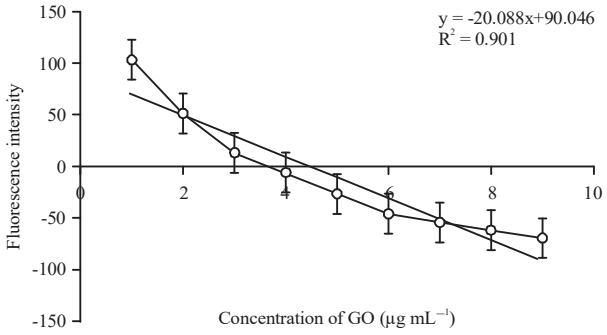

Fig. 1: Effect of GO concentration to the fluorescence intensity of the molecular beacon probe with MB concentration of 5 μg mL^{-1} and GO concentration 1-9 μg mL^{-1}

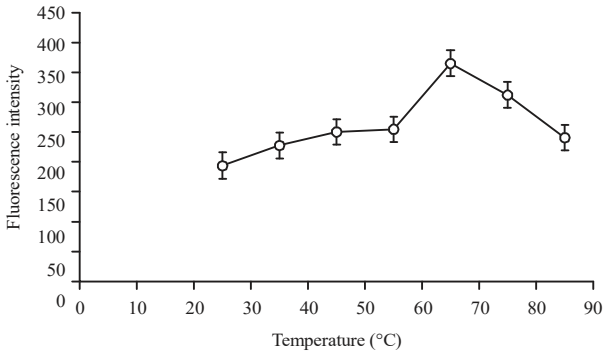

Fig. 2: Effect of incubation temperature to fluorescence intensity. The MB probe concentration is 5 μg mL^{-1} and target DNA is 4 μg mL^{-1}

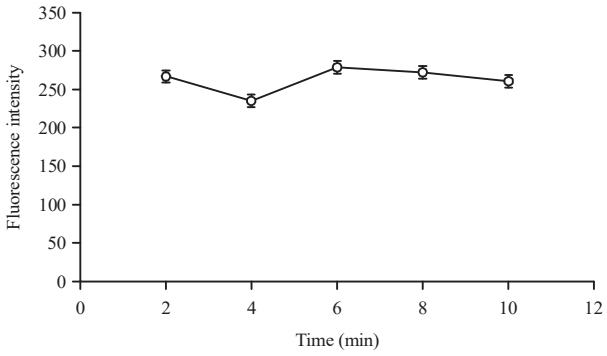

Fig. 3: Effect of incubation time to fluorescence intensity. The MB probe level is 5 μg mL^{-1}

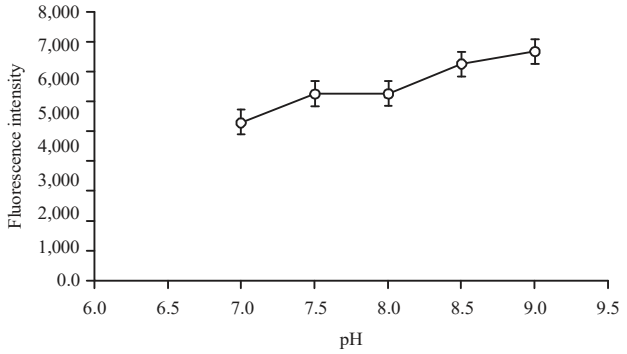

Fig. 4: Effect of pH on fluorescence intensity. The MB probe level is 5 μg mL^{-1}

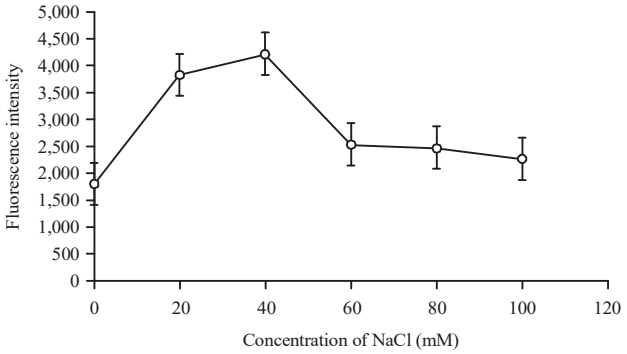

Fig. 5: Effect of ionic strength on the intensity of fluorescence. The concentration of the MB probe is 5 μg Ml^{-1}

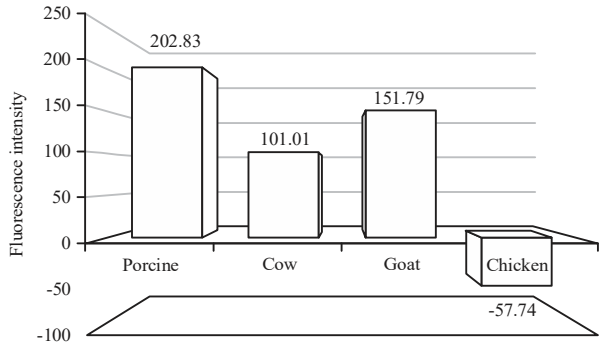

Fig. 6: Selectivity for DNA of porcine, cows, goats and chickens

Effect of pH of solution: The optimum pH of the reaction was determined by testing a number of pHs (7.0-9.0) from the Tris-HCl buffer solution. The results of the pH optimization (Fig. 4) showed that fluorescence intensity tends to increase with the increasing pH of the solution. Intensity of fluorescence tends to increase at pH 7.0-7.5, but slightly decreases at 8.0 and then increases again at pH 8.5-9.0. Therefore, the reaction pH determined in this study was pH 7.5, which was chosen as the optimum pH and not pH 9.0 because, at an increasingly alkaline pH, it can interfere with the stability of the DNA hybridization process.

Effect of ionic strength: The effect of ionic strength was investigated by adding NaCl (0-100 mM) to investigate an increase in hybridization reactions. The measurement results (Fig. 5) show varied changes with the addition of NaCl, including an increase in the intensity of fluorescence within the range of 0-40 mM and a tendency to decrease above 40 mM and up to 100 mM. Therefore, 40 mM of NaCl was added in this study.

Selectivity studies on DNA of porcine, cow, goat and chicken: To find out whether the MB probe can detect porcine DNA contamination for authentication purposes in food products, selectivity testing is carried out on several other DNA targets. Porcine, cow, goat and chicken DNA are used as targets in selectivity testing (Fig. 6). The results showed that the target porcine DNA provides the strongest fluorescence intensity, while the DNA of cattle and goats provide relatively weak fluorescence intensities.

Linear correlation and limit of detection: To determine the linearity of the test, the relationship between the intensity of fluorescence and target DNA at various concentrations was shown. Fluorescence intensity was expressed as ΔI, where, ΔI = I- Io and Io and I are the fluorescence intensities measured without a target and with the addition of the target. Figure 7ab displays the results of the measurements of fluorescence intensity on the target porcine meat DNA and porcine meatball DNA. The measurements of porcine meat DNA were carried out with a series of concentrations from 0-1000 pg mL^{-1}, resulting in a linear regression equation $y = 1.0595x+99.931$ with a correlation coefficient of 0.9668 (R^2) (Fig. 7a). The measurement of the target porcine meatball DNA was carried out with a series of levels from 0-500 pg mL^{-1}, resulting in a linear regression equation $y = 1.8779x+194.36$ with a correlation coefficient of 0.9887 (R^2) (Fig. 7b). These results show that the system is suitable for both DNA sample measurements.

DISCUSSION

The detection principle for porcine meat and meatball DNA in halal authentication was used. The QFMB is assisted by graphene oxide as a quencher while adsorbing ssDNA. The optimum conditions were an incubation temperature of 65°C, incubation time of 6 min for the QFMB probe reaction with the target DNA, reaction pH of 7.5 determined in this study and addition of 40 mM NaCl in this study. The selectivity studies

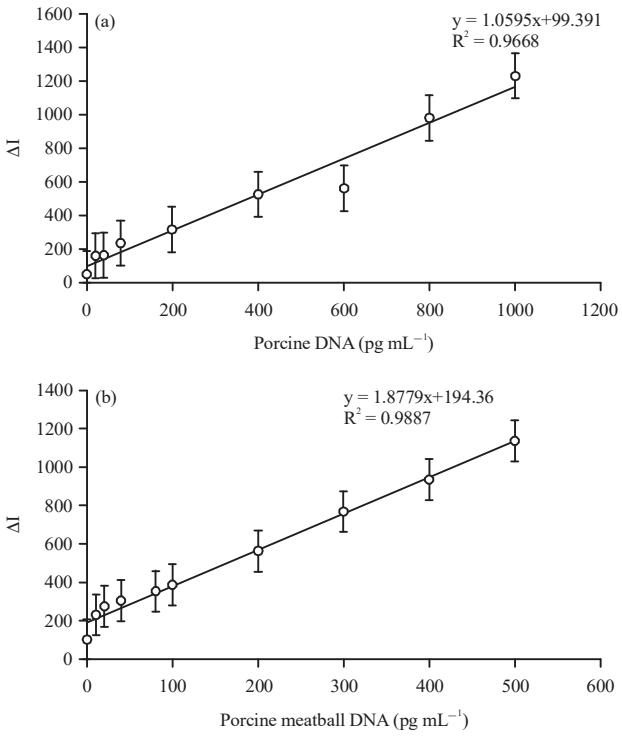

Fig. 7(a-b): Linear regression for measurement of (a) Porcine meat DNA and (b) Porcine meatballs DNA

revealed that the target porcine DNA provides the strongest fluorescence intensity, while the DNA of other animals provides relatively weak fluorescence intensities. As a probe, MB has been applied in various purposes related to DNA detection based on fluorescence resonance energy transfer and complementary pairing[17]. The MB was made as a probe with a fluorescence detection system because it has good sensitivity and can be applied to various testing purposes. Because it is composed of nucleotide strands (DNA), generally, an MB probe is applied to the detection system using PCR. The form of the stem and loop in the MB probe structure and the temperature cycle in the PCR system make it easy to detect the target DNA because it is also supported by the specificity of the developed MB design[8].

Graphene oxide is a nanosized supramolecular hydrocarbon structure produced from graphite sheets through an oxidation process[18]. Graphene oxide has long been explored for its ability to separate ssDNA and dsDNA[19]. This potential provides advantages, especially in the development of DNA biosensors using the label-free system[20]. In this study, graphene oxide was applied to the MB probe, so the developed design was quencher-free.

The design of the quencher-free molecular beacon using graphene oxide, which was developed in addition to saving the use of a quencher in the modification of the MB probe, can also increased the signal-to-background ratio[12]. Graphene oxide as a detection platform can be a very effective fluorescence quencher that can increase detection sensitivity[14]. Furthermore, this system can be developed for the purpose of authenticating animal products in halal matters by developing more selective MB probes.

CONCLUSION

This study aimed to develop a method for detecting pig DNA for halal authentication of food products using MB probes and graphene oxide. The MB probe is a single-strand nucleotide containing a modified stem and loop at the 5' end with fluorescence and a damper at the other end. Using graphene oxide replaces the use of dampers so that the MB probe is only modified with the addition of 5'-FAM as fluorescence.

SIGNIFICANCE STATEMENT

This study discovered the optimum conditions that can be beneficial for the application of the quencher-free system used in MB as a probe. It will help the researchers to uncover the critical areas of the utilization of MB probes in halal authentication of meat products that many researchers were not able to explore. Thus a new theory on halal authentication technique may be arrived at.

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