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Improving the Specificity and Efficiency of Polymerase Chain Reaction using Polyethyleneimine-based Derivatives and Nanocomposites

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Abstract: Polymerase chain reaction (PCR) has been identified as a fundamental technique in molecular biology and clinical medicine. Improvements of the specificity and efficiency of PCR are always required and the understanding of the PCR enhancing mechanism still remains a great challenge. Here we report the use of branched polyethyleneimine (PEI)-based derivatives and nanocomposites as a novel class of enhancing reagents to improve the specificity and efficiency of PCR. We show that the surface charge polarity of PEI and PEI derivatives plays a major role in their effectiveness to improve the PCR specificity and efficiency. Positively charged amine-terminated pristine PEI, partially (50%) acetylated PEI (PEI-Ac_{50}), and completely acetylated PEI (PEI-Ac) are able to improve the PCR efficiency and specificity with an optimum concentration order of PEI $<$ PEI-Ac_{50} $<$ PEI-Ac, whereas negatively charged carboxyl-terminated PEI (PEI-SAH) and neutralized PEI modified with both PEG and acetylation (PEI-PEG-Ac) are unable to improve the PCR specificity and efficiency with the concentrations of 3 orders of magnitude more than that of PEI. In addition, multiwalled carbon nanotubes (MWCNTs) modified with PEI is also able to improve the PCR specificity and efficiency with an optimum concentration much less than that of the MWCNTs without PEI modification. Our data clearly suggests that the PCR enhancing effect is primarily based on the interaction between the PCR components and the PEI derivatives or PEI-based nanocomposites, where electrostatic interaction should play a major role to locally concentrate the PCR components on the backbones of the branched PEI or PEI-based nanocomposites, enabling enhanced PCR specificity and efficiency.
Keywords: Polymerase chain reaction; Specificity; Polyethyleneimine-based derivatives; Nanocomposites

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Introduction

Developed in 1983 by Kary Mullis [1], polymerase chain reaction (PCR) has been identified as a fundamental technique in contemporary molecular biology research and clinical medicine. This gene amplification technique can increase the number of the copies of target genes by 6 orders of magnitude [2]. This unique feature makes PCR widely used in sequencing [3], pathogen detection in food /environment/animals [4-8], genetic analysis [9], and so on. However, the specificity of PCR does not always match its unparalleled sensitivity [10]. It is well known that even with sophisticated optimization processes, PCR specificity and efficiency are not always satisfactory. Due to the fact that the PCR mechanism is rather complicated, certain interference effects would be unavoidable in actual practices. Development of various addi-

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tives to enhance the specificity and efficiency of PCR still remains a great challenge. Besides the conventionally used small molecular additives, such as formamide [11], tetramethylammonium (TMA) chloride [12] and its derivatives [13], and betaine [14], nanoparticles (NPs) have received considerable attention due to their unique physicochemical properties, which is significantly different from their bulk counterparts. Various NP systems, including nanogold [15-18], carbon nanotubes (CNTs) [19], carbon nanopowder [20], magnetic NPs [21], semiconductor nanomaterials [22-23], and Dendrimers [24], have been used as additives to improve PCR specificity and or efficiency. The nonspecific amplification problems can be overcome in the presence of the NPs.

In our previous studies [25], we show that the electrostatic interaction between the PCR components with the positively charged dendrimers or dendrimer-entrapped gold nanoparticles (Au DENPs) plays a major role in optimizing an error-prone two-round PCR system. Our studies show that amine-terminated dendrimers have higher PCR enhancing effect than those with terminal acetyl and carboxyl groups. Similarly, in the presence of Au DENPs, the specificity and efficiency of the PCR can be significantly improved. The strong interaction, especially the electrostatic interaction between NPs and PCR components is believed to be dependent not only on the surface charge density of the particles but also on the particle morphology upon interaction with the PCR components.

In this present study, we explore a new application of branched polyethyleneimine (PEI)-based derivatives and nanocomposites as specificity and efficiency enhancers in PCR. The positively charged amine-terminated pristine PEI were able to be modified with acetic anhydride or succinic anhydride to generate PEI with charge neutral surfaces (PEI-Ac_n, n denotes the ratio of acetylation, Ac denotes acetyl groups; PEI-PEG-Ac, PEG denotes polyethyleneglycol) or negative surface charge (PEI-SAH, SAH denote succinamic acid groups). We systematically investigated the effect of the surface charge of PEI-based derivatives and nanocomposites on the specificity and efficiency of a non-specific PCR system. Possible molecular mechanisms were discussed. To our knowledge, this is the first comprehensive report relating to the optimization of PCR using PEI with different surface charge polarities and nanocomposites.

Experimental

Materials

Amine-terminated PEI was purchased from Aldrich. The derivatives of PEI with positively charged (PEI-Ac_{50}), neutral charged (PEI-Ac, PEI-PEG-Ac), and negatively charged (PEI-100SAH) were synthesized and characterized in the previous work [26]. Besides, multiwalled carbon nanotubes (MWCNTs) and PEI-modified multiwall carbon nanotubes (MWCNT-PEI) were synthesized in our previous work [27-29]. The PEI-stabilized gold NPs (PEI-Au) was synthesized in this work. PEI (6 mg) was dissolved into 10 mL water, and then $HAuCl_4$ (48.6 mg/mL) in 100 μL water was added dropwise to the PEI solution while stirring. The reaction was stopped after 5 d. The synthesized PEI-Au had a diameter of 10 nm as measured by transmission electron microscopy. UV-Vis spectroscopy study confirmed that the synthesized PEI-Au displayed a surface plasma band at 532 nm, which is typical for AuNPs. The obtained PEI-stabilized AuNPs in aqueous solution was restored at 4°C before use. Acetic anhydride and succinct anhydride were obtained from Aldrich. Their structures were schematically illustrated in Fig. 1. The water used in all the experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ cm.

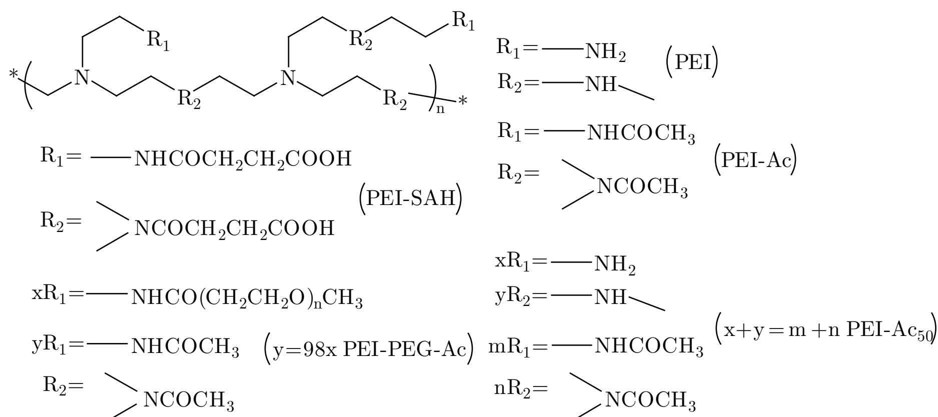

Fig. 1 Schematic illustration of the structures of PEI, PEI-SAH, PEI-Ac, PEI-PEG-Ac and PEI-Ac_{50}. x, y, m, n represent the number of R1 or R2 groups in different molecular.

PCR and its evaluation

In order to test additives' effect on PCR, we set up a non-specific system. In the first-round PCR, a 396 bp target DNA segment was amplified from Pseudomonas aeruginosa genome DNA by using one pair of primers with high specificity. Then the PCR products of the first round were used as the template in the second round PCR, and amplified by the same primers. PCR reagents were mixed in a final volume of 25 μL according to the following conditions: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM primers (Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd.), 0.25 μM each dNTP (TaKaRa Bio. Inc.), 0.025 U/μL Ex Taq DNA polymerase (TaKaRa Bio. Inc.), 0.8 ng/uL Pseudomonas aeruginosa genome DNA. The PCR procedure was: 2 min at 94℃ for pre-denaturation, followed by 27 cycles of: 30 s at 94℃, 30 s at 60℃, and 30 s at 72℃. Then cycling was terminated after 5 min incubation at 72℃. The sequences of the primers were as follows: primer 1: 5'- GACAACGCCCTCAGCATCACCAGC-3', and primer 2: 5'-CGCTGGCCCATTCGCTCCAGCGCT-3'. Amplifications were carried out in $S1000^{TM}$ Thermal Cycler (Bio-Rad Inc.).

PCR products (3 μL) were examined with 1.5% agarose gel electrophoresis with 0.6 μL loading buffer. The effectiveness of PEI derivatives and nanocomposites was described through the assignment of two densitometric quantities, termed specificity and efficiency. From electropherograms, the specificity of amplification was calculated as a ratio of the densitometric value of the specific band and that of all bands amplified by PCR, including undesired nonspecific bands. By definition, maximal value of specificity, in the absence of non-specific bands, equals to 1.0. The efficiency of an additive is defined as a ratio of the densitometric value of the target DNA band determined after PCR to 500 bp of DL2000 DNA marker, which is assigned to a value of 1.0. The concentration of the brightest target band (maximum efficiency) on the gel was identified to be the optimum concentration.

Results and discussion

In our non-specific PCR system, due to the use of the same primers, even though the specific band is observed in the first round, the second round PCR always arise nonspecific amplification. The target lane is 396 bp, and the non-specific lane is lower than it (Fig. 2, lane 1 for all panels). That may be the results of side-reaction products [20].

To study the optimum effect of the PEI derivatives and nanocomposites, eight different materials were tested in this system. Each additive with different concentrations was added into the reaction mixture to optimize the enhancing effect, and the PCR products were analyzed after electrophoretic fractionation on agarose gels.

It was reported that carbon nanotubes (CNTs) can improve the specificity and efficiency of PCR amplification [19-20, 30]. In our previous study, we find both PEI and PEI modified MWCNTs (MWCNT-PEI) can improve the specificity and efficiency in an error-prone two-round PCR system [25]. Thus, MWCNTs, MWCNT-PEI, and PEI were also tested in this non-specific PCR system.

Figure 2 shows the results of PCR optimized using the tested additives. With the addition of the MWCNTs, MWCNT-PEI, and PEI, the original smear bands related to non-specific product disappear, indicating that MWCNTs, MWCNT-PEI, and PEI are all able to improve the specificity and efficiency of the non-specific PCR system. The optimum concentrations of the MWCNTs, MWCNT-PEI, and PEI additives were estimated to be 6.2 mg/L, 94.4 μg/L, and 40 μg/L, respectively (Table 1). This result is in agreement with our previous reports [25]. It is clear that the modification of MWCNTs with PEI makes the require optimum concentration of MWCNTs much lower, indicative of the strong interaction between the negatively charged PCR components and the positively charged MWCNT-PEI. Similarly, after modification onto the surface of MWCNTs, the required optimum concentration of PEI is much lower than that of free PEI, suggesting that the MWCNT-supported PEI may have a stronger interaction with the negatively charged PCR components than free PEI.

The above results appear that the electrostatic interaction between the positively charged PEI/MWCNT-PEI and the negatively charged PCR components is important for improving the PCR specificity and efficiency. To further prove this hypothesis, four additional PEI derivatives were added into PCR reaction mixture, including the 50% acetylated PEI-Ac_{50}, 100% acetylated PEI-Ac, negatively charged PEI.SAH, and PEI modified with both PEG and acetamide (Fig. 3). For each additive, serial concentrations were added into the reaction mixture to optimize the enhancing effect, and the PCR products were analyzed after electrophoretic fractionation on agarose gels and staining with ethidium bromide.

Figure 3 shows the electropherograms of PCR products using PEI derivatives. It is clear that PEI. Ac_{50} and PEI-Ac can improve the efficiency and specificity of the non-specific PCR system. The specificity and efficiency data of each additive were given in Table 1. When PEI (Figure 2), PEI-Ac_{50}, and PEI-Ac were added into PCR mixture, there is a decrease in non-specific bands. However, when the concentrations of additives exceed the optimum concentration, the amplification of target band and the non-specific products

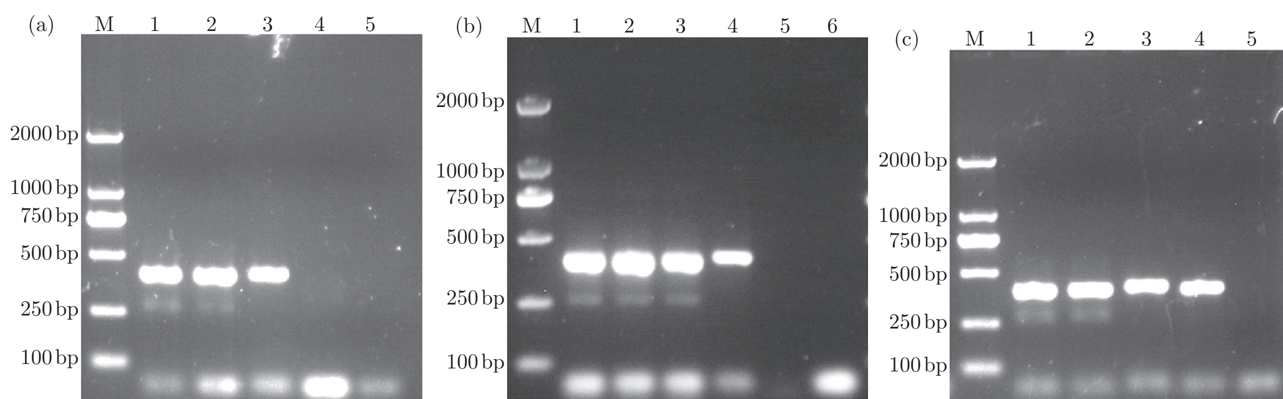

Fig. 2 The effects of MWCNT, MWCNT-PEI, and PEI on the specificity of PCR. Lane M is marker, the rightmost lane is negative control in each panel. (a) MWCNT was added into PCR mixture, from lane 1 to 4, its final concentration is 0, 3.1, 6.2, 12.4 mg/L, respectively. (b) MWCNT-PEI was added into PCR mixture, from lane 1 to 5, its final concentration is 0, 23.6, 47.2, 94.2, 188.8 μg/L, respectively. (c) PEI was added into PCR mixture, from lane 1 to 4, its final concentration is 0, 20, 40, 60 μg/L, respectively.

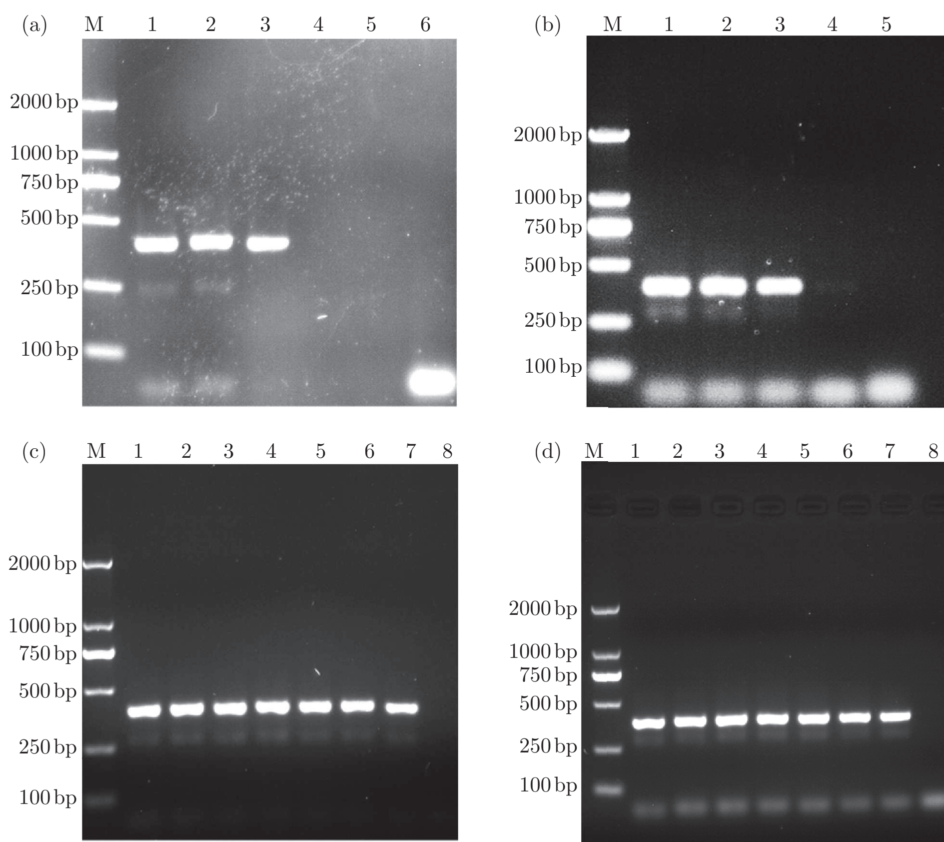

Fig. 3 Effect of PEI-based derivatives on PCR reaction. Lane M is marker, the rightmost land is negative control in every image. (a) PEI-Ac_{50} was added into PCR mixture, from lane 1 to line 5, its final concentration is 0, 9.0, 45, 90, and 135 μg/L, respectively. (b) PEI-Ac was added into PCR mixture, from lane 1 to 4, its final concentration is 0, 36, 360, and 720 μg/L, respectively. (c) PEI-SAH was added into PCR mixture, from lane 1 to 8, its final concentration is 0.0052, 0.041, 0.205, 1.04, 5.2, 21, 102, and 510 μg/L, respectively. (d) PEI-PEG-Ac was added into PCR mixture, Lane M is marker. From lane 1 to 8, its final concentration is 0.256, 1.28, 6.4, 32, 64, 96, 128, and 160 mg/L, respectively.

were significantly inhibited. The optimum concentrations of PEI.Ac_{50} and PEI-Ac were 45 μg/L and 0.36 mg/L, respectively. We can see that optimum concentration of PEI-based derivatives follows the order of PEI<PEI-Ac_{50} <PEI-Ac. With the increase of acetylation degree, the optimum concentration of PEI tends to increase, which is presumably due to the decreased the number of surface amine groups. It is interesting

to note that even for PEI-Ac, the surface amines of PEI cannot be completely acetylated due to the formation of ion pairs between the formed acetic acid and the PEI amines during the acetylation reaction. The negatively charged PEI.SAH and the PEGylated PEI-PEG-Ac could not enhance the efficiency and specificity of PCR even at the concentration of 4 orders of magnitude higher than that of PEI, which is due to the lack of the interaction between the negatively charged PEI-SAH or neutralized PEI-PEG-Ac with antifouling properties [31]. These results imply that the surface interaction between additives and PCR components plays an important role in enhancing specificity and efficiency of PCR.

Table 1 Optimum concentration, efficiency, specificity of additives in PCR reaction

additives	Optimum Concentration (mg/L)	PEI Concentration (μg/L)	Maximal efficiency	Maximal specificity
MWCNTs	6.2	-	1.60	1
MWCNT-PEI (PEI30.6%)	9.44×10^{-2}	28.9	1.25	1
PEI (PEI 100%)	4×10^{-2}	40	1.48	1
PEI-Ac_{50} (PEI 73.5%)	9×10^{-2}	66	1.44	0.97
PEI-Ac (PEI 58.2%)	0.36	209	1.05	1
PEI-Au (PEI 72%)	0.05	36	1.08	0.96

Fig. 4 PEI-Au was added into PCR mixture, Lane M is marker, the rightmost lane is negative control. From lane 1 to 5, its final PEI concentration is 0, 12.5, 25, 36, and 75 μg/L, respectively.

Our above results suggest that when PEI molecules are supported onto the surface of MWCNTs to form MWCNT-PEI, the required optimum concentration of PEI is lower than that of free PEI in terms of the PCR enhancing effect. With the support by MWCNTs, the interaction of PCR components with PEI could be significantly enhanced due to the increased binding sites, making the PCR enhancing effect much more effective. To further prove this mechanism, we prepared PEI-stabilized AuNPs (PEI-Au), where the PEI molecules are supported onto the surface of AuNPs. The addition of PEI-Au is able to enhance PCR, similar to the PEI polymer (Figure 4). The optimum concentration of PEI in PEI-Au is 36 μg/L, which is lower than that of PEI (40 μg/L). We think that similar to the MWCNT-PEI, the AuNP-supported PEI also enables much stronger electrostatic interaction with the negatively charged PCR components, affording enhanced PCR optimization with lower optimum concentration.

Conclusions

In summary, we studied PEI-based derivates and nanocomposites as additives to enhance the specificity and efficiency of PCR. Our data show that the positively charged PEI derivates are able to enhance the specificity and efficiency of the non-specific PCR system, whereas negatively charged PEI derivates and neutralized PEI-PEG-Ac with antifouling property have no such effect to enhance PCR. In addition, the PEI polymer supported onto the surface of MWCNTs or AuNPs appears to have a more pronounced effect to enhance the PCR. Our results clearly suggest that the electrostatic interaction between PEI derivatives with PCR components, especially with DNA polymerase, plays an important role in enhancing the PCR specificity and efficiency.

Acknowledgments

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