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Inhibition of polymerase activity by pristine fullerene nanoparticles can be mitigated by abundant proteins†

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Pristine fullerene nanoparticles are capable of adsorbing polymerase and significantly inhibiting its biologically important replication activity; however, the inhibition can be partially mitigated by abundant proteins through competitive binding.

Since its discovery in 1985,¹ the potential applications of fullerene (C_{60}) have been of intense interest because of its unique physicochemical properties.² Although pristine C_{60} cannot be spontaneously dissolved in water, colloidal aggregates of C_{60} at nanometre scales (nC_{60}) dispersed in aqueous solutions can be prepared by several methods, and are stable for months or years at room temperature.³ Nanometre C_{60} aggregates can migrate throughout the mouse body, accumulate in the liver,⁴ be distributed to the cytoplasm, to the lysosomes, along the plasma membrane, and to the nucleus of human monocyte-derived macrophages.⁵ However, it is still in doubt whether the nC_{60} can cause adverse biological consequences.⁶ Biomolecular interaction studies may further the understanding of the issue.

Fullerene may interact with functionally important but low abundant enzymes, DNA, and abundant proteins.⁷ “Protein corona” can be observed for the copolymer nanoparticles in complex cellular systems.⁸ However, it is not clear whether these interactions play some critical roles in the biological effects of nanoparticles, particularly in the biological matrix. There is a need for measuring the functional change of enzymes while assessing the molecular interactions in the biological matrix. In this work, we developed a real-time polymerase chain reaction (PCR) method to simultaneously measure the enzymatic activity and the interaction of fullerene and large biomolecules (proteins and DNA).

First, we investigated the effect of the nC_{60} on the PCR amplification of template DNA. By taking advantage of real-time PCR technology, both the enzymatic activity (reflected by PCR amplification) and amplified DNA products can be evaluated. The cycle threshold (C_t) value of real time-PCR is low (about 6.7) in the absence of C_{60} , indicating a fast PCR rate. In contrast, the C_t value increases to 14.8 in the presence of $4.0 \mu\text{g mL}^{-1}$ C_{60} , and the fluorescence intensity at the 25th cycle decreases to 28.3% of that of the positive control (Fig. 1). Both the increased C_t value and the decreased fluorescence intensity suggest that the nC_{60} effectively inhibits

PCR amplification. This inhibition is dose-dependent. The inhibition by the nC_{60} is negligible at $0.05 \mu\text{g mL}^{-1}$, and is complete at $5.0 \mu\text{g mL}^{-1}$ (Fig. 1). Polyacrylamide gel electrophoresis (PAGE) analysis of the PCR products (40 cycles) confirms the formation of the correct DNA product (75bp) and the dose-dependent inhibition of PCR activity by the nC_{60} (see the insert of Fig. 1).

PCR requires primed DNA as a template and active polymerase for replication. The PCR amplification may be inhibited due to the interaction of the nC_{60} with either DNA or Taq polymerase, reducing the quantity of the accessible template DNA or the polymerase activity. If the binding of template DNA to the nC_{60} contributes greatly to the inhibition, the addition of excess non-template DNA that is not complemented to the primers may significantly reduce the C_{60} induced PCR inhibition because of their competitive binding to the limited surface of the nC_{60} . However, the PCR inhibition by the nC_{60} is still significant and cannot be eliminated even in the presence of 100 times more non-template ssDNA (Fig. S1, ESI†). Surprisingly, it is also not possible to eliminate the nC_{60} induced PCR inhibition even by increasing template DNA over five times (Fig. 2, C1 to C4). These results suggest that such presumed interaction of C_{60} and DNA should be too weak to eliminate the inhibition of the PCR amplification. On the other hand, they may hint that the inhibition of PCR is due to the interaction of the nC_{60} with the polymerase.

To test this possibility, we gradually increased the amount of Taq polymerase while the primer, template DNA, and C_{60} were kept constant. As shown in Fig. 2 (B1 to B3), the PCR rate (evaluated by the C_t values, the maximum fluorescence

Fig. 1 Real-time PCR curves obtained by plotting SYBR Green I fluorescence intensity versus cycle number. The involved concentrations of C_{60} in the samples were varied as labeled for each curve. The concentrations of Taq polymerase and the template DNA were kept constant. The insert is an electropherogram from polyacrylamide gel electrophoresis analysis of the final PCR products (40 cycles) in the presence of C_{60} at varied concentration (0 – $5.0 \mu\text{g mL}^{-1}$).

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Fig. 2 Compensative experiments by re-addition of polymerase and template DNA in C_{60} inhibited real-time PCR, showing the elimination of C_{60} induced inhibition of PCR by addition of polymerase but not template DNA. (A) Amplification curve for exclusive inhibition by C_{60} (C_{60} : $5.0 \mu\text{g mL}^{-1}$, Taq polymerase: 2.0 units, Template DNA: 0.5 nM); (B1–B3): amplification curves obtained by addition of the Taq polymerase with a total activity of 2.5, 4.0, or 6.0 units (C_{60} : $5 \mu\text{g mL}^{-1}$, Template DNA: 0.5 nM); (C1–C4) amplification curves obtained by addition of the DNA template with a total concentration of 0.75, 1.0, 1.5, or 3.0 nM (C_{60} : $5.0 \mu\text{g mL}^{-1}$, Taq polymerase: 2.0 units).

intensity at the 25th cycle, and the fluorescence intensity *vs.* cycle number) gradually recovered to the level of the positive control on adding more Taq polymerase. This indicates that the inhibition by the nC_{60} can be abolished by the addition of more Taq polymerase, and proves that the inhibition by the nC_{60} can be mainly attributed to the reduced amplification activity of Taq polymerase, which may result from the interaction of the polymerase and the nC_{60} .

There are two possible manners for the nC_{60} to interact with the polymerase: (1) the incorporation of the C_{60} into the catalytic pocket of the polymerase, and (2) the adsorption of the polymerase on the surface of the nC_{60} . The first manner (incorporation) requires the C_{60} to exist as single molecules with much smaller size (0.7 nm). However, our experiment shows that the C_{60} in aqueous solution is dispersed as nanoparticles rather than single molecules. The measured mean hydrodynamic diameter of the nC_{60} is 50–100 nm by TEM (Fig. S2, ESI†). Such nanoparticle sizes are much larger (>20 times) than that of the polymerase (2–3 nm). Therefore, it is impossible for the polymerase to incorporate the nC_{60} into its catalytic pocket. The observed interaction of the polymerase and the nC_{60} should thus be attributed to the adsorption of the polymerase on the surface of the nC_{60} . The interaction of the polymerase and the nC_{60} was further confirmed by our fluorescence quenching experiments (Fig. S3, ESI†).

Besides functional enzymes, some abundant proteins (such as serum albumin) co-existing in biological systems cannot be neglected. It is interesting to know how such abundant proteins affect the inhibition of PCR by the nC_{60} . Serum albumins that are abundant in blood were used as examples. By pre-incubation of the nC_{60} with bovine serum albumin (BSA), the complete inhibition of PCR by the nC_{60} (5.0 mg mL^{-1}) is partially abolished, and the fluorescence intensity at the 30th cycle increases with the increasing concentration of BSA (Fig. 3, B1 to B3). In contrast, BSA itself displays negligible influence on PCR activity in the absence of C_{60} (Fig. 3, A1 to A3; Fig. S1, ESI†). These results indicate that BSA can

Fig. 3 Representative real-time PCR amplification curves, showing the elimination of nC_{60} induced PCR inhibition by addition of BSA. (A1–A3) BSA but no C_{60} was added to normal RT-PCR reaction solution. The final concentration of BSA is 0.01 (A3), 0.05 (A2), or 0.1 (A1) mg mL^{-1} . (B1–B3) BSA was pre-incubated with C_{60} then they were added to RT-PCR together. The final concentration of BSA is 0.01 (B3), 0.05 (B2), or 0.1 (B1) mg mL^{-1} . (C) Only C_{60} was added to RT-PCR. The final concentration of C_{60} used for PCR inhibition is $5.0 \mu\text{g mL}^{-1}$ (B1–B3, C).

partially eliminate the nC_{60} induced PCR inhibition by reducing the adsorption of polymerase on the nC_{60} . Human serum albumin (HSA) has a similar elimination effect (data not shown). The elimination of the PCR inhibition is dependent upon the concentrations of serum albumins. The lowest elimination concentrations are 0.01 mg mL^{-1} for BSA and 0.02 mg mL^{-1} for HSA (Fig. S4, ESI†), respectively.

The function of the polymerase is to amplify the template DNA. To carry out its amplification function, the polymerase must retain its native structure and be accessible to the template DNA. The adsorption of the polymerase on the surface of the nC_{60} may generate a steric barrier for the polymerase to access the template DNA, or denature the active site of the polymerase. Both the limited accessibility of the polymerase to the template DNA and the denaturation of the polymerase can prevent the PCR from proceeding. However, it is not clear which factor plays a major role in the inhibition of PCR by the nC_{60} . To clarify this point, we first incubated the nC_{60} with the polymerase followed by incubation with BSA. Real-time PCR analysis shows that the inhibition of the activity of the polymerase by the nC_{60} can be reversed by later addition of BSA (Fig. 4). This indicates that the adsorbed polymerase molecules are displaceable by BSA molecules from the surface of the nC_{60} , and the adsorbed Taq polymerase was not denatured. Since the inhibition of the PCR activity can be reversed, the displaced polymerase molecules should keep their native structure well in the adsorption state. Therefore, it is reasonable to conclude that the accessibility of adsorbed polymerase to the template DNA is restricted by the nC_{60} adsorption, leading to the PCR inhibition.

Taken together, we propose a simplified model to describe the multiple protein- nC_{60} interactions and the related functional change of the polymerase (Fig. 5). The polymerase chain reaction can be inhibited by water soluble fullerene *via* inhibition of the activity of polymerase. The inhibition of polymerase activity results from high affinity interaction of the nC_{60} and the polymerase, which renders a steric barrier to reduce the

Fig. 4 The influence of the addition sequence of C_{60} , BSA, and polymerase on the elimination of nC_{60} induced PCR inhibition by BSA. The fluorescence signal at the 30th cycle was estimated as the percentage of that of the positive control. The concentration of C_{60} used for PCR inhibition was $5 \mu\text{g mL}^{-1}$. The involved concentration of BSA was 0.05 mg mL^{-1} . (1) Positive control for PCR; (2) PCR in the presence of BSA; (3) PCR in the presence of C_{60} and BSA with a sequence of C_{60} first pre-incubated with BSA, then added to PCR solution containing polymerase; (4) PCR in the presence of C_{60} and BSA with a sequence of C_{60} pre-incubated with PCR solution containing polymerase, and then added BSA.

accessibility of the polymerase to the DNA template. By adding a highly abundant protein (e.g. BSA) that can interact well with the nC_{60} , the adsorbed polymerase can be displaced from the limited nC_{60} surface because of the competitive occupation of the abundant protein on the same nano-surfaces. The released polymerase can function for the PCR amplification again.

In this work, we demonstrate that C_{60} nanoparticles can adsorb polymerase and inhibit PCR amplification. Since the polymerase activity is associated with biologically important DNA replication and repair, the C_{60} nanoparticles induced inhibition of the amplification activity may affect DNA replication, DNA repair, and gene expression, and further change the cellular functions. Therefore, this study has very significant implications in biology. In some sense, the implicated

Fig. 5 A simplified model for illustration of the multiple interactions of nC_{60} , DNA polymerase, and a highly abundant protein (e.g. BSA) and consequent functional change of the DNA polymerase.

change of gene expression pattern suggests that the gene array based study may be useful to the understanding of the nanoparticle–cell interactions.

This is the first report that the lost activity of functional enzymes by C_{60} adsorption can be partially recovered by addition of physiologically relevant abundant proteins, establishing a novel concept that the inhibition of enzyme activity is determined by the balance of multiple protein–nanoparticle interactions. This concept should not be limited to nC_{60} –protein interactions, and should be extendable to the interactions of functional proteins with other nanoparticles. This study demonstrates the protection of functional proteins from the nanoparticles caused adverse effects by highly abundant proteins. Moreover, this work also demonstrates that real-time PCR is a sensitive tool for studying the interactions between proteins and fullerenes, including multiple interactions, and will be useful for research in the fields of biomaterials, drug delivery, and nanotoxicology.

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