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C₆₀ affects DNA replication *in vitro* by decreasing the melting temperature of DNA templates

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ABSTRACT

The effect of C₆₀ on DNA replication *in vitro* was studied by a quantitative real-time polymerase chain reaction (QPCR) system using a designed 110bp single-stranded DNA as the template. The results indicated that the efficiency of QPCR can be dramatically enhanced by C₆₀ at the beginning of the exponential phase, and that QPCR production can be significantly inhibited by C₆₀ at later cycles or at the plateau, which is the period represented by an interesting inverted U-shaped time response curve. Two different sized double-stranded DNA fragments (110bp and 60bp) were used to investigate the possible interaction between C₆₀ and DNA. The melting curve results showed that C₆₀ significantly decreased the melting temperatures (*T_m*) of the DNA fragments. By changing the concentrations of the initial DNA templates or C₆₀ in the QPCR system, we found that the decreased *T_m* value of DNA templates by C₆₀ is the primary reason for the increased QPCR efficiency. Our findings are consistent with the conclusions of other studies that C₆₀ can disrupt DNA replication *in vitro* by binding to DNA and changing the conformation of DNA templates.

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1. Introduction

C₆₀ is a novel carbon allotrope that was first discovered in 1985. It has a polygonal structure made up solely by 60 carbon atoms. During the last few years, the practical applications of C₆₀ as biological and pharmacological agents have been investigated. And its biological activities including antiviral, antioxidant, and chemotactic activities have also been tested on various fullerene-based compounds [1–4]. In recent years, some publications and reports have raised the health and safety concerns about the production and application of nanoparticles, especially in areas of exposure monitoring, personal use, environmental fate, and transport [5–11]. Although early studies showed low toxicity of the C₆₀ molecule itself [12], some recent studies found that C₆₀ is toxic to the biological systems. For example, C₆₀ is cytotoxic to several cell lines, C₆₀ can induce oxidative stress in the brain of juve-

nile largemouth bass and damage the environmentally relevant freshwater and marine species [13–18].

Many molecules can induce extremely negative biological effects by changing the conformation of DNA. By using atomistic molecular dynamics simulations, Zhao et al. [19] demonstrated that C₆₀ could strongly bind to nucleotides, suggesting that C₆₀ molecules have a potentially negative impact on the structure, stability, and biological functions of DNA molecules. Recently, the possible genotoxicity of C₆₀ was demonstrated by using single-cell gel electrophoresis assay [20].

The polymerase chain reaction has been widely used to simulate *in vivo* DNA replication in many fields of scientific research. In recent years, the quantitative real-time polymerase chain reaction (QPCR) was developed to monitor the process of amplification by using fluorescence [21]. In QPCR, an additional melting curve step is usually conducted following the conventional PCR program to evaluate the veracity of

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amplification products. Since the melting temperature (T_m) of DNA is strongly related to the length, guanine (G)/cytosine (C) frequency, sequence, and conformation of the DNA molecules, T_m can serve as the indicator of the conformational changes of DNA [22].

Previous research has shown that different nanomaterials can strongly bind to DNA, increase the specificity and efficiency of PCR, and selectively induce special DNA conformation formation [23–25]. Our hypothesis is that the nanomaterials can bind to DNA and induce DNA damage, inhibit or improve DNA replication, or cause other unknown negative biological effects *in vivo*. In this study, a simple and efficient method was established to evaluate the interaction between C_{60} and DNA conformation stability. A single-stranded DNA (ssDNA) was designed and artificially synthesized, and was used as templates in QPCR for QPCR efficiency evaluation. It was found that the efficiency can be dramatically enhanced by C_{60} at relatively lower concentrations of the DNA template. The melting curve analysis clearly showed that the T_m values of DNA fragments were significantly decreased by C_{60} . Therefore, the increased quantity of effective ssDNA templates in QPCR denaturation significantly improved the QPCR efficiency. Our results strongly suggested that C_{60} can bind to DNA and decrease the thermal stability of DNA.

2. Materials and methods

2.1. Water-dispersible C_{60}

C_{60} (99.5%, Alpha Aesar) was suspended in water as described by Yamakoshi et al. [26]. First, 8 mg C_{60} was completely dissolved in 10 ml toluene. Then 20 ml $CHCl_3$ and 1 g polyvinylpyrrolidone (PVP k-30, 95%, Shanghai Chemical Reagents Co) were added into the toluene solution. After adequate mixing, the solvent was thoroughly evaporated under vacuum for at least 1 h. The residue was re-dissolved in 20 ml Milli-Q water. The brown aqueous solution was obtained by ultrasonication. The final concentration of C_{60} is 400 $\mu\text{g/ml}$.

2.2. Single-stranded DNA template and primers

The sequence of the 110bp ssDNA template is 5'-GTTG-CCAGTGGGGGTTGGGGGGT TGGGGGTTGGGGGTTGGGGG-GTTGGGTCTCTGCTCTTGGTTTTTGGTTTTTGGTTTTTGG-TTTTTTGGTTTTGGTATAGCGC-3'. The forward primer sequence is 5'-GTTGCCAGTG-3' (for 110bp) or 5'-GTCTCTG-CTC-3' (for 60bp), and the reverse primer sequence is 5'-GCGCTATACC-3'. The amplified DNA fragments were designed to have different lengths, G-C contents, and theoretic T_m values for the purpose of this study. The DNA template and primers were all purchased from Sangon (Shanghai, China) and were diluted with Milli-Q water.

2.3. QPCR and the establishment of target fragment standard curves

QPCR was performed in a PTC-200 thermal cycler equipped with a Chromo4 real-time fluorescence detector (MJ

Research). The fluorescent agent SYBR Green I can emit fluorescent light when randomly intercalated into the minor groove of double-stranded DNA (dsDNA) and was used to monitor the production of double-stranded PCR products. Amplifications were carried out in Bio-Rad 0.2 ml white low tube strips with flat caps. Each reaction mixture contained 1 μl DNA template, 1 μl of 2 μM forward primer, 1 μl of 2 μM reverse primer, 10 μl of 2 $\times$ SYBR QPCR Master mix (Toyobo) and 1 μl or 2.5 μl of C_{60} solution or PVP solvent control, with a total volume of 20 μl . The final concentration of C_{60} in the reaction mixture was 20 or 50 $\mu\text{g/ml}$, and the final concentration of PVP in the QPCR tube was 2.5 or 6.25 mg/ml. The QPCR reaction mixture without C_{60} or PVP was used as the internal control, and deionized water was used as the negative control in all experiments. The synthesized DNA template was dissolved in Milli-Q water with a final concentration of 50 μM , and then diluted to 100, 50, 25, 20, 10, 5 and 1 nM. This dilution series of the DNA template was used to generate standard curves for 110bp fragment and 60bp fragment.

The QPCR reaction course consists of pre-denaturation at 94 $^{\circ}\text{C}$ for 1 min, denaturation at 94 $^{\circ}\text{C}$ for 20 s, annealing at 45 $^{\circ}\text{C}$ (for 110bp) or 40 $^{\circ}\text{C}$ (for 60bp) for 20 s, extension at 72 $^{\circ}\text{C}$ for 30 s, with a total of 45 cycles. During each cycle, the fluorescence intensity at 520 nm was recorded after the completion of extension at 72 $^{\circ}\text{C}$. The excitement wavelength is 490 nm.

2.4. Melting curve analysis

At the end of the QPCR program, a melting curve analysis was performed to study the possible interaction between C_{60} and DNA. During the process of cooling to 70 $^{\circ}\text{C}$ and ramping to 94 $^{\circ}\text{C}$, the fluorescence intensity of amplified products was recorded at every 0.1 $^{\circ}\text{C}$ increase. The recorded data was analyzed using Opticon Monitor software (MJ Research). The T_m at which half of the DNA base pairs disassociated was observed as a sudden decrease in the SYBR Green I fluorescence. The melting peak (T_m value) was obtained by plotting the negative first derivative of fluorescence ($-dF/dT$) against temperature (T) [22].

2.5. Conventional PCR

The synthesized 110bp ssDNA was used as the template for conventional PCR (TC-312, TECHNE, Cambridge, UK). The reaction was carried out in a final volume of 20 μl , including 1 μl DNA template, 1 μl of 2 μM forward primer, 1 μl of 2 μM reverse primer, 10 μl 2 $\times$ PCR Taq Master mix (Tiangen, China), 1 μl C_{60} solution, or 1 μl PVP solvent control. The PCR reaction program consists of pre-denaturation at 94 $^{\circ}\text{C}$ for 1 min, 25 or 45 cycles of 94 $^{\circ}\text{C}$ for 30 s (denaturation), 45 $^{\circ}\text{C}$ for 30 s (annealing) and 72 $^{\circ}\text{C}$ for 40 s (extension) followed by a final extension at 72 $^{\circ}\text{C}$ for 2 min.

PCR products were size-fractionated by agarose gel electrophoresis (2.5%). Gels were stained with ethidium bromide (Sigma), visualized and photographed by the gel imaging system (UVP). The intensities of ethidium bromide stained bands were analyzed with JD-801 gel imaging system (Jieda tech, Nanjing).

2.6. Statistical analyses

All statistical analyses were performed by using the SPSS statistical package (Version 10.0; SPSS Company, Chicago, IL, USA). Results were presented as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and Dunnett's test were used to compare the treatment means with controls. The statistically significance level was pre-set at 0.01.

3. Results

3.1. Effects of C_{60} on DNA replication *in vitro*

DNA conformational change is a common process in DNA replication. PCR, a method that simulates DNA replication *in vivo*, was employed to study the effects of C_{60} on DNA replication. Because the actual amount of initial templates generated in pre-denaturation could be different due to the non-constant heating efficiency of PCR machines, the ssDNA was used as the template in DNA amplification to minimize that effect on PCR amplification efficiency at the beginning of the exponential phase.

The initial optimal QPCR conditions were established in the absence of C_{60} or PVP. According to the quantitative theory of QPCR, the threshold was manually set at 0.1 and the cycle threshold (Ct) was defined as the cycle number at which a sample's fluorescence crossed this threshold. Theoretically, there is a quantitative relationship between the initial amount of DNA template and the amount of PCR product at any reaction cycle. Our standard curves showed that the Ct

values were linear with respect to the logarithm of initial DNA template concentrations (Fig. 1).

Various amounts of C_{60} were added into the QPCR reaction system, and the Ct value was chosen to evaluate the efficiency of QPCR amplification. A typical QPCR result of C_{60} treatment is shown in Fig. 2. The QPCR amplification curve of C_{60} treatment plateaus earlier and the final fluorescence intensity of C_{60} treatment was much lower than that of the control group.

C_{60} can significantly enhance the efficiency of QPCR at the beginning of the exponential phase. For 110bp and 60bp fragment amplification, both 20 $\mu\text{g/ml}$ C_{60} and 50 $\mu\text{g/ml}$ C_{60} significantly decreased the Ct values (Figs. 3 and 4). Because the Ct values were linear relative to the logarithm of initial DNA template concentrations, it is possible that the QPCR efficiency can be enhanced by increasing the amount of ssDNA template at the beginning stage of amplification. Our results showed that the enhancement induced by C_{60} was minimal in the highest concentration of the initial DNA template tested (2.5 nM). In lower concentrations (0.25 and 0.5 nM), the effect of C_{60} was much more obvious. In addition, 6.25 mg/ml PVP decreased the Ct values, and 2.5 mg/ml PVP had no effect on the efficiency of QPCR. However, the effects of C_{60} were more significant than that of PVP. The change of Ct value was dependent on the concentration of C_{60} and the quantity of initial DNA templates in the QPCR reaction system.

In order to evaluate the effects of C_{60} on QPCR amplification, the fluorescence intensity of all treatments were normalized to the level of the DNA template control group.

Fig. 1 – The amplification plots of 110bp DNA (top) and 60bp DNA (bottom). The inset figures are the standard curves of 110bp and 60bp DNA amplification with relative coefficients of 0.995 and 0.960, respectively.

Fig. 2 – A typical QPCR amplification plot of C_{60} treatment (0.25 nM 110bp DNA template, 20 $\mu\text{g}/\text{ml}$ C_{60}). The threshold was manually set at 0.1.

Fig. 3 – Effect of C_{60} on the Ct value of 110bp DNA template. The numbers on the bar are the average Ct values ($n = 4$). The asterisk denotes significant differences from the control at $P < 0.01$.

Fig. 4 – Effect of C_{60} on the Ct value of 60bp DNA template. The numbers on the bar are the average Ct values ($n = 4$); the asterisk denotes significant differences from the control at $P < 0.01$.

Each data point in the plot was the mean value of at least four individual experimental measurements (Figs. 5 and 6). The results showed that the efficiency of QPCR can be dramatically enhanced by C_{60} nanoparticles at the beginning of the exponential phase, and the PCR production can be significantly inhibited at later cycles or at the plateau phase. Similar to Ct results, in 0.25 and 0.5 nM initial DNA template groups, the enhancing property of C_{60} was stronger than that of the 2.5 nM control group. But in all test groups, C_{60} significantly inhibited PCR production at the plateau phase. At the late stage of PCR amplification, the curve of C_{60} group was lower than the contrast group and the ratio was about 0.5 or lower. The effect of C_{60} on DNA replication *in vitro* showed an interesting inverted U-shaped time response curve. On the other hand, 6.25 mg/ml PVP could enhance the PCR amplification at the beginning of QPCR but had no effect on PCR production at the plateau phase.

To further understand the impact of C_{60} on QPCR, conventional PCR was conducted under similar conditions. The agarose gel staining result showed that the quantity of 110bp

DNA PCR product was obviously increased by 20 μ g/ml C_{60} after 25 PCR cycles (Fig. 7). For the relatively lower initial DNA template group (0.5 nM), the addition of C_{60} in PCR reaction solution increased the yield amplification two fold compared to the control. On the other hand, like QPCR, C_{60} inhibited the generation of PCR products after 45 PCR cycles in a dose-dependent manner as C_{60} concentrations increased in the solution (Fig. 8). These results revealed that C_{60} could disrupt DNA replication *in vitro* and that the DNA template and Taq DNA polymerase are possibly the binding targets of C_{60} .

3.2. Effect of C_{60} on the T_m value of DNA template

The effect of C_{60} on QPCR amplification was further explored by performing a melting curve analysis. This additional time-temperature program provides a melting curve to detect the presence of non-specific PCR products. The non-specific PCR products showed melting peaks different from the target amplification product with regard to different DNA lengths,

Fig. 5 – Time response curves of 20 μ g/ml C_{60} (A, C, E) and 50 μ g/ml C_{60} (B, D, F) on the 110bp DNA template group.

Fig. 6 – Time response curves of 20 µg/ml C₆₀ (A, C, E) and 50 µg/ml C₆₀ (B, D, F) on the 60bp DNA template group.

G/C frequencies, sequences, etc. In contrast, the T_m value is a constant physical character for a specific DNA sequence. The change of T_m value is always related to the variation of micro-environment of the DNA. Therefore, the change of T_m value would indicate the conformational change of DNA since weak interactions are a major factor in determining DNA conformation stability.

Two different sized PCR products were used to evaluate the possible interrelation between the C₆₀ and DNA conformation stability. Because the T_m value is not related to the amount of DNA, the T_m values of same sized PCR products collected from various initial DNA template concentration groups were evaluated together. For the 110bp DNA, A/T content equal to G/C content and significantly greater T_m value than 60bp DNA, the T_m was 82.9 °C and was decreased to 82.2 °C by 50 µg/ml C₆₀. For the 60bp DNA, high A/T content and relative low T_m value, the T_m was 77.8 °C and was gradually decreased to 76.4 °C by increasing C₆₀ concentration (Figs. 9 and 10). Both

50 µg/ml and 20 µg/ml C₆₀ significantly decreased the T_m value of amplified DNA templates. The PVP had no apparent effects on the T_m value. These results suggested that the decrease of T_m value was related to the concentration of C₆₀ and the length of DNA.

To further confirm the results, another experiment was carried out to examine the effect of C₆₀ on the T_m of DNA template. Different amounts of C₆₀ were added into the QPCR reaction solution after amplification, and the resulting melting curve analysis was similar (data not shown).

4. Discussion

In this study, a special DNA template and different PCR primers were employed to generate an 110bp full fragment and a 60bp partial fragment. The two fragments were very different at G/C content, which provided two DNA templates with different T_m to study the impacts of C₆₀. The results showed that

Fig. 7 – Twenty microlitre per millilitre C_{60} increased 110bp DNA conventional PCR efficiency. The light intensity of each C_{60} treatment was normalized to the level of DNA template control group and represented as the ratio ($n = 4$). The inset is a typical electrophoresis result and the asterisk denotes significant differences from the control at $P < 0.01$.

Fig. 8 – Decrease of 110bp DNA conventional PCR efficiency in a C_{60} dose-dependent manner. The light intensity of each C_{60} treatment was normalized to the level of DNA template control group (2.5 nM) and represented as the ratio ($n = 4$). The inset is a typical electrophoresis result and the asterisk denotes significant differences from the control at $P < 0.01$.

C_{60} could decrease the T_m of DNA templates, higher concentration of C_{60} led to more decline in the T_m value of DNA template and that shorter DNA templates resulted in more significant impacts of C_{60} . This phenomenon can be explained by the assumption that C_{60} affects DNA conformation through a combination with its hydrophobic region.

The changes of Ct value in this study could directly reflect the quantitative differences of initial DNA templates. Due to the absence of biological feedback mechanisms in an *in vitro* experimental model, impacts of C_{60} on PCR may be the result from a variety of the interactions. At the early stage of PCR amplification, Ct value of C_{60} group decreased, which indicates that the DNA amplification products are larger than the control group after the same cycle of DNA amplification.

By comparing these results with the control group results processed under the same denaturation conditions, we proposed that the enhanced efficiency of PCR caused by C_{60} is due to the increased amount of single DNA templates at the beginning of the exponential phase. To test this hypothesis, different amounts of initial DNA template were amplified with C_{60} . The results provide positive evidence that the increase of PCR efficiency induced by C_{60} was significantly related to the quantity of the initial DNA templates. In the test groups with relatively low initial DNA template concentrations, the increase of PCR efficiency induced by C_{60} was much more significant than the 2.5 nM initial DNA template groups. The amount ratio of C_{60} and DNA template is the key factor in the PCR reaction, and the concentration of C_{60} remains

Fig. 9 – Effect of C_{60} on the T_m value of 110bp PCR products. The numbers on the bar are the average of T_m values and the sample size. The asterisk denotes significant differences from the control at $P < 0.01$. The inset is a typical melting curve for 110bp PCR product.

Fig. 10 – Effect of C₆₀ on the T_m value of 60bp PCR products. The numbers on the bar are the average of T_m values and the sample size. The asterisk denotes significant differences from the control at P < 0.01. The inset is a typical melting curve for 60bp PCR product.

constant during amplification. For the 2.5 nM initial DNA template groups, however, the amount of ssDNA templates in the PCR reaction solutions is much higher than the 0.25 or 0.5 nM initial DNA template groups after several exponential increases. Therefore, the results could not reveal the few change of ssDNA templates induced by C₆₀ in 2.5 nM initial DNA template groups after several amplification. Even the 20 µg/ml C₆₀ and 50 µg/ml C₆₀ could not significantly decreased the Ct values of 2.5 nM initial DNA template groups, but the final PCR products were significantly inhibited by C₆₀.

The modified C₆₀ could deliver DNA plasmid into cell by endocytosis, and the derivatives of C₆₀ could inhibit human immunodeficiency virus (HIV) reverse transcriptase or moloney murine leukemia virus (MMLV) reverse transcriptase activity [3,12,27,28]. Although the actual biological targets of these approaches are not clear, it was suggested to be related with DNA or enzymes. Our findings indicate that C₆₀ can significantly inhibit PCR production at later cycles or at the plateau. Our initial idea is that the DNA polymerase activity can be inhibited by C₆₀. In the PCR reaction system, the quantity of DNA polymerase is adequate for all amplification reactions. As PCR proceeds, the quantity of DNA template was greatly increased, and the activity of total DNA polymerase was decreased with the frequently changing temperatures. So at later cycles or at the plateau of PCR, the ratio between DNA polymerase, substrate, C₆₀, and DNA templates changed greatly. If the activity of total DNA polymerase was inhibited by C₆₀ at these times, the final PCR products should decrease greatly.

A separate experiment was carried out to determine the effect of C₆₀ on the DNA polymerase activity. The Taq DNA polymerase (Promega) was incubated with C₆₀ at 37 °C for several hours, and centrifuged at 10,000 g for 5 min to remove C₆₀ from the solution. The conventional PCR results showed that the activity of treated DNA polymerase was significantly

inhibited by increasing the amount of C₆₀. On the other hand, by increasing the amount of Taq DNA polymerase in the PCR reaction system, the inhibition induced by C₆₀ could be eliminated (data not shown). Therefore, we concluded that the decrease of PCR production is related to the lower activity of DNA polymerase caused by C₆₀. Considering the hydrophobic character of C₆₀, we suggest that the active site of Taq DNA polymerase is the actual binding site of C₆₀.

Exploring the valid method within the next 5–15 years to evaluate the toxicity of engineered nanomaterials is one of the greatest challenges in nanomaterials application [11]. The *in vitro* assays will be employed widely, since these assays are able to test specific biological and mechanistic pathways under controlled conditions and can be designed to be pragmatic and mechanism-based [29]. In this study, a DNA *in vitro* replication method was employed to evaluate the possible effects of C₆₀ on DNA replication. We demonstrated that C₆₀ can affect DNA replication *in vitro*. Understanding the effects of C₆₀ on DNA replication in cells and the biological effects of C₆₀ on DNA and enzyme conformational changes will need further investigation.

5. Conclusions

A simple and efficient method was developed to evaluate the effect of C₆₀ on DNA replication *in vitro*. We found that the efficiency of PCR can be dramatically enhanced by C₆₀ at the beginning of the exponential phase by decreasing the T_m of DNA template, and the PCR production could be significantly inhibited at later cycles or at the plateau due to the inhibited activity of DNA polymerases. These results suggest that C₆₀ may interfere with normal DNA replication *in vivo*, serving as a guide to identify potential adverse biological effects including toxicity of carbon nanoparticles in DNA damage.

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REFERENCES

- [1] Lyon DY, Adams LK, Falkner JC, Alvarez PJ. Antibacterial activity of fullerene water suspensions: effects of preparation method and particle size. *Environ Sci Technol* 2006;40(14):4360–6.
- [2] Chen YW, Hwang KC, Yen CC, Lai YL. Fullerene derivatives protect against oxidative stress in RAW 264.7 cells and ischemia-reperfused lungs. *Am J Physiol Regul Integr Comp Physiol* 2004;287(1):R21–6.
- [3] Nakamura E, Isobe H. Functionalized fullerenes in water. The first 10 years of their chemistry, biology, and nanoscience. *Acc Chem Res* 2003;36(11):807–15.
- [4] Wang IC, Tai LA, Lee DD, Kanakamma PP, Shen CK, Luh TY, et al. C(60) and water-soluble fullerene derivatives as antioxidants against radical-initiated lipid peroxidation. *J Med Chem* 1999;42(22):4614–20.
- [5] Dreher KL. Health and environmental impact of nanotechnology: toxicological assessment of manufactured nanoparticles. *Toxicol Sci* 2004;77(1):3–5.
- [6] Thomas K, Sayre P. Research strategies for safety evaluation of nanomaterials, part I: evaluating the human health implications of exposure to nanoscale materials. *Toxicol Sci* 2005;87(2):316–21.
- [7] Holsapple MP, Farland WH, Landry TD, Monteiro-Riviere NA, Carter JM, Walker NJ, et al. Research strategies for safety evaluation of nanomaterials, part II: toxicological and safety evaluation of nanomaterials, current challenges and data needs. *Toxicol Sci* 2005;88(1):12–7.
- [8] Colvin VL. The potential environmental impact of engineered nanomaterials. *Nature Biotechnol* 2003;21(10):1166–70.
- [9] Moore MN. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ Int* 2006;32(8):967–76.
- [10] Wiesner MR, Lowry GV, Alvarez P, Dionysiou D, Biswas P. Assessing the risks of manufactured nanomaterials. *Environ Sci Technol* 2006;40(14):4336–45.
- [11] Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, et al. Safe handling of nanotechnology. *Nature* 2006;444(7117):267–9.
- [12] Jensen AW, Wilson SR, Schuster DI. Biological applications of fullerenes. *Bioorg Med Chem* 1996;4(6):767–79.
- [13] Sayes CM, Fortner JD, Guo W, Lyon D, Boyd AM, Ausman KD, et al. The differential cytotoxicity of water-soluble fullerenes. *Nano Lett* 2004;4(10):1881–7.
- [14] Oberdorster E. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environ Health Perspect* 2004;112(10):1058–62.
- [15] Oberdorster E, Zhu S, Blickley TM, McClellan-Green P, Haasch ML. Ecotoxicology of carbon-based engineered nanoparticles: effects of fullerene (C60) on aquatic organisms. *Carbon* 2006;44(6):1112–20.
- [16] Lovorn SB, Klaper R. Daphnia magna mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles. *Environ Toxicol Chem* 2006;25(4):1132–7.
- [17] Isakovic A, Markovic Z, Todorovic-Markovic B, Nikolic N, Vranjes-Djuric S, Mirkovic M, et al. Distinct cytotoxic mechanisms of pristine versus hydroxylated fullerene. *Toxicol Sci* 2006;91(1):173–83.
- [18] Usenko CY, Harper SL, Tanguay RL. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. *Carbon* 2007;45(9):1891–8.
- [19] Zhao X, Striolo A, Cummings PT. C60 binds to and deforms nucleotides. *Biophys J* 2005;89(6):3856–62.
- [20] Dhawan A, Taurozzi JS, Pandey AK, Shan W, Miller SM, Hashsham SA, et al. Stable colloidal dispersions of C60 fullerenes in water: evidence for genotoxicity. *Environ Sci Technol* 2006;40(23):7394–401.
- [21] Jung R, Soondrum K, Neumaier M. Quantitative PCR. *Clin Chem Lab Med* 2000;38(9):833–6.
- [22] Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* 1997;245(2):154–60.
- [23] Li H, Huang J, Lv J, An H, Zhang X, Zhang Z, et al. Nanoparticle PCR: nanogold-assisted PCR with enhanced specificity. *Angew Chem Int Ed Engl* 2005;44(32):5100–3.
- [24] Li X, Peng Y, Ren J, Qu X. Carboxyl-modified single-walled carbon nanotubes selectively induce human telomeric i-motif formation. *Proc Natl Acad Sci USA* 2006;103(52):19658–63.
- [25] Cui D, Tian F, Kong Y, Titushkin I, Gao H. Effects of single-walled carbon nanotubes on the polymerase chain reaction. *Nanotechnology* 2004;15(1):154–7.
- [26] Yamakoshi YN, Yagami T, Fukuhara K, Sueyoshi S, Miyata N. Solubilization of fullerenes into water with polyvinylpyrrolidone applicable to biological tests. *J Chem Soc, Chem Commun* 1994;23(4):517–8.
- [27] Mashino T, Shimotohno K, Ikegami N, Nishikawa D, Okuda K, Takahashi K, et al. Human immunodeficiency virus-reverse transcriptase inhibition and hepatitis C virus RNA-dependent RNA polymerase inhibition activities of fullerene derivatives. *Bioorg Med Chem Lett* 2005;15(4):1107–9.
- [28] Foley S, Crowley C, Smaih M, Bonfils C, Erlanger BF, Seta P, et al. Cellular localisation of a water-soluble fullerene derivative. *Biochem Biophys Res Commun* 2002;294(1):116–9.
- [29] Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science* 2006;311(5761):622–7.