The scavenging of reactive oxygen species and the potential for cell protection by fullerenols with different isoelectric points

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ABSTRACT

Fullerenols were known as the major water-soluble derivatives of fullerene that possess particular significance biological effects such as anticancer active and free radical scavengers. We have reported a novel method for purifying nanoparticles of fullerenols, which based on different isoelectric points (pIs). Consistent with their cytoprotective abilities, these samples can scavenge the stable 2,2-diphenyl-1-picryhydrazyl (DPPH) radical in vitro with the following relative potencies: pI 7.0 > pI 5.29> pI 4.40> pI 2.81. The observed differences in free radical-scavenging capabilities support the hypothesis that physicochemical properties, such as surface chemistry induced differences in electron affinity, and degree of aggregation, influence the biological and biomedical activities of fullerenols with different pIs. These purified fullerenols with pI 2.81, pI 4.40, pI 5.29 and pI 7.0 were performed to determine the effect of purified fullerenols on cytotoxicity and DNA stability in Raw 264.7 cells. There no significantly different by statistical product and service solutions (SPSS) analysis. But only the sample of fullerenols with pI 2.81 caused obvious DNA damage to RAW264.7 cells in the continuous buffer system.

Keywords: Fullerenols, Isoelectric points, Reactive oxygen species, Biological effect in vitro

1 INTRODUCTION

Fullerenols were known as the major water-soluble derivatives of fullerene that possess particular significance biological effects such as anticancer active and free radical scavengers. Owing to their unique physical and chemical properties and the possibilities for various surface modifications, fullerenes based materials have attracted much attention in recent years[1]. In particular, nanoparticles of fullerenols, which are fullerenes with hydroxyl functionalized carbon cage, were found to exhibit surprising properties and biocompatibility. These nanoparticles can block apoptosis and inhibit lipid peroxidation by scavenging reactive oxygen species (e.g., H_2O_2, $O_2^{\bullet}$), making them ideal candidates for the treatment of neuro-degenerative disorders (e.g., Parkinson's and Alzheimer's disease) as well as the prevention of damages caused by ischemia reperfusion[2, 3]. Therefore, In this study, electron spin resonance (ESR) spin trap technique is used to provide direct evidence that fullerenols nanoparticles with different pIs can efficiently scavenge the stable free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and the nanoparticles protected cells from oxidative stress in vitro was discussed.

2 EXPERIMENTAL

2.1 Materials and methods

C60(OH)n nanoparticles were prepared as described previously[4]. (Xing et al., 2004) The nanoparicle characterizations have been described by our group (Juan Li et al,2012). The nanoparticles were aggregated in saline solution, which was measured by high-resolution transmission electron microscopy[5]. DPPH were purchased from Sigma-Aldrich (St. Louis, MO). All of the other reagents were at least of analytical grade.

2.2 Preparation of Fullerenols

Fullerenols were synthesized by the alkaline reaction[4]. Briefly, a solution of C_{60} in toluene was added with an aqueous solution containing NaOH and 40% tetrabutylammonium hydroxide (TBAH) as the catalyst [6, 7]. The mixture was stirred at room temperature for 24 h. The color of the solution changed from deep violet to colorless, while a brown sludge precipitated on the bottom of the beaker. The aqueous phase was then separated and evaporated under vacuum to obtain the crude product, which was further washed by methanol. The crude product was also passed through a Sephadex G-25 column and eluted with double distilled water. Then C60(OH)n nanoparticles were separated by Isoelectric focusing and purified. The structures of fullerenols were analyzed and characterized by Fourier transforminfrared spectroscopy and X-ray photoelectron spectroscopy in the paper (Juan Li et al,2012)[5].

2.3 DPPH. Scavenging Activity

Following our previously established methodology[8], ESR spectroscopy was used to measure scavenging of the DPPH free radical by fullerenol nanoparticles. Although DPPH· is a stable, nitrogen-centered radical that has no involvement in physiological processes, attenuation of the ESR signal for DPPH is one of the methods widely used to demonstrate the ability of a chemical to scavenge reactive oxygen species through donation of a hydrogen atom, or, in some cases, electron transfer (Huang et al., 2005). To measure scavenging of DPPH, the reaction mixture contained 50 µL of fullerenol nanoparticles in 0.85% NaCl and 50µL of 0.50 mM DPPH dissolved in ethanol. The final concentration of fullerenol nanoparticles used for measurements was 100 µM). ESR spectra were recorded at 1 and 11 min after the initiation of the scavenging reaction by adding fullerenol nanoparticles. Spectra were obtained using 20-mW incident microwave power and 100-kHz field modulation of 2 G, at room temperature. The scavenging effect was determined by comparison with a control group lacking fullerenol nanoparticles.

3 RESULTS

The scavenging activity of fullerenol nanoparticles with pI 2.81, pI 4.40, pI 5.29 and pI 7.0 for DPPH·, a stable nitrogen-centered radical, was determined. As shown in Fig. 1, DPPH· produced a characteristic ESR profile. Treatment with 100 µM fullerenol nanoparticles for 1 min resulted in reduction of ESR signals induced by DPPH·. Besides, addition of fullerenol nanoparticles caused a time-dependent decrease in the intensity of the DPPH· signal for up to 11 min. These results clearly indicate that under experimental conditions, the fullerenol nanoparticles are capable of scavenging the DPPH· stable radical in a time-dependent manner(see Fig 2). Clearance of hydroxyl radical(%) was calculated by the the change of the maximum of samples/ the change of the maximum of the controls.

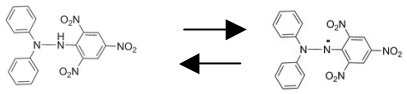

Figure 1: DPPH. produced a characteristic ESR profile

Clearance of hydroxyl radical(%) was calculated by the the change of the maximum of samples/ the change of the maximum of the controls. Consistent with their cytoprotective abilities, these samples can scavenge the stable 2,2-diphenyl-1-picryhydrazyl (DPPH·) radical in vitro with the following relative potencies: pI 7.0 > pI 5.29> pI 4.40> pI 2.81. The observed differences in free radical-scavenging capabilities support the hypothesis that physicochemical properties, such as surface chemistry induced differences in electron affinity, and degree of aggregation, influence the biological and biomedical activities of fullerenols with different pIs.

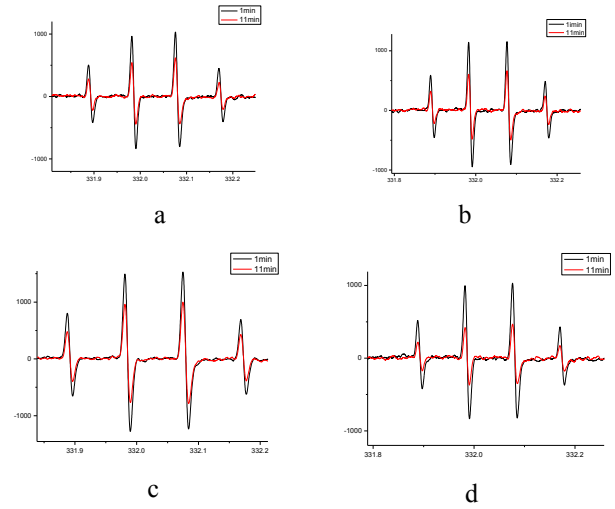

Figure 2: Scavenging of DPPH. by fullerenols with pI 2.81 (a), pI 4.40 (b), pI 5.29(c), pI 7.00 (d). For the samples contained 100 mM fullerenols and 0.1 mM DPPH. in 20% ethanol; and ESR spectra were recorded 1 and 11 min after sample mixing at room temperature.

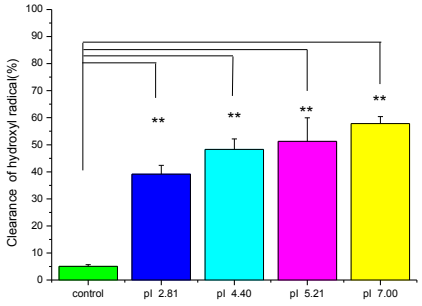

Figure.3: Clearance of hydroxyl radical by fullerenols with pI 2.81, pI 4.40, pI 5.29, pI 7.00. For each kind of treatments, three independent experiments were carried. All values are means ± standard error. **P < 0.01 when compared with control.

4 DISCUSSION

In this study, we used the ESR technique to provide direct evidence that fullerenol nanoparticles can markedly scavenge DPPH·. Some researchers have indicated that fullerenols would not induce cytotoxicity[9, 10]. Cytotoxicity assay was performed on RAW264.7 cell line using CCK-8 Kit (Supporting data Figure S2) and LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) by staining live cells with Calcein acetoxymethyl ester (calcein AM) (fluorescent green) and dead cells with ethidium homodimer (ED) (fluorescent red) (Fig 4). The cells were

treated with various concentrations of the each purified fractions of fullerenols. The results of cytotoxicity showed that the cells remained viable after exposure to the nanoparticles at 50-200 μM. Compared to the control, the results were not significantly different by statistical product and service solutions (SPSS) analysis. However, the more number of cells were stained by ED as cells were exposed to the fullerenols with pI 2.81 (Fig 4).

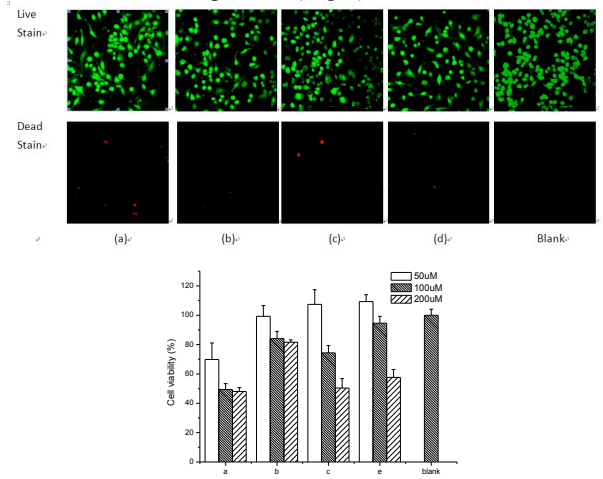

Figure 4: RAW264.7 cells were exposed to the fullerenols for 48h. The live and dead stains were shown by calcein AM (live cells fluoresce green) /ED (dead cells fluoresce red) fluorescent. Cells were treated by fullerenols with pI 2.81 (a), pI 4.40 (b), pI 5.29 (c), pI 7.00 (d) and blank is without fullerenol. For each kind of treatments, three independent experiments were carried. All values are means ±standard error. **P<0.01 when compared with control.

But in our previous work, we applied single-cell gel electrophoresis (SCGE), which is a sensitive high-throughput screening assay for detecting DNA strand breaks on the single cell level[11, 12], to determine if the purified fullerenols induced any DNA damaging effects and the sample of fullerenols with pI 2.81 maybe caused obvious DNA damage to RAW264.7 cells in the continuous buffer system(see fig 5).

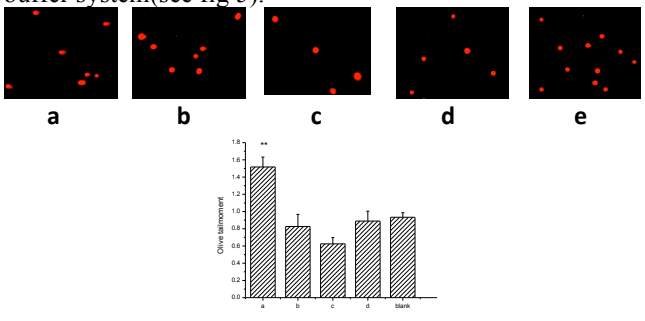

Figure 5. DNA damage is assayed by SCGE. RAW264.7 cells were exposed to the fullerenols for 24 h. Cells were treated by fullerenols with pI 2.81 (a), pI 4.40 (b), pI 5.29 (c), pI 7.00 (d) at a concentration of 50 lM and the blank is without fullerenol. For each kind of treatments, three independent experiments were carried. All values are means ± standard error. **P < 0.01 when compared with control.

Maybe the reason is that the radical-scavenging capabilities of fullerenol nanoparticles with pI 2.81 is the worst among the four samples. The samples of fullerenol nanoparticles with pI 2.81 were a mixture of fullerenols with different chemical structures, while the fullerenols in this study were purified with specific structures.Fullerenes has very strong ability of electrophilic and the reaction of free radicals is priority to the multiple addition reaction with C = C bond. In the process of fullerenols synthesis, some of the C = C bond on fullerene molecules were open and hydroxylated, but there were still a lot of C = C bonds, so fullerenols still have certain ability of electrophilic and the scavenging of free radical may open a C = C bond and add two –OH. On the other hand, the different electronegativities of C=O and C-OH on surface of carbon cage provide probability to separate fullerenols. There more electronegativities C=O on the structure of fullerenols with pI 2.81. Therefore, fullerenols with low degree of C=O functionalization are required for biocompatibility and application, which could be obtained by using the IEF purification method we have developed.

5 ACKNOWLEDGMENT

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