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Radical scavenging activities of α-alanine C_{60} adduct

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Abstract—Water-soluble α-alanine C_{60} adduct was synthesized, and its scavenging abilities for superoxide anion $O_2^{\cdot-}$ and hydroxyl radical ·OH were studied by the spectrophotometry and chemiluminescence. It was found that α-alanine C_{60} adduct showed an excellent efficiency in eliminating superoxide anion and hydroxyl radical. The 50% inhibiting concentration (IC_{50}) for superoxide was 184 μg/mL by spectrophotometry and 292 μg/mL by chemiluminescence. The IC_{50} for hydroxyl radical was 42 μg/mL. In different test systems, the results showed that α-alanine C_{60} adduct had comparable radical scavenging abilities as thiourea and ascorbic acid, and was proved to be an effective scavenger for superoxide anion and hydroxyl radical. It can be prospected that water-soluble α-alanine C_{60} adduct will be useful in radical related biomedical fields.

The peculiar cage structure of C_{60} has attracted most attention since its discovery. Many research achievements have been gained in the fields of superconductivity, magnetics, photology, catalysis, and polymer science.[1] Among them, it is very important the biological activity study of C_{60} and its derivatives. Many experiments have affirmed that C_{60} derivatives have prospective applications in many fields such as enzymatic inhibition, anti-HIV activity, neuroprotective, antibacterial, DNA cleavage, and photodynamic therapy.[2] Neuroprotective properties are based on the fact that C_{60} derivatives can scavenge free radicals.[3] Chiang and Zhu have separately reported the scavenging effect of fullerenols on superoxide anion $O_2^{\cdot-}$ and hydroxyl radical ·OH.[4,5] Recently, many water-soluble C_{60} derivatives have been found to possess effective free radical scavenging activities.[6–12]

It is known that many phenomena in biological systems are interrelated to free radicals. Excessive free radicals will cause cell trauma, pathological changes, and unusual death. Superoxide anion is the essential free radical and can form other free radicals through a series of reactions. The chemical activity of hydroxyl radical is the strongest, which can easily react with biomolecules such as amino acids, proteins, and DNA.[13] So it is very important to search highly effective scavengers for superoxide anion and hydroxyl radical. C_{60} has thirty C=C double bonds and is regarded as 'sponge for radical absorbing'.[14] Considering its insolubility in water, so it is essential to synthesize water-soluble C_{60} derivatives for researches on their biological activities. In this paper, we report the synthesis of water-soluble α-alanine C_{60} adduct and its scavenging ability for superoxide anion and hydroxyl radical by spectrophotometry and chemiluminescence.

α-Alanine (3.5 g) and sodium hydroxide (1.5 g) were dissolved in 4.0 mL deionized water, 40.0 mL ethanol was added, and the resulting solution was added dropwise to a stirred dry toluene solution (100 mL) containing C_{60} (⩾99.9%, purchased from Wuhan University, 100 mg). α-Alanine C_{60} adduct was synthesized in alkali heterogeneous phases. After stirring at room temperature for five days, the aqueous layer was separated from the colorless organic layer, diluted with 5.0 mL H_2O, 50.0 mL ethanol was then added to cause the precipitation of product. The precipitation was purified several times by dissolving in water, precipitated from absolute ethanol, then dried under a vacuum. Ninhydrin test showed no free α-alanine present in the product. The product was separated by HPLC and showed one peak.[15] FTIR and ^1H NMR (500 MHz) were used to characterize α-alanine C_{60} adduct.[16] The incorporation of α-alanine group in C_{60} was confirmed by absorption bands (IR) at 1406 [$\gamma_{sym}(COO^-)$] and 1589 cm^{-1}[$\gamma_{as}(COO^-)$], and proton absorption (^1H NMR) at $\delta = 1.91$–2.01 and

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3.94–4.12 ppm. The broad bands at $\delta = 1.27$–1.39 and 3.58 ppm may be due to protons on the C_{60} molecule. Compared with C_{60}, α-alanine C_{60} adduct has different soluble properties. It cannot dissolve in benzene, absolute methanol or ethanol. α-Alanine C_{60} adduct (1.5 mg) can dissolve in 1.0 mL dimethylsulfoxide and 2.0 mg in 1.0 mL water.

Superoxide anion scavenging activity was determined by spectrophotometry according to the following procedure. 0.3 mL of 3 mmol/L pyrogallol, 4.5 mL of 100 mmol/L buffer solution (Tris–HCl, pH 8.2), and 4.2 mL redistilled water were mixed intensively after pre-equilibrated at 25 °C. The absorbance of the above mixture at 325 nm was measured every 30 s on an ultraviolet–visible spectrophotometer (WFZ 800-D 2C). The autoxidation rate of pyrogallol, calculated from the change of absorbance at 325 nm, was controlled about 0.070 by adjusting the concentration of pyrogallol. The autoxidation rate of pyrogallol was represented by the increment of absorbance every minute in the linear range. The scavenging rate of α-alanine C_{60} adduct to superoxide anion radical was calculated as per the following formula: $SR = (K_0 - K_1)/K_0 \times 100\%$, where K_0, K_1 are autoxidation rates of pyrogallol without and with α-alanine C_{60} adduct, respectively.

Superoxide anion was generated by a luminol-enhanced autoxidaton of pyrogallol and determined by chemiluminescence. 0.8 mL of luminol (1×10^{-3} mol/L) and 0.2 mL $Na_2CO_3/NaHCO_3$ buffer solution (0.05 mol/L, pH 10.3) were mixed vigorously, and the background intensity was measured on a bio-chemical luminescence-measuring instrument (SGH-C, made in Shanghai). Then 0.1 mL pyrogallol (6.25×10^{-4} mol/L) was added to initiate the luminescence reaction. The luminescence kinetics curve chemiluminescence (CL–*t*) was measured with an integral time of 6 s and accumulated continuously for 150 s.The relative content of superoxide anion radical $O_2^{\cdot -}$ in the system was calculated as the abstraction value of the peak value of curve CL–*t* and the background. α-Alanine C_{60} adduct was added into the above system, and at the same time the volume of the buffer solution was reduced to keep the volume constant. The scavenging rate for superoxide anion $O_2^{\cdot -}$ was calculated as: $SR = (CL_0 - CL_1)/CL_0 \times 100\%$, where CL_0 and CL_1 represent peak values in the CL–*t* curves of the control group and test group, respectively. Every data point was obtained from three parallel determinations. The tolerance was no more than 3%. The free radical produced in the system was proved to be superoxide anion tested by superoxide dismutase (SOD), catalase, and mannitol.

Hydroxyl radical scavenging activity was determined by chemiluminescence. 0.4 mL $CuSO_4$ (2.0 mmol/L), 0.2 mL ascorbic acid (2.0 mmol/L), 0.6 mL of 50.0 mmol/L phosphoric acid buffer solution (or α-alanine C_{60} adduct), and 0.2 mL zymosan (25.0 mg/mL) were mixed intensively and the background intensity was measured on a bio-chemical luminescence-measuring instrument (SGH-C, Shanghai). H_2O_2, 0.6 mL, (66.0 mmol/L) was added to initiate the luminescence reaction, and the chemiluminescent emission from the resulting mixture was counted at an interval of 15 s for 100 times. The amount of hydroxyl radical in the system was represented by the chemiluminescence intensity. The scavenging rate for hydroxyl radical was calculated according to the following formula: $SR = (CL_0 - CL_1)/CL_0 \times 100\%$, where CL_0, CL_1 are the chemiluminescence intensities in the system without and with scavenger, respectively. The free radical produced in this system was proved to be hydroxyl radical tested by superoxide dismutase (SOD), catalase, and mannitol. Every data point was obtained from three parallel determinations. The tolerance was no more than 3%. Thiourea and ascorbic acid were used as a control. α-Alanine was used as a radical scavenger in the above three antioxidant evaluation systems.

The autoxidation of pyrogallol has been widely used in the determination of active of SOD and valuation of antioxidative properties of some substance.[17,18] Figure 1, lines a and b shows the change of absorbance *A* with the increase of time *t* when no and 17 μg/mL α-alanine C_{60} adduct were added, respectively. Compared with line a, the slope of line b decreased obviously, which indicated that the content of superoxide anion decreased, that is, α-alanine C_{60} adduct has scavenged superoxide anion in the system.

The superoxide anion scavenging effect of α-alanine C_{60} adduct at different concentrations is shown in Figure 2. The scavenging rate increased with the increase of concentration of α-alanine C_{60} adduct. At the concentration of 184 μg/mL, a superoxide anion scavenging rate of approximately 50% was achieved, that is, the 50% inhibition concentration (IC_{50}) was 184 μg/mL. The maximum scavenging rate of α-alanine C_{60} adduct against superoxide anion was 74.8% when the concentration was 255 μg/mL. In the above system, the IC_{50}'s of tea polyphenols, thiourea, and ascorbic acid were 80, 205, and 312 μg/mL, respectively. So, α-alanine C_{60} adduct has comparable scavenging ability as thiourea and ascorbic acid, and is an effective superoxide anion scavenger.

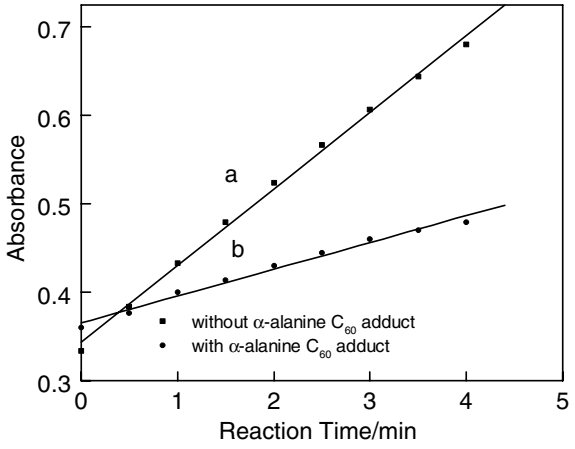

Figure 1. Absorbance of autoxidation of pyrogallol without scavengers and with scavenger concentration of 17 μg/mL.

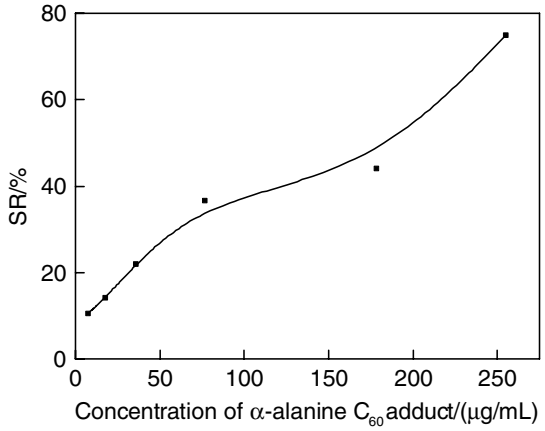

Figure 2. Scavenging rate of α-alanine C_{60} adduct against superoxide anion at different concentrations by spectrophotometry.

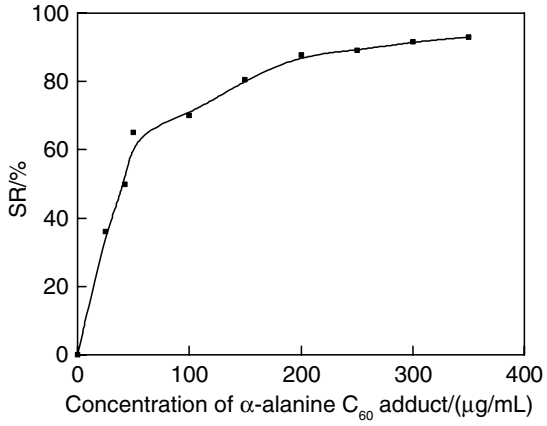

Figure 4. Scavenging rate of α-alanine C_{60} adduct against hydroxyl radical at different concentrations by chemiluminescence.

In the luminol-enhanced antioxidation of pyrogallol, the content of superoxide in the system can be measured by the intensity of chemiluminescence. When free radical scavengers were added into the reaction system, the amount of superoxide anion would decrease, and thus the chemiluminescence intensity would be inhibited. The stronger the scavenging ability, the more the chemiluminescence intensity would be inhibited. As shown in Figure 3, the scavenging rate increased with the increase of concentration of α-alanine C_{60} adduct. The IC_{50} was 292 μg/mL. The maximum scavenging rate of α-alanine C_{60} adduct against superoxide anion was 84.1% when the concentration was 1050 μg/mL. In this system, the IC_{50}'s of tea polyphenols, thiourea, and ascorbic acid were 113, 258, and 253 μg/mL, respectively. Though in different determined systems, the results showed that α-alanine C_{60} adduct has comparable scavenging ability as thiourea and ascorbic acid, and is an effective superoxide anion scavenger.

Similar to scavenging of superoxide anion, in the copper-catalyzed Haber–Wiess reaction, the scavenging rate for hydroxyl radical may be calculated by the ratio of chemiluminescence intensity with and no α-alanine C_{60} adduct added. The scavenging rate of α-alanine C_{60} adduct against hydroxyl radical at different concentrations is shown in Figure 4. The IC_{50} is about 42 μg/mL, and the maximum scavenging rate was 93% at the concentration of 350 μg/mL. As compared, the IC_{50}'s of thiourea, benzoic acid, and mannitol were 20, 201, and 123 μg/mL, respectively. The results showed that α-alanine C_{60} adduct was an effective scavenger for hydroxyl radical.

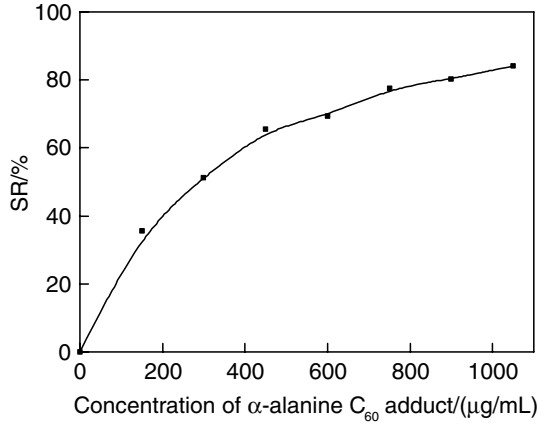

Figure 3. Scavenging rate of α-alanine C_{60} adduct against superoxide anion at different concentrations by chemiluminescence.

In the above three determined systems for superoxide anion and hydroxyl radical, α-alanine was added into the systems as radical scavenger but showed no scavenging effect on superoxide anion or hydroxyl radical. This indicated that the scavenging ability of α-alanine C_{60} adduct might be concerned with the C=C double bonds in C_{60}. C_{60} with thirty C=C bonds is typical alkene that is lacking electron, and has the ability of capturing electron, so it is called 'sponge of absorbing free radical'.[14] But C_{60} has poor water solubility, which restricted its application in biomedical fields. After the addition of water-soluble amino acid chains, the adduct still has some C=C double bond and retains the scavenging ability for superoxide anion and hydroxyl radical. It can be prospected that water-soluble amino acid C_{60} adduct will be a kind of effective radical scavenger and will be applied in the radical related fields such as neutroprotection.

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15. The α-alanine C_{60} adduct was solved in redistilled water to prepare a solution of 400 ppm and separated in high-performance liquid chromatograph HP1100. The flow phase was made of H_2O (300 mL), CH_3OH (200 mL), and 2.5 mL CH_3COOH and the detection wavenumber was 278 nm.
16. A Fourier-transform infrared (FTIR) spectrometer (Nicolet NEXDS 470) was employed to confirm the structures of α-alanine C_{60} adduct. ^1H NMR spectrum was measured on a Bruker DMX 500 spectrometer. α-Alanine C_{60} adduct was dissolved in D_2O. ^1H chemical shifts were expressed in parts per million downfield from the signal for sodium 3-trimethylsilyl-1-propanesulfonate (DSS) as an internal reference.
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