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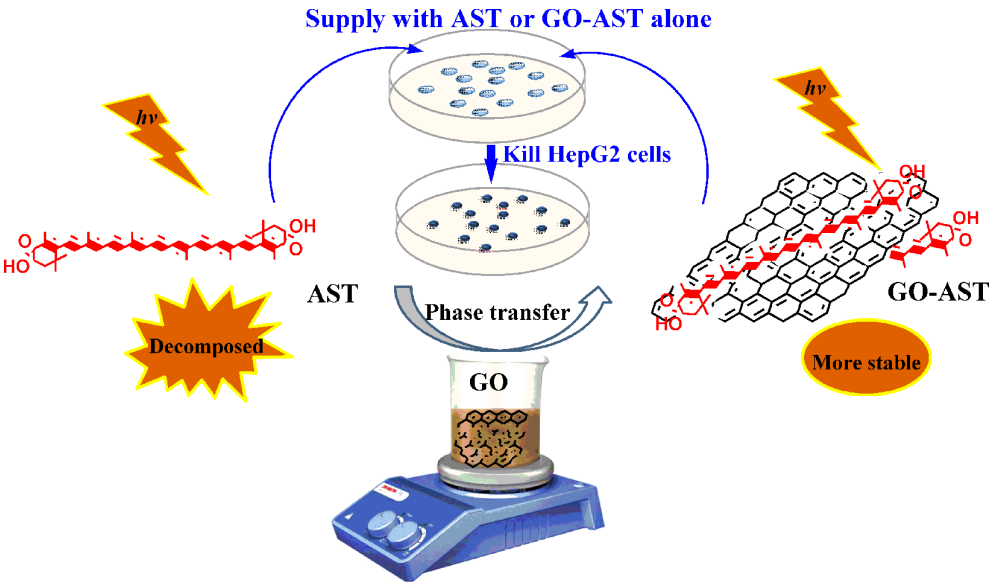
Graphical Abstract:



Schematic to illustrate the facile phase-transfer of AST by GO via simple stir to improve the property of AST, such as poor water solubility, storage stability and antitumor activity.

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"Pulling" π-conjugated polyene biomolecules into water: with the enhancement of light-thermal stability and bioactivity by a facile graphene oxide-based phase transfer approach

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This work demonstrated graphene oxide (GO) could not only be exploited as a nanovector to efficiently transfer π-conjugated polyene biomolecules from organic phase to aqueous one, but also benefit for the enhancement of light-thermal stability and bioactivity of the transferred π-conjugated polyene biomolecules.

Graphene oxide, emerged as an allotrope of carbon atoms tightly packed into a two-dimensional (2D) crystalline lattice of hexagonally arranged, consists of intact graphitic regions interspersed with sp^3-hybridized carbons containing -COOH, -OH, and epoxide functional groups on surfaces of each GO sheet and sp^2-hybridized carbons on the aromatic network.[1, 2] Due to its intrinsic and unique structure and physical, mechanical, and optical properties,[3,4] GO has attracted enormous attention and shown great potential in biomedical and biological applications, such as cell imaging,[5] photothermal therapy,[5, 6] and biomedical devices.[7] Particularly, the π conjugated hexagonal cells of GO can form π-π stacking interaction with the aromatic ring structure of molecules. Add that into the consideration with good aqueous dispersity, recently, GO based layers were demonstrated to be able to serve as efficient carrier systems for the delivery of water insoluble chemical drugs and biomolecules with aromatic structure in many papers.[1, 8, 9] Thus far, little has been done to explore the phase-transfer of π-conjugated polyene biomolecules without aromatic structure by GO, despite some efforts in the area of carbon nanotubes to encapsulate β-carotene and investigate the mechanism of incorporation, mainly *via* π–π interaction between its conjugated bonds and π conjugated hexagonal cells of nanotube.[10, 11]

Astaxanthin (AST), as a typical π-conjugated polyene biomolecule, is synthesized by phototrophic organisms and many non-phototrophic species (except animals). AST possesses various pharmacological activities both in *vivo* and in *vitro*, such as antioxidant activity, *anti*-inflammatory properties and enhancing cognitive function and immune response.[12] With various superior functions, AST has widespread applications in medical, nutraceutical, cosmetic and aquaculture industry. However, the highly unsaturated structure of AST results in that AST is hydrophobic and susceptible to degradation during the manufacture and storage, which severely debases its bioavailability and hampers its extensive application.[13] To solve this problem, several delivery systems, such as incorporation into emulsions, microencapsulation into chitosan matrix and encapsulation into polymeric nanospheres[12], have been proposed. However, these methods need complex synthesis progress of phase-transfer systems and have low transfer efficiency. An easy transfer system with high efficiency of AST loading for large-scale application is thus highly demanded.

Herein, we report a facile, low-cost and efficient approach, based on the phase-transfer capability of GO, to improve the aqueous dispersity of AST. Although there is no report about transferring π-conjugated polyene biomolecules by GO, from a structural point of view, phase-transfer is possible as GO can form π-π stacking interaction in the same way that nanotube encapsulates β-carotene,[10] as well as the hydrophobic interaction between them. Additionally, several -OH on biomolecules can form a strong hydrogen bond interaction with the -OH and -COOH groups on the GO sheet. Thus, it is possible to realize phase transfer of π-conjugated polyene biomolecules from organic phase to aqueous phase *via* non-covalent interaction between GO and AST to form GO-AST complex (Fig. 1).

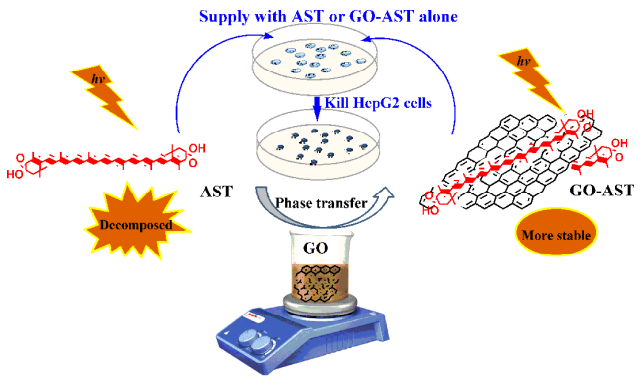

Fig.1 Schematic to illustrate the facile phase-transfer of AST by GO via simple stir to improve the property of AST, such as poor water solubility, storage stability and antitumor activity.

To demonstrate the feasibility of the proposed approach for phase transfer of π-conjugated polyene biomolecules, GO was prepared by oxidizing graphite using a modified Hummer's method.[1] Then the phase-transfer of AST was carried out by mixing AST dissolved in ethyl acetate with a GO water solution under a nitrogen atmosphere and stirred at 25ºC in the dark for 48 h. The mixing solution was stood overnight for stratification (Shown in Fig. S1b), and then the aqueous phase was collected. The obtained solution was monitored by UV-vis spectroscopy and the result was displayed in Fig. 2a. It reveals the characteristic AST peaks (near 491 nm) superimposing with the typical absorption of GO (at 230 nm), suggesting that AST was transferred by GO, forming GO-AST phase-transfer complex. At the same time, different colors of AST, GO-AST and GO are observed from the inset of Fig. 2a, which visibly shows the information of GO-AST phase-transfer complex.

To further demonstrate the formation of the GO-AST complex, FTIR experiment was then carried out. The FTIR spectrum of GO (Fig. 2b) reveals the characteristic bands of C=O stretching vibrations from carbonyl and carboxyl groups around 1730 cm^{-1}. The band at 1628 m^{-1} corresponds to C=C stretching and skeletal vibrations from un-oxidized graphitic domains. 1380 cm^{-1} is for absorption band of O-H bending vibrations from hydroxyl groups. The peaks around 1229 cm^{-1} and 1073 cm^{-1} are attributed to C-OH stretching vibrations and stretching vibration of C-O in ethers and/or epoxides, respectively.[14] FTIR spectrum of pure AST shows characteristics of very strong absorption bands at about 1656 cm^{-1} for C=O stretching vibration, 1554 cm^{-1} for stretching vibration of C=C in the hexatomic ring, and 1281 and 1075 cm^{-1} for stretching vibrations of C-OH. 1367 cm^{-1} and 1037 cm^{-1} were for the symmetric deformation (CH deformation in plane) and rocking of methyl, respectively. 966 cm^{-1} was assigned to C-H stretching vibration in π conjugate system and 671 cm^{-1} belongs to C-H stretching vibration in methyl.[15] After GO interacted with AST, it could be seen that the FTIR spectrum of the GO-AST exhibits both the typical AST and GO absorption features. Additionally, FESEM and TEM were also used to confirm the fabrication of the complex. As presented in Fig. 2c, a number of flake-like nanostructures with smooth surface of GO are observed. For the GO-AST sample, however, the surface roughness increases (Fig. 2d), indicating that AST is loaded onto the surface of GO. TEM image (Fig. 2e) illustrates the morphology of GO, which clearly exhibits the flake-like shapes of GO on the plane of which some corrugations and scrolling are observed. Whereas, when GO interacted with AST, as shown in Fig. 2f, some visually speckles-like nano-structures are clearly illustrated on the surface of GO. The reason might be the fact that AST attached on the surface of GO sheet is carbonized at a high applied potential difference during the measurement process of FESEM and TEM.[16, 17] Taken together, all these results clearly confirm that AST molecules have been successfully transferred from ethyl acetate to water by the formation of GO-AST complex.

After having successfully transferred AST from organic phase to aqueous one by assembling GO-AST complex, the phase-transfer capacity of GO for AST was further evaluated. It should be pointed out that the amount of AST in the GO-AST complex could not be directly quantitated by the adsorption spectrum of GO-AST complex because of the severe background interferance of GO. Thus, AST extraction from freeze-dried GO-AST (~1.7 mg), as complete as possible, was conducted with dichloromethane prior to the spectrometric analysis (Shown in Fig. S1c). The extraction was then concentrated to dry under nitrogen, and redissolved in DMSO for the UV-vis analysis of AST according to the standard curves of AST. Based on the regression equation and the absorbance value (0.520) of AST extracted from GO-AST complex in Fig. 3a, the concentration of AST in the final DMSO solution is 2.506 μg/mL.

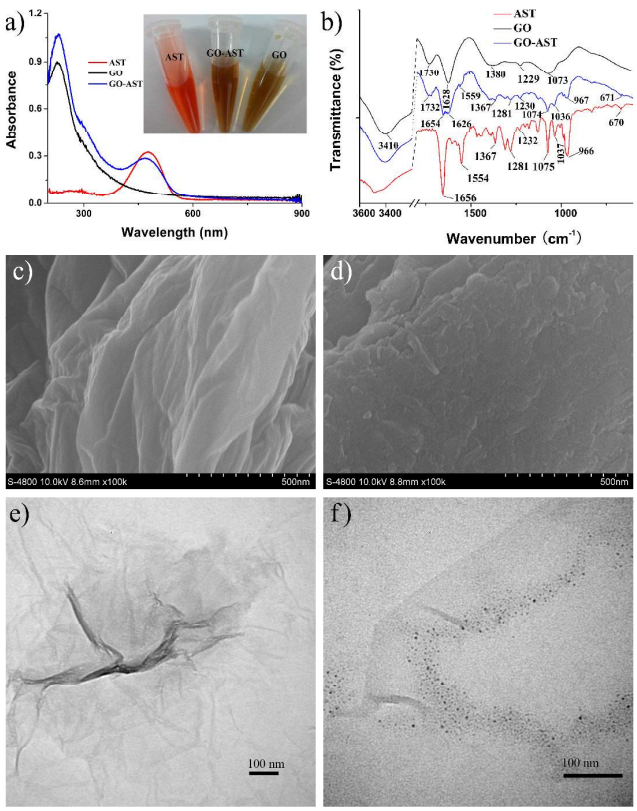

Fig. 2 Characterizations of as-prepared materials by UV-vis spectroscopy, FTIR, FESEM and TEM. (a) The UV-vis spectra and (b) FTIR spectra of AST, GO and GO-AST. (Inset) A photo of GO, AST and GO-AST in solution. FESEM photographs of GO (c) and GO-AST (d); and TEM photographs of GO (e) and GO-AST (f).

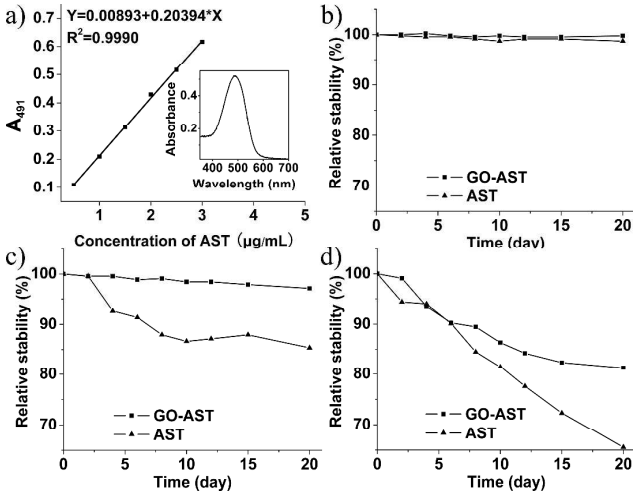

Fig. 3 Normalized curve of AST and storage stability of two ASTs. (a) The normalized curve of AST in DMSO (0.5~3.0 μg/mL) (Y is the UV absorbance at 491 nm, X is the concentration of AST), (inset) the UV-vis spectrum of extracted AST redissolved in DMSO. Relative storage stability of AST and GO-AST stored at 4 ºC (b) and 37 ºC in dark (c), and 37 ºC in natural light (d).

Therefore, the concentration of AST in GO-AST stock solution is 50.12 μg/mL, and 1 g GO can transfer ~58.96 mg AST, which is similar to that of AST/β-cyclodextrin inclusion complexes prepared at 25 ºC.[18] Thus, AST can be efficiently transferred from the organic phase to the aqueous one by GO. The strong phase-transfer of GO could be attributed to two aspects. On one hand, AST molecules are largely carried on the surface of GO by π-π stacking interaction mainly and hydrophobic interactions. On the other hand, the abundant -COOH and -OH on the surface of GO promises GO can still disperse in water after the loading of AST. That is to say, the solubility of AST in water is highly improved with the aid of GO, which can make great contribution to broaden its potential applications restricted by its poor water-solubility.

The special structure of π-conjugated polyene biomolecules gives AST potent bioactivities, unfortunately, it is also the main reason to limit the application of AST because this special structure is easily degraded by light and heat during the process of manufacture and storage of AST.[12] An interested phenomenon found here is that the light-thermal stability of AST is obviously enhanced by the information of GO-AST complex. The stability profile was examined by monitoring changes of absorbance at 491 nm at known time intervals and results were shown in Fig. 3b–d. As shown in Fig. 3b, when stored at 4 ºC in dark for 20 days, free AST and AST in GO-AST complex decay with tiny degradation rates of 1.29% and 0.23%, respectively. When stored at 37 ºC in dark for 20 days (Fig. 3c), 14.66% of native AST is decomposed, meanwhile, only 2.87% of AST in the complex is degraded. When stored at 37 ºC in natural light for 20 days (Fig. 3d), more severe decay occures. The free AST is decreased to 65.52%, while only 18.72% of AST in the complex is degraded. Although AST molecules from both of samples are degraded during the process of storage, there are remarkable differences in the degradation rate. In other words, AST immobilized on the GO shows higher stability and lower degradation rate than that of free AST, which well agrees with the result of Chen et al..[19] The enhanced thermal stability could presumably be attributed to the presence of non-covalent bond between AST and GO suppressing the mobility of AST chains and preventing the conformational denaturation of AST at higher temperature, which is similar with the protection effect of nanomaterials for pea starch and redox enzyme.[20, 21] Furthermore, it is reported that GO has remarkable optical absorptivity over a wide wavelength range (visible-IR). [22] Hence, when surrounded by light and heat, GO can be a structure suppresser and a photon harvester to avoid the destruction of AST and improves its light-thermal stability, which could also benefit for extending the applied field of AST.

Although we have demonstrated GO can greatly enhance the solubility and light-thermal stability of transferred AST, biological properties of the transferred AST in GO-AST is still questionable. AST is assumed to be the natural substance that shows the strongest antioxidant effect.[23] Thus, the antioxidant activity of the as-prepared GO-AST complex was firstly evaluated by DPPH method. As illustrated in Fig. 4a, the maximum absorbance of DPPH appeared near 520 nm is gradually decreased with the increase of GO-AST concentration. The DPPH scavenging activity of free AST was also assayed and compared with that of AST in GO-AST (Fig. 4b). The DPPH scavenging rates of AST and AST in GO-AST are 8.13% and 8.55% at 0.2 μg/mL, respectively, and increase to 56.48% and 59.55% accordingly, when the concentration increased to 2.0 μg/mL. The DPPH scavenging ability of both AST and AST in GO-AST shows a positive correlation with their concentration among the evaluated range. However, comparing the antioxidant activity of AST and AST in GO-AST at the same concentration, AST in GO-AST presents slightly stronger antioxidant capacity than that of pure AST [Note: the DPPH scavenging rate of AST in GO-AST in Fig. 4b was calculated by Equation (2) shown in the Electronic Supplementary Information to eliminate the interference of GO]. The slight enhancement of antioxidant capacity could be attributed to the formation of the intermolecular hydrogen bond between the hydroxyl and carboxyl group of GO and AST, which inhibits the formation of the five-membered intramolecular hydrogen bond of AST, and thus enhances the antioxidant activity of AST in complex (Shown in Fig. S2). [24, 25]

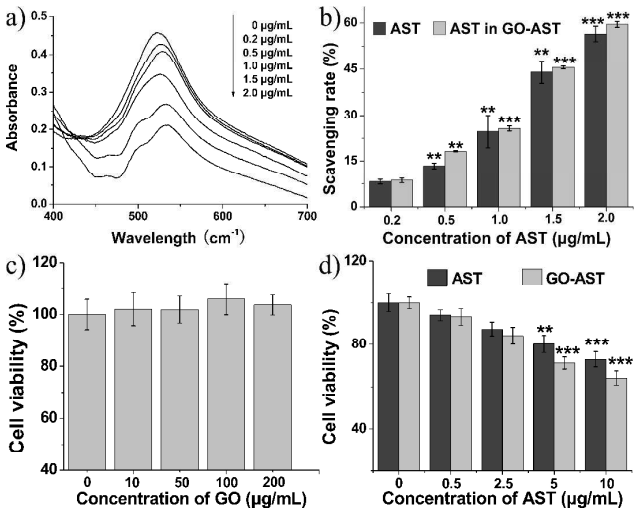

Fig. 4 The antioxidant and antitumor activity of two ASTs. (a) UV-vis spectra of DPPH scavenged by different concentrations of GO-AST. (b) The scavenging rates of DPPH by different concentrations of AST or AST in GO-AST, data were presented as means ± SD of three independent experiments, and the significant difference was set at $p < 0.05$. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with 0.2 μg/mL AST or GO-AST treatment. (c) Relative cell viability data of HepG2 cells after incubation with GO and (d) AST with or without GO, results were shown as the means ± SD of six separate experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control.

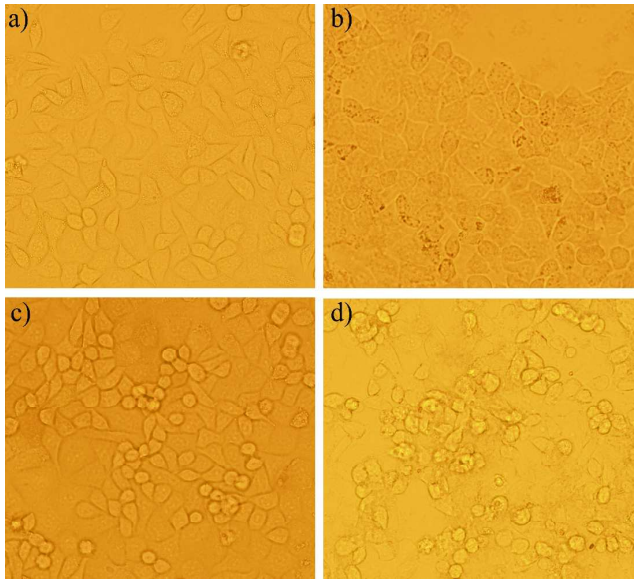

Fig. 5 Optical microscopy images of HepG2 cells with different treatment, (a) the control; (b) GO; (c) AST; (d) GO-AST.

Finally, the cytotoxicity test for the GO-AST complex was carried out by using human hepatoma cell line (HepG2) to further investigate the bioactivity of AST in the complex via CCK-8 assay. At the same time, the cell morphological changes after each of treatments were imaged to in situ track the status of cells. Firstly, the toxicity of dialyzed GO was investigated. As shown in Fig. 4c, no obvious cytotoxicity was observed for GO even at a concentration of 200 μg/mL. Consistently, no obvious morphological difference between the GO-treated cells (Fig. 5b) and the control cells is observed (Fig. 5a). These results indicate that GO sheets themselves have no cytotoxicity to HepG2 cells and would not interfere the anticancer activity determination of AST, which well accords with previous reports.[26, 27] The antitumor activity of GO-AST was then explored. The optical microscopic observation displayed in Fig. 5c and d clearly shows that both AST and GO-AST-treated cells become circular, and are smaller than control cells, which indicates that AST and GO-AST have obvious effect on cells. Moreover, as shown in Fig. 4d, both AST and GO-AST result in a dose-dependent toxicity in HepG2 cells. However, the treatment with GO-AST markedly reduces the cell viability by 35.81% at 10 μg/mL compared to the control cells, while only 28.1% is reduced by AST. It means GO-AST possesses potent cancer cell killing ability, which is stronger than that of pure AST. The improved cytotoxic effect on the cancer cell maybe due to the adsorption of GO onto the cellular surface and higher cell penetration of GO, related to amphiphilicity of GO for improved cell wall interaction compared with absolute hydrophobic AST.[26, 27] These results indicate GO could not transfer AST from organic phase to aqueous one, but also enhance the bioactivity of AST by the formation of AST-GO complex.

Conclusions

To sum up, we report for the first time that the phase-transfer activity of GO can make contributions to transfer π-conjugated polyene biomolecules (such as AST) from organic phase to aqueous one by simple mixing of biomolecules dissolved in ethyl acetate with a GO water solution with the assistance of stir, resulting in the increasing dispersity of π-conjugated polyene biomolecules in water (50.12 μg AST/mL). Besides, GO can also protect the π-conjugated polyene biomolecules against harsh storage conditions. Most importantly, the bioactivity of biomolecules were not only unhampered but slightly enhanced by GO. Thus, this work might broaden the potential applications of π-conjugated polyene biomolecules, restricted by their poor water-solubility and volatility, such as biomedicine and future photonics applications.[10] Further research is needed to fully recover variations of molecules after transferred by GO.

Acknowledgements

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Notes and references

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