

Functionalization of multiwalled carbon nanotubes with β -lactoglobulin

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ABSTRACT

β -Lactoglobulin (β -LG) can be successfully immobilized on the multiwalled carbon nanotubes (MWNTs) to form nanohybrid via noncovalent bonding with 1-pyrenebutanoic acid succinimidyl ester (PSE) as connector. PSE was first attached to the MWNTs by the π - π stacking interaction. Then, β -LG was immobilized on the MWNTs in an aqueous solution by a reaction between amine group of β -LG and N-hydroxysuccinimide of the PSE. The resultant functionalized MWNTs were characterized by UV-vis, fluorescence spectroscopy, confocal microscopy and scanned electron microscopy (SEM). Results showed that the functionalized MWNTs were quite soluble in aqueous medium. The effects of conjugation of carbon nanotubes to protein backbones on the rheological properties of protein-carbon nanotube hybrids were also determined using a dynamic rheometer. The core-shell structure of nanohybrid can be observed from SEM images.

Keywords: MWNTs, β -LG, nanohybrid, core-shell structure, encapsulation and delivery

1 INTRODUCTION

Carbon nanotubes have been researched as potential carriers for the delivery of biological molecules into cells. Noncovalent functionalization of the carbon nanotubes (CNTs) has proven to be a promising tool because of undamaged sp² nanotube structure of the CNTs[1]. Pyrene-containing compounds have been widely used to functionalize the CNTs[1-4].

The immobilization of proteins on CNTs is attracting much attention because the resultant hybrid nanomaterials are potentially useful in many aspects such as biomolecular nanosensors and novel encapsulation and delivery materials. Many proteins have been immobilized on single-walled carbon nanotubes by the nonvalent sidewall functionalization methodology[5].

β -LG, the major whey protein component in the milk of ruminants, is a small globular protein widely used as food ingredient because of its nutritional value and functionalities. β -LG exhibits ligand binding properties and appears to be resistant to degradation of the pepsin in the stomach in its native structure[6, 7].

The major goal of the present paper is to immobilize β -LG on MWNTs using the 1-pyrenebutanoic acid

succinimidyl ester (PSE) as a connector to synthesize the novel β -LG/MWNTs hybrids. The resulted hybrids may possess the advantages of both protein and carbon nanotube, exhibiting unique structure and encapsulation properties for targeted delivery of bioactive and pharmaceutical compounds.

2 EXPERIMENTAL SECTION

2.1 Materials

MWNTs, PSE and β -LG were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade. Milli-Q water (resistivity 18.2 M Ω cm) and N, N-dimethylformamide (DMF) were used in the experiments for suspension preparation.

2.2 Preparation of MWNTs/PSE

10 mg MWNTs, 20 mg PSE and 5 mL DMF were added into a 10 mL three-necked tube. After sonication for 30 min, the suspension reacted overnight at room temperature. Then, the product was centrifugally separated and was washed by DMF three times. The resultant solid was dried overnight under vacuum at 40 °C.

2.3 Functionalization of MWNTs

2 mg of previously modified MWNTs and 20 mg β -LG were dispersed in 10 mL water in a test tube under sonication for 30 min. The dispersion was kept stirred overnight at room temperature to ensure complete reaction. After reaction, dispersion was separated centrifugally and was washed by water three times. The resulting β -LG/MWNTs hybrids were isolated and dried overnight under vacuum at 40 °C.

2.4 Conjugation characterization

UV-vis spectrophotometer (UV-1601PC, Shimadzu Corporation) was used to investigate the π - π stacking between MWNTs and modified β -LG. Fluorescence spectroscopy was carried out on a fluorescence spectrometer (LS 55, Perkin Elmer). Microstructure of the β -LG/MWNTs conjugates suspension were studied using a confocal scanning laser microscope (CLSM, Zeiss Axiovert

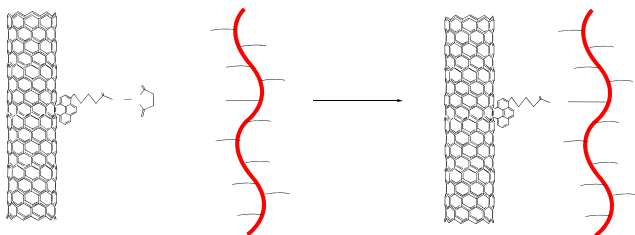
200M) with 100x oil-immersed objective lens. Scanning electronic microscopy (SEM, Hitachi S900) was used to observe the surface structure of β -LG/MWNTs nanohybrid.

2.5 Rheological characterization

The apparent viscosity of the β -LG/MWNTs hybrid dispersions was measured by steady shear viscometry in a shear rate range of 0.1 to 10 s^{-1} . 0.1g β -LG was dispersed into 2 mL solvent containing 1 mL deionized water and 1 mL DMF. Certain amount of MWNTs was then added to β -LG dispersion to synthesis β -LG/MWNTs hybrids with relative concentration of 1 wt% and 2 wt% β -LG/MWNTs hybrids according to the method described above. The viscoelastic behaviors of β -LG/MWNTs hybrids were evaluated by dynamic small-amplitude oscillatory shear (SAOS) tests via frequency sweep and time sweep. Steady shear and SAOS tests were performed using a controlled stress dynamic rheometer with cone and plate measurement cell (Bohlin CVO-R, Malvern Instruments Inc.). Rheological properties of β -LG, β -LG/MWNTs mixture and β -LG/MWNTs hybrids were compared.

3 RESULTS & DISCUSSION

Dai et al. [1] reported PSE could combine carbon nanotubes and all kinds of protein together. Here, we adopted the PSE as a connector preparing the β -LG/MWNTs hybrids, as illustrated in **scheme 1**.



Scheme 1 Functionalization of multiwalled carbon nanotubes with β -LG

UV-vis spectrum was used to characterize the β -LG/MWNTs hybrids. **Fig. 1** shows the UV-vis spectra of MWNTs/PSE, β -LG and β -LG/MWNTs hybrids. From **Fig. 1**, β -LG has an adsorption at 293 nm. For the β -LG/MWNTs hybrids, a similar adsorption at 293 nm. This result verified the hybrid structure of β -LG/MWNTs.

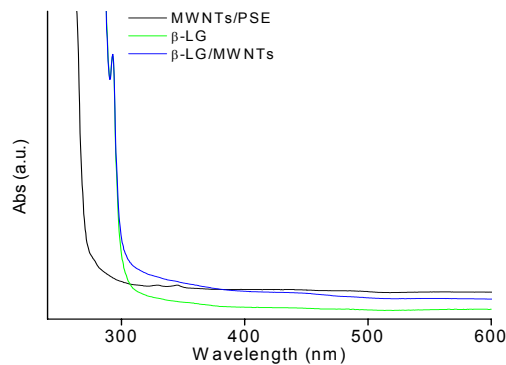


Fig. 1 UV-vis spectra of MWNTs/PSE, β -LG and β -LG/MWNTs hybrids

Fluorescence spectroscopy is one of the powerful techniques to investigate the structure of protein/carbon nanotube hybrids. It is well known that carbon nanotubes can quench the fluorescence efficiency of many proteins. In our experiments, the fluorescence quenching phenomena of the modified MWNTs on β -LG was also observed obviously, as shown in **Fig. 2**. β -LG exhibited strong characteristic adsorption at 330 nm. After forming hybrid, fluorescence efficiency of β -LG has been completely quenched by MWNTs.

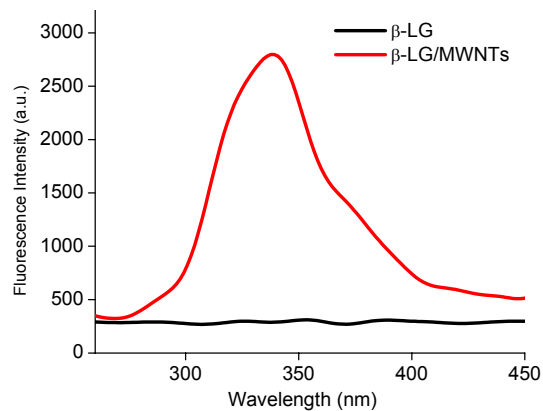


Fig. 2 Emission spectra of β -LG and β -LG/MWNTs in D_2O (λ excitation = 235nm)

Suspensions of synthesized β -LG/MWNTs hybrids showed clear conjugation between MWNTs and protein molecules as observed by CLSM from **Fig. 3**. Some aggregates of hybrids are also observed, which is in good agreement with the rheological study described below.

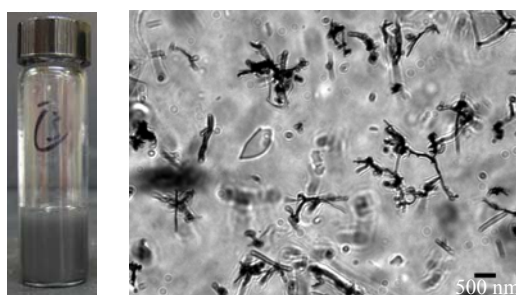


Fig. 3 Photograph and Confocal microscopy of β -LG/MWNTs hybrids in suspension (Scale bar stands for 500 nm).

The core-shell structure of β -LG/MWNTs hybrids (Fig. 4) can be observed from SEM images, where protein molecules (A) surround the carbon nanotube (B) surfaces. From Fig. 4-2, partially coated β -LG/MWNTs hybrids (C) and fully coated hybrid (D) can be observed. When the β -LG concentration reach the gelation critical point, the incorporation of carbon nanotube may reinforce the gel structure by offering higher mechanical strength.

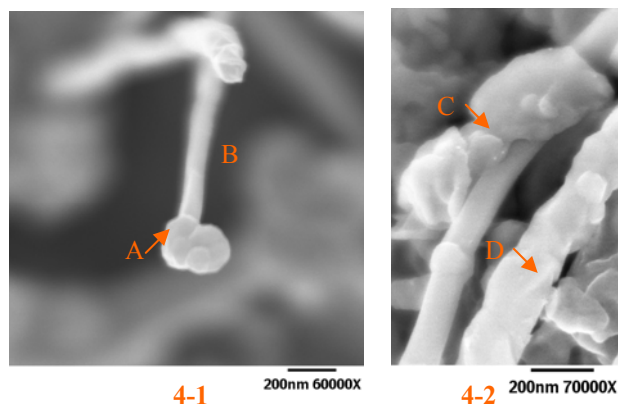


Fig. 4 SEM images of β -LG/MWNTs nanohybrids

Shear-thinning behavior of all the suspensions (**Fig. 5**) were identified across the experimental shear rate ranges, which may account for the breakdown of aggregates in the suspension due to the hydrodynamic forces generated during shear. The existence of MWNTs tends to raise the viscosity at higher shear rate compared to that of protein suspension, indicating the mechanical strength of MWNTs brought to the protein molecules resulting in the less protein molecular rearrangement under hydrodynamic forces. It therefore showed higher resistance to flow exhibiting the higher viscosity.

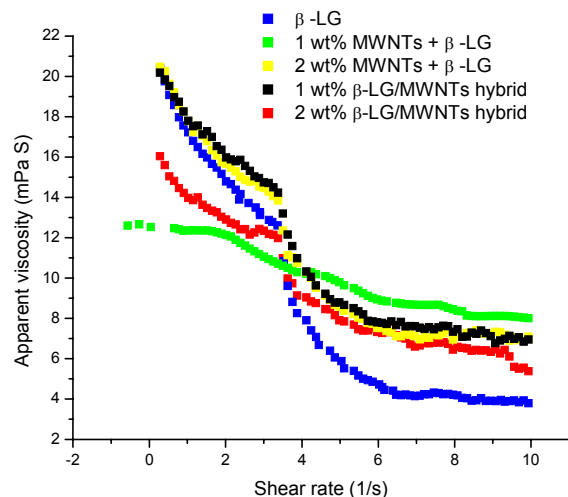


Fig. 5 Steady shear viscosity of β -LG, β -LG/MWNTs mixture and β -LG/MWNTs hybrid suspensions

The elastic modulus, G' , from **Fig. 6** showed strong frequency-dependence behavior across the full experimental frequency range, indicating the existence of relaxation processes occurring even at short time scales. The moduli showed an approximately power-law scaling, $G' \sim \omega^n$, with $n=0.86$ for β -LG/MWNTs hybrids dispersion. Since the concentration of protein in this study is low, there is less opportunity to form cross-linking among protein chains. Although MWNTs have been reported to exhibit strong elastic properties[8], the magnitude of G' is significantly dependant on concentration and network/aggregates structure. The low concentration of MWNTs (0.05~0.1%) in our samples may not form crosslinking structure but some aggregates, which in turn is significantly frequency-dependent. The G' of MWNTs added β -LG and MWNTs- β -LG hybrids are one magnitude lower than that of β -LG dispersion, suggesting that β -LG make more contributions on G' than MWNTs and the decrease of the moduli may attribute to the steric effect of MWNTs in diluted protein dispersions. It can also be seen that the higher concentration of MWNTs showed higher G' under the low frequency whereas the difference diminished as the frequency increased, indicating the loose aggregates structure formed by MWNTs exist.

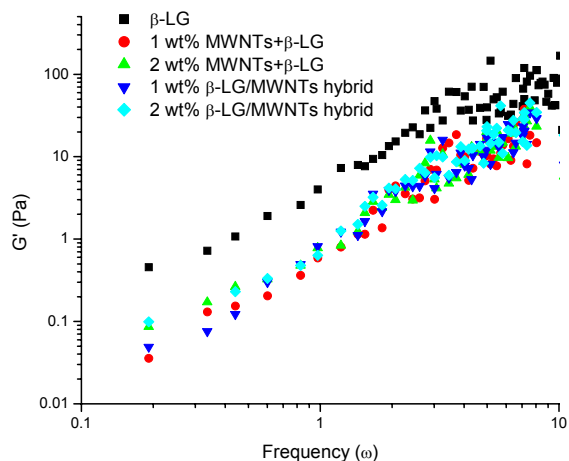


Fig. 6 Dynamic shear storage modulus of β -LG/MWNTs hybrids suspension

4 CONCLUSIONS & FUTURE WORK

β -LG can be successfully immobilized on the MWNTs to form nanohybrid via noncovalent bonding with PSE as connector. Core-shell structure of the hybrids was identified and shear-thinning behavior was found. Conjugating MNNTs with β -LG affects the rheological properties and the hybrids in suspension showed strong frequency dependency. Further studies on concentrated suspension of β -LG/MWNTs need to be carried out and the effects of carbon nanotube incorporation on β -LG functionalities, such as emulsification and gelation are worth to be investigated in future. The introduction of carbon nanotube to functional proteins may enhance their encapsulation properties for targeted delivery of bioactive and pharmaceutical compounds.

REFERENCES

- [1] Chen, R.J., et al., *Noncovalent Sidewall Functionalization of Single-Walled Carbon Nanotubes for Protein Immobilization*. *J. Am. Chem. Soc.*, 2001. **123**(16): p. 3838-3839.
- [2] Ou, Y.Y. and M.H. Huang, *High-Density Assembly of Gold Nanoparticles on Multiwalled Carbon Nanotubes Using 1-Pyrenemethylamine as Interlinker*. *J. Phys. Chem. B*, 2006. **110**(5): p. 2031-2036.
- [3] Guldi, D.M., et al., *Functional Single-Wall Carbon Nanotube Nanohybrids-Associating SWNTs with Water-Soluble Enzyme Model Systems*. *J. Am. Chem. Soc.*, 2005. **127**(27): p. 9830-9838.

- [4] L. Hu, Y.L.Z., K. Ryu, C. Zhou, J.F. Stoddart, G. Gruner *Light-Induced Charge Transfer in Pyrene/CdSe-SWNT Hybrids*. *Advanced Materials*, 2008. **20**(5): p. 939-946.
- [5] Patrick G. Holder and M.B. Francis, *Integration of a Self-Assembling Protein Scaffold with Water-Soluble Single-Walled Carbon Nanotubes*. *Angewandte Chemie International Edition*, 2007. **46**(23): p. 4370-4373.
- [6] Morr, C.V. and E.Y.W. Ha, *Whey-Protein Concentrates and Isolates - Processing and Functional-Properties*. *Critical Reviews in Food Science and Nutrition*, 1993. **33**(6): p. 431-476.
- [7] Kontopidis, G., C. Holt, and L. Sawyer, *Invited Review: beta-lactoglobulin: Binding properties, structure, and function*. *Journal of Dairy Science*, 2004. **87**(4): p. 785-796.
- [8] Fan, Z.H. and S.G. Advani, *Rheology of multiwall carbon nanotube suspensions*. *Journal of Rheology*, 2007. **51**(4): p. 585-604.