Labeling Polymeric Nanoparticles with Copper Chlorophyll as Contrast Agent for Electron Microscopy

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Copper chlorophyll (CC), the hydrophobic pigment applied in food, is employed as a contrast agent instead of Au nanoparticles (NPs) to label polymeric NPs for in vivo observation by electron microscopy. Using the nanoprecipitation method without surfactants CC is aggregated in coordinately saturated form and synchronously encapsulated in the matrix of polymeric NPs without other complex pretreatments, which may ensure the stability of CC in NPs.

Polymeric nanoparticles (NPs) are representative highlights in drug delivery systems.[1] Drugs are loaded to realize the accurate delivery especially via the blood stream and lymphatic system with some advantages, such as specific targeting and sustained release action.[1] To elucidate kinds of mechanisms concerned with those NPs, it is necessary to trace their fate in vivo by electron microscopic analyses in histological sections.[2] Both polymeric NPs and tissues are composed of light elements, however. Hence, the present methods suffer from a lot of practical limitations when applied to observe the intratissuelar distribution of NPs with electron microscopy.[2]

Usually heavy metals have strong signal in the electron microscopy.[3] Qiu et al.[2] once tried to label poly-dl-lactide (PLA) NPs with Au NPs. Nevertheless, in order to stabilize Au NPs, a kind of PLA end-capped by a protected thiol (PLA-SH) had to be aforehand synthesized to pretreat Au NPs together with $C_{11}SH$.[2] Copper chlorophyll (CC) is a derivative of chlorophyll, only the central Mg in the macrocyclic porphyrin ring replaced by Cu.[4] Similar to Au, Cu is either a trace mineral in the mammals[5] or a typical heavy metal. As a hydrophobic pigment applied in food,[4b] it may be possible to directly load CC in the polymeric NPs without other treatments. Hence, we are prompted to label NPs with CC as a contrast agent instead of Au NPs for in vivo observation by electron microscopy.

A typical kind of CC labeled NPs (LNPs) was produced using the nanoprecipitation method without surfactants.[6a] On the basis of the mechanisms of Maragoni effect and interfacial polymer deposition, nanoprecipitation is most suitable for compounds having a hydrophobic nature, without the heat treatment, comminuting forces from high-energy source, unacceptable toxic organic solvents and even surfactants.[6b] Briefly, 50 mg of PLA ($M_w = 5000$, Shandong Province Institute of Medical Equipments, China) was dissolved in 10-mL freshly prepared acetone solution of CC (8.5 μM, Qingdao Green Source Bioengineering Co., Ltd., China, purified with the reported method[7]) at room temperature. To get homogeneous LNPs, the solution was added dropwise into 40 mL of aqueous solution of ethanol (50% v/v) with agitation. An aqueous suspension of LNPs with a concentration of ca. 2.5 mg/mL was then acquired after eliminating organic solvents in a rotative vacuum evaporator. The LNPs suspension can keep stable on shelf for at least 3 months. In order to produce LNPs powders for other physical characterizations, some of the LNPs suspension was further lyophilized after centrifugation.

To determine the loading of CC in LNPs, LNPs powders with known weight were dissolved in acetone. The loading of CC was calculated according to the amount of CC in acetone determined in a UV-2102PC spectrophotometer (Unico (Shanghai) Instrument Co., Ltd., China) at 415 nm at 25 °C: 1.41 wt %, i.e., the concentration of Cu in LNPs is 95.3 μg/g, much higher than that in body.[5] It's very attractive that CC is synchronously loaded when LNPs are formed without other complex pretreatments. Zeta-potential measurements were performed as reported in a Zeta Pals zetasizer (Brookhaven Instruments, U.S.) It shows that LNPs (−29.28 mV) are negatively charged, very close to that of the empty PLA NPs (prepared just like LNPs only without CC) (−30.14 mV). PLA carry the negative charge for the negatively charged carbonyl group,[6a] while CC is neutral. The results show that CC doesn't obviously affect the surface property of LNPs. Because of the electrostatic stabilization of negatively charged carbonyl group on the surface of NPs,[8] serious amount of surfactants, required to control the size of the NPs and stabilize the dispersion system containing the NPs in conventional preparation methods,[8b] are avoided. Those unnecessary surfactants may lead to some side effects in body and are hard to be removed when remaining at the surface of the NPs,[8b] which may greatly affect the followed surface modification that may be performed.

The morphology was measured with an SPA 400 AFM atomic force microscope (Seiko Instruments Industry, Co., Ltd., Japan) in the tapping mode and with a Tecnai G2 20 transmission electron microscope (TEM) (FEI Company) at 200 kV, respectively. AFM and TEM of LNPs are shown in Figures 1 and 2, respectively. From Figure 1, it is found that the LNPs are particles ranging from 20 to 50 nm. It is indicated that there are some aggregates with the enhanced signal, similar to Au NPs,[2] in LNPs through the comparison between the empty PLA NPs and LNPs illustrated in Figures 2a and 2b. According to the procedures aforecited, LNPs are only composed of CC and PLA. Hence, the results suggest that the encapsulated aggregates are from CC. Combined with the results of zeta potential, it is indicated that CC is mainly distributed in the LNPs. The intravenously injected LNPs were traced in tissues by electron microscopy (see Supporting Information).

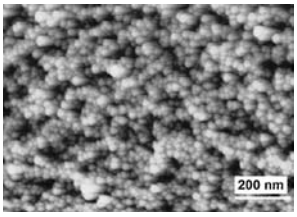


Figure 1. AFM image of LNPs deposited on the freshly cleaved mica.

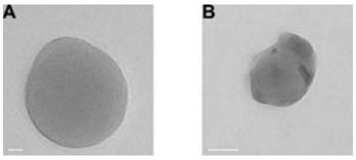


Figure 2. TEM images of single NPs taken from the suspensions by C-coated stainless steel (S304) grips: A) empty PLA NPs, B) LNPs. The scale bars in A and B are 10 and 20 nm, respectively.

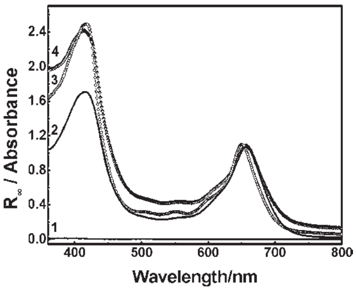
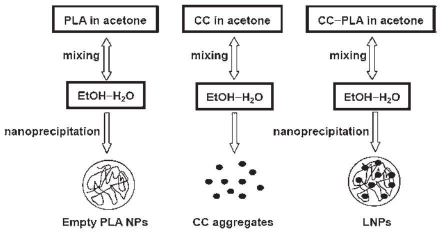


Figure 3. Visible spectra of CC in different environments. Curves 1 and 2, DRS of the PLA powders and LNPs powders, respectively. Curves 3 and 4, the adsorption spectra of CC (ca. 3.4 μM) in "dry" acetone (treated with anhydrous $CaCl_2$) and the acetone–water–ethanol (10:45:45 in volume) mixture, respectively. Curves 3 and 4 are both magnified by fourfolds.



Scheme 1. Comparison of the formation of empty PLA NPs, CC aggregates and LNPs.

Spectroscopical experiments were further carried out to determine the state of CC in LNPs in a UV-2550 spectrophotometer (Shimadzu, Japan) (1-nm resolution) equipped with conventional integrating spheres at 25 °C. The diffuse reflectance spectrum (DRS) measurements were performed as reported method.[9] In Figure 3, curves 1 and 2 are DRS of PLA powders and LNPs powders, respectively. There is nearly no absorbance in curve 1. Thus, curve 2 displays the state of CC in LNPs powders. Curves 3 and 4 are the adsorption spectra of CC in pure acetone and the acetone–water–ethanol mixture (10:45:45 in volume), where water (73% in mole) predominately presents in the mixed solvent. It is shown the adsorption maxima of the principle Soret band and Q band in curve 2 are located at 412 and 657 nm, respectively, as the same as those in curve 4, while in curve 3 the principle Soret band and Q band are located at 419 and 649 nm, respectively. Furthermore, in curve 3, not only the principle Soret band and Q band are narrower than those in curves 2 and 4 correspondingly, but also the weak bands between the two bands are more obvious.

Studies have shown that chlorophylls are dissolved in pure polar solvents as monomers, while aggregates are formed in polar solvent–water mixtures with the increment of water.[10] Curve 3 may represent the situation of the monomeric CC, while curve 4 may describe the aggregated CC. During that process of preparing LNPs, the mole fraction of water in the mixed solvents is never lower than 70% in the preparation, i.e., water predominates in the preparation. Meanwhile PLA is not a nucleophile that may lead to the formation of monomeric chlorophylls.[10] These results indicate that in LNPs CC is aggregated in the matrix of PLA like that situation of CC in the acetone–water–ethanol mixture, where water predominates in the ternary solvent mixture (see Scheme 1).

As a marker employed to label NPs, the stability of CC in LNPs is very important. In Figure 3 there is no strong red shift in the principle Q band in both curves 2 and 4, similar to the spectra of chlorophyll *a* in highly polarizable solvent–water mixtures, where aggregated chlorophyll *a* presents in predominantly six-coordinated state.[10a] Notably, it has been known that the central divalent atoms of chlorophylls with coordination number 4 are coordinately unsaturated,[11] while 6 is the most common coordination number for Cu^{2+}.[12] The results suggest that the aggregated CC presents in the more stable state when compared with the monomeric CC. Furthermore, the encapsulation by PLA may further improve the stability of CC.

In summary, CC can be employed as a contrast agent to label polymeric NPs for electron microscopy. Using the nanoprecipitation method without surfactants CC is aggregated in coordinately saturated form and synchronously encapsulated in the matrix of polymeric NPs without other complex pretreatments, which may ensure the stability of CC in NPs.

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