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ARTICLE TYPE

Biodegradable PLGA Nanoparticles Loaded with Hydrophobic Drugs: Confocal Raman Microspectroscopic Characterization

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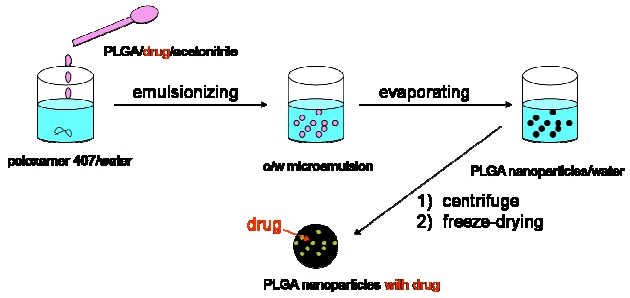
Poly(lactic-co-glycolic acid) (PLGA) nanoparticles with bicyclol (5%) and 3-n-butyl-6-bromophthalid (Br-NBP) (3%) were prepared by an emulsification-solvent evaporation technique. The PLGA nanoparticles were for the first time successfully characterized by a laser trapping/confocal Raman spectroscopic technique only using individual PLGA nanoparticles. This technique allowed us to selectively obtain Raman spectra of optically trapped PLGA nanoparticles (~ 10 nanoparticles) in solution. The Raman spectrum of PLGA nanoparticles loaded with hydrophobic drugs showed that these drugs were certainly incorporated in the nanoparticles.

Poly(lactic-co-glycolic acid) (PLGA) is one of the most promising biodegradable and biocompatible polymers thanks to its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid, which are easily metabolized by the human body, and also thanks to a minimal systemic toxicity when use of PLGA for drug delivery or biomaterial applications [1]. PLGA had been approved by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) in various drug delivery systems in humans, which is not necessarily scientifically meaningful but is practically important for development of drugs [2]. PLGA is commercially available with different molecular weights and copolymer ratios of lactic acid and glycolic acid units. Interestingly, the degradation time of PLGA, which controls sustained release of drug, can vary from several months to several years, depending on the molecular weight and copolymer ratio of the lactic acid and glycolic acid units [3].

Drug delivery system with nanoparticles of biodegradable and biocompatible polymer is an option for controlled drug delivery and drug targeting [4-7]. PLGA nanoparticles are widely employed for the sustained or targeted release of drugs [8]. PLGA nanoparticles loaded with hydrophobic and poorly water-soluble drugs are most commonly formulated by nanoprecipitation. Several methods have been reported to prepare PLGA nanoparticles [8]. The most common preparation method of PLGA nanoparticles is an emulsification-solvent evaporation technique. This method allows the encapsulation of hydrophobic drugs on the PLGA matrix "nanosphere" through dissolving the PLGA polymer and the compound (drug) in an organic solvent. The emulsion of oil (O) in water (W) i.e. O/W is prepared by adding water and surfactant to the PLGA polymer solution. The nano-sized droplets are induced by ultrasonication or homogenization. The organic solvent is then evaporated and the nanoparticles collected from colloidal dispersion by centrifugation.

The average particle size and the polydispersity index of the PLGA nanoparticles can be measured by dynamic light scattering. This technique is based on the dispersion of the light caused by the Brownian motion of the particles [8]. The zeta potential (ζ) of the nanoparticles is measured by an electrical potential via the mobility of charged particles monitored. Depending on the polymer and the surface modification, the zeta potential values may be positive, neutral or negative [8]. Microscopic techniques such as scanning (SEM) or transmission electron microscopy (TEM) or atomic force microscopy (AFM) provide information on the shape and size of the nanoparticles.



Scheme 1. Flow chart of preparation of drug-loaded PLGA nanoparticles.

Precise determination of the drug content is not easy because nanoparticles are colloidal systems [3]. Therefore, the most relevant way to separate nanoparticles from non-encapsulated or non-adsorbed drug is the ultracentrifugation. The drug-loaded PLGA nanoparticles are usually obtained as powder which is provided for analysis of drug content. To date, microscopy such as TEM, SEM, AFM have been reported as only analytic tool for a few individual PLGA nanoparticles level. The analysis only provides insights mainly related with shape and size. To best our knowledge no analysis of a few individual nanoparticles has been

reported for chemical structure and composition of the PLGA nanoparticles and the loaded drugs.

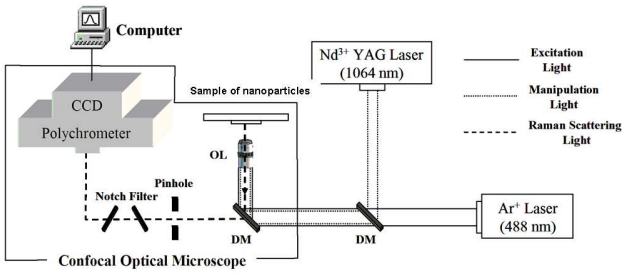


Figure 1. Setup of the laser-trapping/Raman spectroscopic measurements on the PLGA nanoparticles.

Kitamura et al. have reviewed in detail the optical trapping/chemical analysis of single microparticles or vesicles/liposomes in solution [9]. This analytic technique has been greatly improved since Ashkin first demonstrated the optical trapping/manipulation of a single microparticle [10]. Optical trapping/microspectroscopy (absorption, fluorescence, and Raman spectroscopy) is now a powerful analytic tool for studying microgels and micro- or even nano-particles in solution [11-13]. This technique can trap the nanoparticles in solution at the focal point of the laser beam using radiation force. Confocal Raman microspectroscopy obtains vibrational information of the optically trapped nanoparticles. Herein the PLGA nanoparticles with hydrophobic drugs were characterized by a laser trapping/Raman spectroscopic technique only using about ten individual PLGA nanoparticles in order to confirm drug-loading at the individual particle level. Based on these results, the individual nano-scopic objects were confirmed to be PLGA and loading of designed hydrophobic drugs. Br-NBP and bicyclol were used as model compounds of the hydrophobic drugs in this work. The Br-NBP was synthesized using *o*-phthalic anhydride as a starting materials through six-step synthesis, while the bicyclol (CAS no. 118159-48-1) was commercially available from Santa Cruz Biotechnology (see ESI).

Table 1. **Particle sizes, Zetal potentials and drug loadings of the PLGA nanoparticles**

Sample No.	PLGA	Drug	Particle size	Zeta potential	Drug-loading
H1	50 mg	Non	264 nm	-23.41 mV	-
H2	50 mg	bicyclol, 6.4 mg	282 nm	-22.60 mV	5%
H3	50 mg	Br-NBP, 6.4 mg	384 nm	-30.50mV	3%

PLGA nanoparticles were prepared using the nanoprecipitation method (Scheme 1) [14]. Particle size, zeta potential and entrapment efficiency were measured for typical formulation, i.e., organic phase (acetonitrile), concentration of Poloxamer 407 as stabilizer (0.5% w/v), ratio of organic phase and aqueous phase (1:5), drug:polymer ratio (1:8 for Br-NBP; 1:4 for bicyclol). Typically, the optimized formulation was prepared by dissolving PLGA (250 mg) and drug (6.2 mg) in 10 ml of acetonitrile [14]. This organic phase was added dropwise to 10 ml of 0.5% w/v Poloxamer 407 solution with continuous stirring on a magnetic stirrer at room temperature. Stirring was continued for 3–4 h to allow complete evaporation of the organic solvent. Finally, traces of organic solvent were eliminated under reduced pressure in a rotary flask evaporator at 40 ^{o}C for 30 min. The PLGA nanoparticle-suspension was centrifuged at 25,000 rpm for 30 min at 10 ^{o}C (Avanti J-26 XP, Beckman, USA), supernatant was decanted. The PLGA nanoparticle pellets were re-dispersed in water (10 ml) and then freeze-dried (Heto PowerDry LL3000) using sucrose as a cryoprotectant. Empty PLGA nanoparticles were prepared by the method described above with the exception of adding drug.

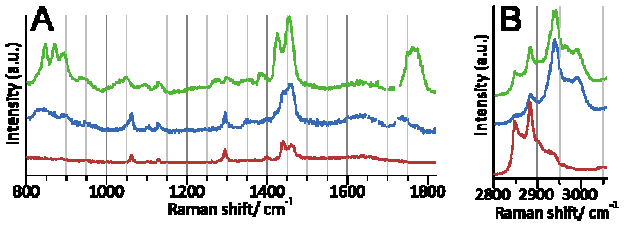


Figure 2. Raman spectra of PLGA nanoparticles trapped by optical tweezers; H1 (PLGA only, red), H2 (PLGA + bicyclol, blue), H3 (PLGA + Br-NBP, green). Spectra are baseline-corrected.

A confocal Raman microspectroscopy combined with an optical tweezing system (Figure 1) [12] was used for the characterization of the individual PLGA nanoparticles. Two laser beams: a continuous-wave (cw) Ar^{+} laser (λ = 488 nm, Coherent, Inova 70, 30 mW) and a cw Nd^{3+}: YAG laser beam (λ = 1064 nm, Spectron Laser System, SL-902T, 0.4 W laser power at the nanoparticle sample) were used as the excitation light source for Raman scattering and the light source for the optical tweezers, respectively. These laser beams were tightly focused into PLGA-dispersed water with an immersion objective lens (× 100, N.A. = 1.30) of an inverted optical microscope (Nikon, ECLIPSE TE300). Raman scattered light from the optically trapped PLGA nanoparticles (~ 10 particles, which were counted under bright-field observation and the backscattering image of Ar^{+} laser) was spatially selected by passing through a pinhole (diameter: 100 μm) to ensure a confocal optical arrangement, and was detected by a cooled CCD camera (Andor Tec.) equipped with a polychromator (grating: 1200 grooves/mm).

The size analysis and polydispersity index of the NP were determined using a Malvern Zetasizer Nano ZS-90 (Malvern Instruments, UK). The averaged particle sizes of the PLGA nanoparticles without drug (H1), with bicyclol (H2) and Br-NBP (H3) were 264, 282 and 384 nm, respectively (Table 1 and ESI). The PLGA nanoparticles were negatively charged, i.e., the zeta potentials of H1, H2 and H3 were -23.4, -20.6 and -30.5 mV, respectively (Table 1 and ESI), indicating stably dispersed in water. The Br-NBP loaded particles have significantly lower zeta potential than drug-free and bicyclol loaded particles. It is considerable that the particle size is largely dependent on the size of oil-drop in the O/W emulsion coupled with the polymer concentration in the oil-drop. The study on controlling the particle size is under way by us.

The drug loading in the PLGA nanoparticles was measured by dissolving 2.7 mg of the freeze-dried PLGA nanoparticles in 10 ml of acetonitrile. Absorbance of the solution after filtration

and appropriate dilution with acetonitrile was then measured UV spectra at wavelength of 284 nm for Br-NBP, 228 nm for bicyclol, respectively, and the drug concentration was calculated from the calibration curves (see Figs. S15, 16 in ESI). As a result, the

Table 2. Raman peaks (cm^{-1}, (relative intensity)) assignment of PLGA nanoparticles (NPs) trapped by optical tweezers[a]

Assignment	H1/ PLGA NPs		H2/ PLGA NPs with bicyclol		H3/ PLGA NPs with Br-NBP	
δ(CH)			829	(16)		
δ(CH)			845	(16)		
δ(CH)					846	(19)
δ(CH)					870	(18)
δ(CH)					892	(14)
ν (CC)$_T$	1062	(4)	1062	(5)	1049	(5)
ν_s(COC)	1100	(< 1)	1102	(< 1)	1093	(3)
r_w (CH_3)	1129	(2)	1130	(2)	1130	(4)
ν_a(COC)					1272	(3)
δ(CH)	1296	(6)	1298	(8)	1296	(4)
δ_s (CH_2)	1400	(2)	1395	(3)	1386	(5)
ν(=CH)			1424	(7)	1425	(22)
δ(CH_2)	1439	(9)	1439	(16)	1439	(9)
δ_a(CH_3)	1460	(7)	1457	(20)	1454	(31)
ν(C=O)	1635	(2)	1635	(4)	1635	(4)
ν(C=O)			1737	(5)		
ν(C=O)					1764	(20)
ν_s(CH_2)	2850	(75)	2850	(17)	2850	(32)
ν(CH)	2884	(100)	2884	(40)	2884	(60)
ν_s(CH_3)	2930	(36)	2940	(100)	2940	(100)
ν_a(CH_3)	2960	(19)	2960	(56)	2960	(59)
ν_s(CH)			2990	(59)	2990	(57)

[a] NOTE: ν, stretch; τ, twist; δ, bend and/or scissor; r, rocking; subscripts: a, asymmetric; s, symmetric; T, trans. Assignment taken from Ref 16,17.

PLGA nanoparticles with bicyclol (H2) and Br-NBP (H3) have 5 and 3 w% of drug loading, respectively (Table 1). In the preliminary investigation on the releasing profiles of the drug-loaded PLGA nanoparticles in water, the accumulated releasing amount of the drug kept in the range of 0.1-0.3 mg ml^{-1} for Br-NBP, 0.2-0.45 mg ml^{-1} for bicyclol (see Fig. S14 in ESI).

The thermal analysis of the PLGA nanoparticles was carried out by using powder samples. Thermal analysis (TG/DSC) of the PLGA nanoparticles with or without drug did not show any sharp endothermic peaks, indicating that the PLGA nanoparticles are amorphous and the drug both bicyclol and Br-NBP homogeneously loaded in the PLGA domain (see ESI). The sharp endothermic peak (melting) of sucrose (cryoprotectant), however, was observed for all powder samples. Preliminary TEM observation showed sphere shape of the PLGA nanoparticles at least for the Br-NBP-loading (see Fig. S17 in ESI).

Figure 2 shows Raman spectra of PLGA nanoparticles loading with bicyclol (H2) and Br-NBP (H3), and the spectral assignments of the most intense peaks are listed in Table 2. The Raman spectrum of PLGA (H1, red line) was ascribed to vibrational modes of lactic (LA) or/and glycolic (GA) units [12]. Raman peaks at 2930, 2960 cm^{-1} were assigned to the stretching modes of the CH_3 (LA), while the stretching modes of the CH (LA) appeared at 2884 cm^{-1}. The stretching mode of CH_2 in GA units was observed at 2850 cm^{-1}. Other Raman peaks in 1000 ~ 1650 cm^{-1} were identified as the vibration modes of PLGA (Table 2). This assignment of the Raman spectrum was further supported by IR spectra of PLGA polymer and nanoparticles (see ESI). In the Raman spectra of PLGA loaded with bicyclol (H2, blue line) and Br-NBP (H3, green line), the peak at 2990 cm^{-1} was assigned to the =C−H stretching vibration in aromatic hydrocarbons of bicyclol and Br-NBP. Raman spectrum of H2 has weak peaks at 829, 845 (C−H out-of-plane deformation in benzene ring), and 1737 cm^{-1} (C=O stretching in methyl ester group). These characteristic peaks indicated that bicyclol was certainly loaded in PLGA nanoparticles. In Raman spectrum of H3, the strong peak at 1764 cm^{-1} was assigned to the C=O stretching vibration in γ-butyrolactone group of Br-NBP. Furthermore, out-of-plane deformation bands of 1,2,4-trisubst benzene group of Br-NBP were clearly observed at 846, 870, and 892 cm^{-1}. Thus, Raman microspectroscopy combined with optical tweezers demonstrated that bicyclol and Br-NBP were incorporated in PLGA nanoparticles.

As mentioned above, to the best of our knowledge there are several analytic techniques such as TEM, SEM and AFM for particle size, shape and crystalline structure of individual nano-scale objects while there are no any reports on analysis of chemical structure for individual nano-scale objects, especially, of organic materials. This is the first example for drug-loaded nano-scale objects. The use of the confocal Raman microscopy is considered to have certain advantages for chemical structure of organic materials such as drug-loaded polymer nanoparticles compared with other techniques. For example, TEM-EDX or electron diffraction in TEM may provide useful insights of elemental and crystal analysis to identify a substance for individual nano-scale objects of inorganic materials but not true for organic materials, while the confocal Raman microscopy can give more detailed insights for chemical structure of organic materials like the case presented in this work.

In conclusion the PLGA nanoparticles with bicyclol (5%) and Br-NBP (3%) were synthesized by the emulsification-solvent evaporation technique. The PLGA nanoparticles were for the first time characterized by a laser trapping/confocal Raman spectroscopic technique only using about ten individual PLGA nanoparticles. The Raman spectra of optically trapped ten individual PLGA nanoparticles in water were selectively obtained. The confocal Raman microspectroscopy indicated that the hydrophobic drugs were incorporated in the PLGA nanoparticles. This technique would be a promising tool of non-destructive qualitative and quantitative analysis for these the polymer nanoparticles loaded with drugs. The drug-loaded PLGA nanoparticles are expected to be potentially utilized for nanocarriers for sustainable releasing and target-releasing drugs.

Notes and references

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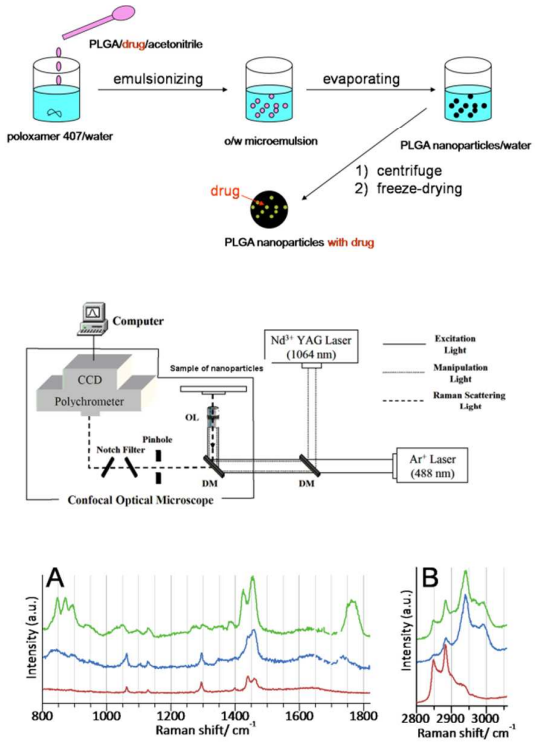
Graphic Abstract



Poly(lactic-co-glycolic acid) (PLGA) nanoparticles with bicyclol (5%) and 3-n-butyl-6-bromophthalid (Br-NBP) (3%) were prepared by an emulsification-solvent evaporation technique. We have characterized the PLGA nanoparticles by a laser trapping/confocal Raman spectroscopic technique only using individual PLGA nanoparticles. This technique allowed us to selectively obtain Raman spectra of optically trapped PLGA nanoparticles (~ 10 nanoparticles) in solution. The Raman spectrum of PLGA nanoparticles loaded with hydrophobic drugs showed that these drugs were certainly incorporated in the nanoparticles.