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Optimization of Production of PLA microbubble ultrasound contrast agents for Hydroxycamptothecin delivery

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Abstract

In this paper, ultrasound contrast agents based on a high molecular polymer-poly lactic acid (PLA) and loaded with Hydroxycamptothecin (HCPT) were prepared by combining ultrasound method and a Shirasu Porous Glass (SPG) membrane emulsification technique. A special focus was on the optimization of production of HCPT-PLA microbubbles. Different factors, such as the power and the time of ultrasonic action, the ratio of inner aqueous phase against outer oil phase, and the concentration of PLA were evaluated, and the average size of HCPT-PLA microbubbles, the drug carrying efficiency, as well as the acoustically-triggered drug release at 3kHz ultrasound were determined. The study showed that the HCPT-PLA microbubbles prepared using our optimized conditions, were sphere-like in shape with a mean diameter of 1-7μm. The drug loading efficiency reached up to 56.48%. In vitro, the drug release of HCPT-PLA microbubbles increased significantly at 3kHz ultrasound for 30s compared with that of ultrasound-free condition. In conclusion, the HCPT-PLA microbubbles has the characteristics desirable for an intravenously administered ultrasound contrast agent for further clinical use.

Keywords. Ultrasound contrast agent; Poly lactic acid (PLA); Hydroxycamptothecin (HCPT);Drug delivery

1.Introduction

Ultrasound contrast agents (UCA) can provide enhanced scattering signatures to distinguish the acoustic scattering signatures of blood from the surrounding tissues [1, 2]. The first-generation UCA were not ideal for the large size and instability. In recent years, microbubble encapsulated by a thin layer coat become the most extensive application compared with other contrast agents [3]. The coat-forming materials of microbubbles have phospholipids compounds, albumin, carbohydrate, non-ionic surfactant and the biodegradable polymeric-molecule polymer and so on[4,5]. Microbubbles encapsulated with polymer have uniformly and tight size distribution, compressibility, more stability in vivo, high natural resonance frequency, and acquired ideal scattering intensity under high emitted frequency [6,7,8,9]. It is assumed as the best contrast agents now and become a study hotspot.Microbubbles not only used for ultrasound clinical diagnostics, but also used for targeting or controlling drug release, because it will result in bubbles collapse ,and cell membrane permeability or absorption increase when the focus insonated by ultrasound [10,11,12].

There are many advantages when microbubble ultrasound agents were used in drug-carrying delivery system. Drug carried by stabilized microbubble could extend the half time in vivo and enhance the targeting property, eliminate unwanted immune response, stabilize the drug concentration in blood and reduce the toxicity and side effects.[13,14].

Poly lactic acid (PLA) is a popular biodegrade-able and bioconsistent polymer, that has been approved by the FDA for use in sutures, microcapsule, micobubble and others in intravenous injection.PLA can slowly be degraded by non-enzymatihydrolysis into lactic acids which is a natural body metabolite and can be degraded by enzymes to carbon dioxide and aqueous[15,16].

The drug loading microbubbles targeted in vivo is determined by their size. Microbubbles exceed 7μm in diameter are removed from blood in the lung circulation; while below 5μm they can flow across the capillary and are captured by the liver or spleen finally. So to use for drug loading, microbubble must be prepared with suitable sizes

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depended on different targetingtissues or organs[17,18].

Hydroycamptothecin(HCPT) is cytotoxic anti-tumor drug and a cell period restraining drug. It mainly acts on S period, and has selective inhibition on Toposomerases(TOPO1), It is effect-tive against idopathic liver cancer, stomach cancer, adenoid epithelium on head or neck ,leukemia, cancer of rectum, and bladder cancer et al[19]. However it has a short half-life and some adverse effects. In this paper, we optimized the preparation of encapsulated-HCPT, gas-filled PLA microbubbl es ultrasound contrast agents and integrated its drug releasing behivor in order to set up a drug targeting delivery model for toxic and short half-life drugs based on microbubble combining ultrasound.

2. Material and methods

2.1.Preparation of PLA microbubble by ultrasound double emulsion technique

Preparation process: 1ml inner aqueous phase (W1) was injected into 10ml CH_2Cl_2 oil phase in which dissolved PLA10000 and 1% Span80, while the ultrasound generator was used to form a w1/o primary emulsion. Then, this emulsion was injected into outer aqueous phase(w2) which contained 0.5%PVA, while the ultrasound generator was used to form w1/o/w2 double emulsion. Whereafter, the double emulsion was poured into 10 times volume of 10% PVA solution. Then, the liquor was churned up at 200rpm/min over night holding at 40□ on a magnetic force blender before centrifugated at 2000g for 20min. The microbubbles sediment were washed 3 times with distilled water after collected, and then were dried in vacuum refrigerant drier(Labconco Free Zone）before filled with air. The size distribution and figure of microbubbles were observed and measured with electron microscope (Philips XL-30) and Zetasizer (zetasizer nano series).

The power and time of ultrasound to prepare the w1/o emulsion, the concentration of PLA10000 , the rate of inner aqueous phase against outer oil phase, the power and time of ultrasound to prepare w1/o/w2 double emulsion, and the concentration of NH_4HCO_3 in the inner aqueous phase were all investigated just as the table1 shows.

Table 1. The investigated factors for the preparation of PLA microbubbles

Num.	U.p.t. for w1/o (w.s.t)	U.p.t.for w1/o/w2 (w.s.t)	$C_{PLA10000}$ （g/ml）	$R_{w1/o/w2}$	$C_{NH4HCO3}$ （g/ml）
1	80/10/2	200/10/2	0.0125	1：5：100	0.0025
2	80/10/5	200/10/5	0.025	1：10：100	0.005
3	80/10/10	200/10/10	0.05	1：15：100	0.01
4	80/10/15	200/10/15	0.1	1：20：100	0.02

Num.: Number.

U.p.t. for w1/o E.(w.s.t): ultrasound power and time for w1/o emulsion (watt.second.times).

U.p.t. for w1/o/w2 (w.s.t): ultrasound power and time for lipiodol double emulsion (watt.second.times).

$C_{PLA10000}$（g/ml）:Concentration of PLA10000（g/ml）in outer oil phase.

$R_{W1/O/W2}$: Rate of inner aqueous: outer oil: outer aqueous.

$C_{NH4HCO3}$（g/ml）: Concentration of NH_4HCO_3 in the inner aqueous phase（g/ml）.

2.2.Preparation of PLA microbubbles by the method of ultrasound cooperated with microporous membrane emulsifier

Preparation process: the preparation of the w1/o emulsion was based on the optimized method of 2.1.Then, the w1/o emulsion was poured into the pressure cabin of microporous membrane emulsifier which was fixed a SPG membrane of 1.1μm. Appropriate and equal pressure was given by the high pressure and high purified N2 to press the w1/o emulsion through SPG membrane into 20 times volume of circularly flowing 1% PVA solution. Then we churned it up at 200rpm/min over night holding at 40□ on a magnetic force blender. Collected the microbubbles sediment and washed 3 times with distilled water after centrifugated at 2000g for 20min. Dried it in vacuum refrigerant drier before filled with air. The size distribution and figure of microbubbles were observed and measured with electron microscope and zetasizer.

2.3.Preparation of HCPT–PLA microbubbles

The microbubbles were prepared with the optimized method of 2.2, and the inner aqueous phase was replaced by 0.9% sodium chloride and 0.0025g/ml NH_4HCO_3 solution into which was dissolved HCPT in different concentration of

1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml respectively. And then try to probe the relationship between HCPT concentration and microbubbles drug loading capacity or encapsulation rate.

A passel of 50mg PLA microbubbles was dissolved into 0.5ml CH_2Cl_2, then the HCPT was extracted out with 0.1mol/l sodium hydroxide solution to mensurate OD384nm. The concentration of HCPT was calculated referring to the standard curve between HCPT concentration and OD384nm. The calculation methods of the drug loading capacity and encapsulation rate of microbubble were refered to follow formulae.

Drug loading capacity = WHCPT / Wm×100%;
Encapsulation rate = Mdlc / Tdlc×100%
WHCPT: Weight of loading HCPT
Wm: Weight of microbubbles
Mdlc:Mensurated drug loading capacity
Tdlc: Theoretic drug loading capacity

2.4.Drug releasing behavior in vitro of insonated with ultrasound or not

A passel of 50mg PLA microbubbles was dispersed into 10ml of 0.9% sodium chloride solution, and was churned up on the magnetic force blender keeping the speed of 100rpm/min in 37□. 0.5ml solution was extracted every time after 0.5h, 3h, 24h, 72h and 168h, respectively, then the samples were centrifugated at 2000g for 20min to extract the upper liquid. The HCPT concentration of extraction was calculated by the standard curve between HCPT concentration and OD_{384nm}. Drug spontaneous releasing behavior in vitro was described with the relationship between cumulative concentration of released HCPT and time. The microbubbles dispersive buffer was prepared by the same way as upwards, and 0.5ml solution was extracted from each before the first operated and after operated by ultrasound in the 4frequency of 3.5MHz, and the average intensity per cross section of 1.0W/cm_2 for 30s,60s or 90s every time.

The HCPT concentration of extraction was calculated by the same way as upwards, and drug releasing behavior in vitro of being insonated with ultrasound was described with the relationship between cumulative concentration of released HCPT and insonated times.

3.Results and discussions

3.1.Preparation of PLA microbubbles by ultrasound double emulsion technique

Character of the prepared PLA microbubbles was effected by the ultrasound power and time for w1/o emulsion, ultrasound power and time for double emulsion, concentration of PLA10000, the volume rate of inner aqueous:outer oil: outer aqueous and the concentration of NH_4HCO_3 in the inner aqueous phase.

When the power/time/times of ultrasound for w1/o emulsion was low to 80w/10s/2t, the emulsion was unstable and the prepared microbubbles were too big and distributed in a large range. While it was up to 80w/10s/5t, the diameter of microbubbles can be controlled in 7μm, but too high of power/time/times over 80w/10s/10t will decreased the uniformity of microbubbles size.

Microbubbles size were effected observably by the power/time/times of ultrasound for w1/o/w2 double emulsion. The higher power/time/times always leaded to smaller size of microbubble. When the power/time/times was 200w/10s/2t,the size of some microbubbles were over 50μm, while it was up to 200w/10s/5t,the size was controlled in 7um, and the size decreased to about 0.1μm while the power/time/times increased to 200w/10s/15t.

Concentration of PLA10000 was the major element to affect the thickness and smoothness of the microbubbles' shell. when it was 0.0125g/ml, some of the microbubbles' shell were too thin to split, however, when it was increased to 0.05g/ml,the outer oil phase seems viscid and the microbubbles were out of sphere and smooth. While the concentration was up to 0.1g/ml, the PLA couldn't be dissolved into CH_2Cl_2 entirely, and no Microbubble but something nubbly were prepared. The results showed the best concentration of PLA10000 was about 0.025g/ml.

The diameter and amount of the pores on microbubbles' shell were both decided by the concentration of NH_4HCO_3 .when NH_4HCO_3 concentration was 0.0025g/ml，the great mass of microbubbles were just one pore on the shell. The more NH_4HCO_3 was dissolved into inner aqueous phase the more pores would be distributed on the microbubbles'shell.

The thickness of the microbubbles' shell was effected by the rate between the volume of inner aqueous phase and outer oil phase as well as the concentration of PLA10000. When the rate was 1：5, a part of the microbubbles was breaked which would certainly decrease the drug loading capacity and encapsulation rate of microbubbles. While the rate was low to 1:15, the shell of microbubbles was too thin and coarse as well as the rate's of 1:10. while the rate was 1:20, the microbubbles were out of sphere because the shell was too thin.

According to the results upwards, the feasible

method of preparing PLA microbubbles with a tight size distribution (from 1 to 7μm) and smooth surface was got as follows: adopting the same preparation processing as 2.1 described, dissolving 0.0025g NH_4HCO_3 into 1ml distilled water as inner aqueous phase, and 0.25g PLA10000 into 10ml CH_2Cl_2 as outer oil phase to which was dispersed 0.1ml Span80, and keeping the ultrasound working at 25kHz for 80w/10s/5t while prepare the first w1/o emulsion and working at 25kHz for 200w/10s/5t while prepare the w1/o/w2 double emulsion, filling air into the prepared microbubbles after dried in vacuum refrigerant drier, observing and measuring the size distribution and figure of microbubbles with electron microscope and zetasizer.

The electron microscope photograph (figure.1)

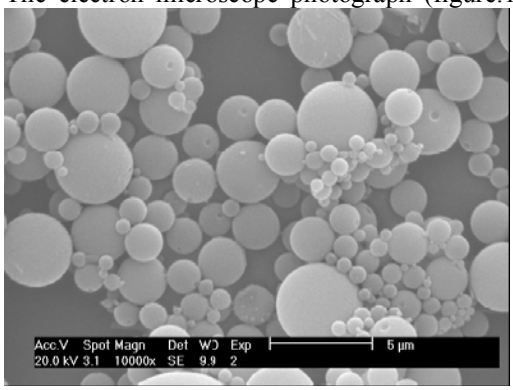
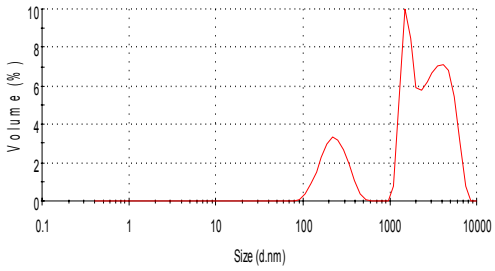


Figure 1.Electron microscope photograph of PLA microbubble produced by the method of supersonic w/o/w double emulsion



Graph1.Size distribution by volume of PLA microbubbles produced by the method of ultrasound.

shows that the diameter of microbubbles was distributed from 1 to 7μm, and the surface of the microbubbles is smooth as well as just one or no pore is on the shell. The microbubbles size distribution measured by zetasizer shows as graph1.The size distribution of the microbubbles was showed as graph1.19.5% of the microbubbles were blonged to peak 1 with the mean diameter of 238.1nm,and 34.3% were blonged to peak 2 of 1713nm,and 46.2% were blonged to peak 3 of 3961nm and no microbubble's diameter was bigger than 7000nm.

3.2.Preparation of PLA microbubbles by the method of ultrasound combining a Shirasu Porous Glass (SPG) membrane emulsification technique

The prime w1/o emulsion was prepared by ultrasound emulsification， dissolving 0.0025g NH_4HCO_3 into 1ml distilled water as inner aqueous phase, and 0.25g PLA10000 into 10ml CH_2Cl_2 as outer oil phase to which was dispersed 0.1ml Span80, and keeping the ultrasound working at 25kHz for 80w/10s/5t while injecting the inner aqueous phase into outer oil phase.

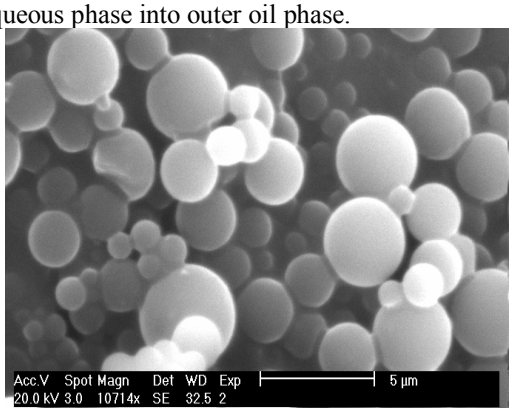
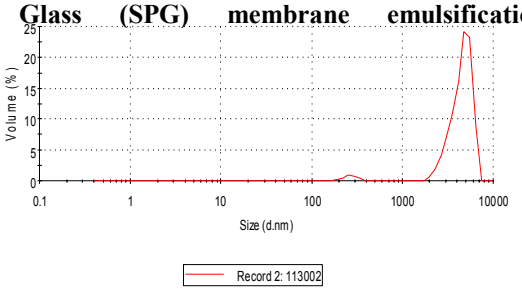


Figure 2. Electron microscope photograph of PLA microbubbles produced by the method of ultrasound combining a Shirasu Porous Glass (SPG) membrane emulsification



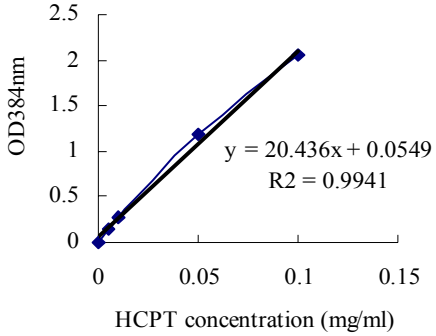
Graph2: Size distribution of PLA microbubble prepared by the method of ultrasonic cooperated with micromembrane emulsifier.

The size distribution of microbubbles was dominated by the N_2 pressure while the diameter of pores in SPG microporous membrane were aptotic of 1.1μm. The appropriate pressure of N_2 was about 30kPa, on which the prime w1/o emulsion can equably permeate the SPG microporous membrane, and the microbubbles were prepared with a narrow size distribution. results showed as fig2 and graph2 that 93.6% microbubbles blonged

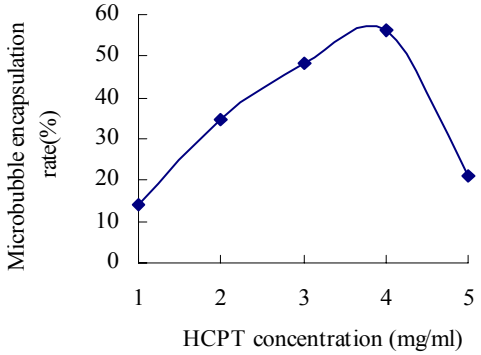
to peak2 of the mean diameter of 4124nm without microbubbles bigger than 7000nm and 6.4% microbubbles blonged to peak1 of 274nm.

3.3.Preparation of PLA microbubbles carried HCPT

3.3.1.Relationship between inner aqueous phase HCPT concentration and drug loading capacity or encapsulation rate of microbubbles

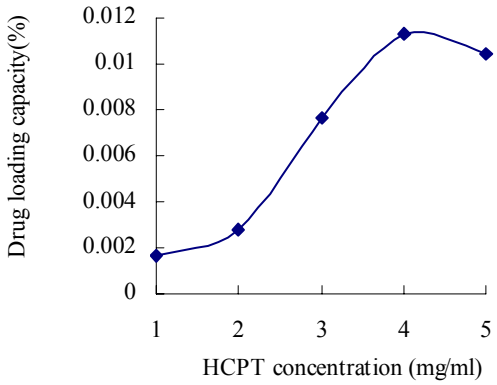


Graph 3.Standard curve between HCPT concentration and OD384nm



Graph 4.Relationship between HCPT concentration of inner aqueous phage and microbubble encapsulation rate

The standard curve and equation between HCPT concentration and OD_{384nm} was showed as graph3. The relationship between HCPT concentr-ation of inner aqueous phase and microbubbles drug loading capacity or microbubbles



Graph 5. Relationship between HCPT concentration of inner aqueous phage andmicrobubble drug loading capacity rate

encapsulation rate of PLA microbubbles were showed on table2, graph4 and graph5. Results show that both the drug loading capacity and encapsulation rate were increased with the HCPT concentration before 4mg/ml, at which point them both on the top of 1.13% and 56.48%, and then them decreased together against to the increasing of HCPT concentration.

Table 2.Relationship between HCPT concentration and drug loading capacity or microbubbles encapsulation rate

$Concentration_{HCPT}$ (mg/ml)	1	2	3	4	5
Encapsulation rate(%)	14.02	34.79	48.08	56.48	20.89
Drug loading capacity(%)	0.17	0.28	0.77	1.13	1.04

3.3.2.Drug releasing behavior in vitro insonated with ultrasound or not

Drug releasing behavior in vitro of microbubbles was showed as table3 and graph6 that the cumulative percent of drug spontaneous released in vitro was increased with the prolonger of time when the time was prolonged to 168h. The cumulative percent of drug released was 64.14%. The cumulative percent of drug released was increased with the enhancing of ultrasound work. While the microbubbles were treated with ultrasound for 4 times of 30sec (total 120sec.) every time at 3.5MHz ,the percent of drug released was cumulated to 68.49%. While it was treated for 4 times of 60sec. (total 240sec.) every time, the percent of drug released was cumulated to 86.97%. and while the it was treated for 3 times of 90sec(total 270sec.) every time, the percent of drug released was cumulated to 94.37%. However, treated it for 4 times of 90sec (total 360sec.) every

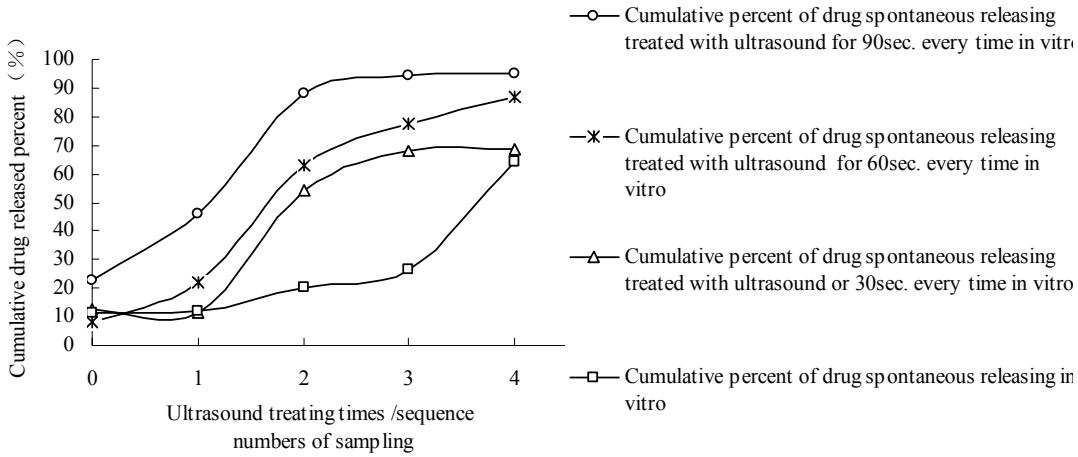
time, the percent of drug released was only cumulated to 94.81%, which seems no more drugs could be released from microbubbles.

Table 3.Drug releasing behavior in vitro of insonated with ultrasound or not

T/S	$P_{D.S.R.(\%)}$	$P_{D.R.S.30}$ (%)	$P_{D.R.S.60}$ (%)	$P_{D.R.S.90}$ (%)
0	11.52	12.61	8.26	22.40
1	12.17	11.09	21.96	45.88
2	20.44	54.14	62.84	87.84
3	26.53	67.84	77.62	94.37
4	64.14	68.49	86.97	94.81

T/S: ultrasound treating times of sequence of sampling.
PD.S.R.: cumulative percent of drug spontaneous releasing in vitro.
PD.R.S.30: cumulative percent of drug releasing treated with B-ultrasound for 30sec. every time in vitro.
PD.R.S.60: cumulative percent of drug releasing treated with B-ultrasound for 60sec. every time in vitro.
PD.R.S.90: cumulative percent of drug releasing treated with B-ultrasound for 90sec. every time

Graph 6.Microbubble drug releasing behavior in vitro



4.Conclusions

Drug carrying ultrasound microbubble has been deemed as a new carrier for drug targeting and controlling delivery. To target in vivo, the microbubbles must be controlled under 7μ m in diameter to pass the lung circulation. When drug carrying ultrasound microbubbles were injected into vein and focus was scanned by ultrasound in a appropriate frequency, the drug will be delivered mostly to the targeted focus. So, the focus has a high drug concentration against the low drug concentration in blood.

Our research shows that the diameter of the microbubbles prepared with ultrasound double emulsion technique were controlled under 7μm, therein, the diameter of 19.5%, 34.3% and 46.2% microbubbles were around 238.1nm, 1713nm, and 3961nm respectively. However, 93.6% microbubbles prepared by the method of ultrasound cooperating with microporous membrane emulsifier was around 4124nm and no one bigger than 7000nm besides 6.4% microbubbles was around 274nm.

So, we consider that the microbubbles can be used as drug carrier both prepared by the method of ultrasound double emulsion and the method of ultrasound cooperating with microporous membrane emulsifier. While, the microbubbles prepared by the latter are better than the first's. we also find that the time of HCPT spontaneously releasing from PLA microbubbles in vitro can be kept over 1 week and ultrasound can evidently accelerate the HCPT releasing from PLA microbubbles in vitro.

Based on the results of upwards we are sure that the drug releasing behavior in vivo insonated with ultrasound or not should be more interesting and complex. The further investigation of drug releasing behavior in vivo is in process.

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