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Resveratrol Incorporated Liposomes Prepared from Pegylated Phospholipids and Cholesterol

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***Abstract*—**Liposomes and pegylated liposomes were widely used as drug delivery system in pharmaceutical field since a long time. However, in the former time, polyethylene glycol (PEG) was connected into phospholipid after the liposomes were already prepared. In this paper, we intend to study the possibility of applying phospholipids which already connected with PEG and then they were used to prepare liposomes. The model drug resveratrol was used because it can be applied against different diseases. Cholesterol was applied to stabilize the membrane of liposomes. The thin film technique in a laboratory scale was a preparation method. The liposomes were then characterized by nanoparticle tracking analysis (NTA), photon correlation spectroscopy (PCS) and light microscopic techniques. The stable liposomes can be produced and the particle sizes after filtration were in nanometers. The 2- and 3-chains-PEG-phospholipid (PL) caused in smaller particle size than the 4-chains-PEG-PL. Liposomes from PL 90G and cholesterol were stable during storage at 8 °C of 56 days because the particle sizes measured by PCS were almost not changed. There was almost no leakage of resveratrol from liposomes PL 90G with cholesterol after diffusion test in dialysis tube for 28 days. All liposomes showed the sustained release during measuring time of 270 min. The maximum release amount of 16-20% was detected with liposomes from 2- and 3-chains-PEG-PL. The other liposomes gave max. release amount of resveratrol only of 10%. The release kinetic can be explained by Korsmeyer-Peppas equation.

***Keywords*—**Liposome, NTA, resveratrol, pegylation, cholesterol.

I. Introduction

LIPOSOMES and pegylated liposomes were intensively studied by other research groups [1], [2]. In our paper, we intent to study the PLs that have been synthesized by our cooperation partner (Celares GmbH. Germany, [3]) in order to compare the results with PLs that commercial available until now. The model drug resveratrol was used because it was reported to be useful as anticancer, anti-aging etc. [4]. The NTA technique works under the principle of dynamic light scattering including the light microscope [5], [6]. Therefore, the particle size, size distribution, concentration (numbers of particles/milliliter) as well as image of particles can be observed. The NTA technique therefore was used in combination with the classical dynamic light scattering (PCS) technique to study the particle size.

II. Materials and Methods

Different PLs and mixture thereof were studied. Table I shows the commercial available PL and abbreviations that have been used.

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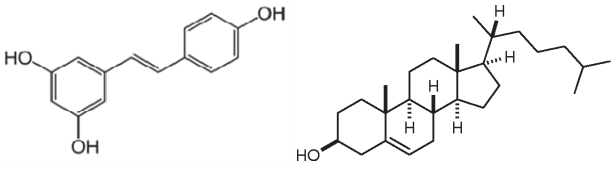
TABLE I
Type of PLs Used

Type of PL	Symbol	Component
Phospholipon ® 90G	PL 90G	Phosphatidylcholine (PC) 94.0%-102.0% and Lysophosphatidyl choline max. 4.0% and Tocopherol max. 0.3%. PC was stabilized with 0.1% ascorbyl palmitate
Phospholipon ® 90NG	PL 90NG	Phosphatidylcholine 90.0% and Lysophosphatidylcholine max. 6.0% and Tocopherol max. 0.3 %
Phospholipon ® 85G	PL 85G	Phosphatidylcholine min. 85.0% and Lysophosphatidylcholine 3%

TABLE II
Components of Different Samples

Sample Number	Type of PL	Drug	Cholesterol	Pepylated PL
1	PL 90G	--	--	--
2	PL 90NG	--	--	--
3	PL 85G	--	--	--
4	PL 90G	--	Cholesterol	--
5	PL 90NG	--	Cholesterol	--
6	PL 85G	--	Cholesterol	--
7	PL 90G	Resveratrol	--	--
8	PL 90NG	Resveratrol	--	--
9	PL 85G	Resveratrol	--	--
10	PL 90G	Resveratrol	Cholesterol	--
11	PL 90NG	Resveratrol	Cholesterol	--
12	PL 85G	Resveratrol	Cholesterol	--
13	PL 90G	--	--	2-chains-PEG-PL
14	PL 90G	--	--	3-chains-PEG-PL
15	PL 90G	--	--	4-chains-PEG-PL
1a	PL 90G	Resveratrol		--
2a	PL 90G	Resveratrol	Cholesterol	--
3a	PL 90G	Resveratrol	--	2-chains-PEG-PL. 1%
4a	PL 90G	Resveratrol	--	2-chains-PEG-PL. 10%
5a	PL 90G	Resveratrol	--	3-chains-PEG-PL. 1%
6a	PL 90G	Resveratrol	--	3-chains-PEG-PL. 10%
7a	PL 90G	Resveratrol	--	4-chains-PEG-PL. 1%
8a	PL 90G	Resveratrol	--	4-chains-PEG-PL. 10%



(a) (b)

Fig. 1 Chemical structures of (a) resveratrol and (b) cholesterol [7]

The formulations that have been studied are shown in Table II. The thin film technique in a laboratory scale was used to prepare liposomes. The amount of cholesterol was 5%w/w and

PL (Phospholipon®) was 95%w/w. If pegylated PL was used, it was calculated to be 1%w/w and Phospholipon was 99%w/w.

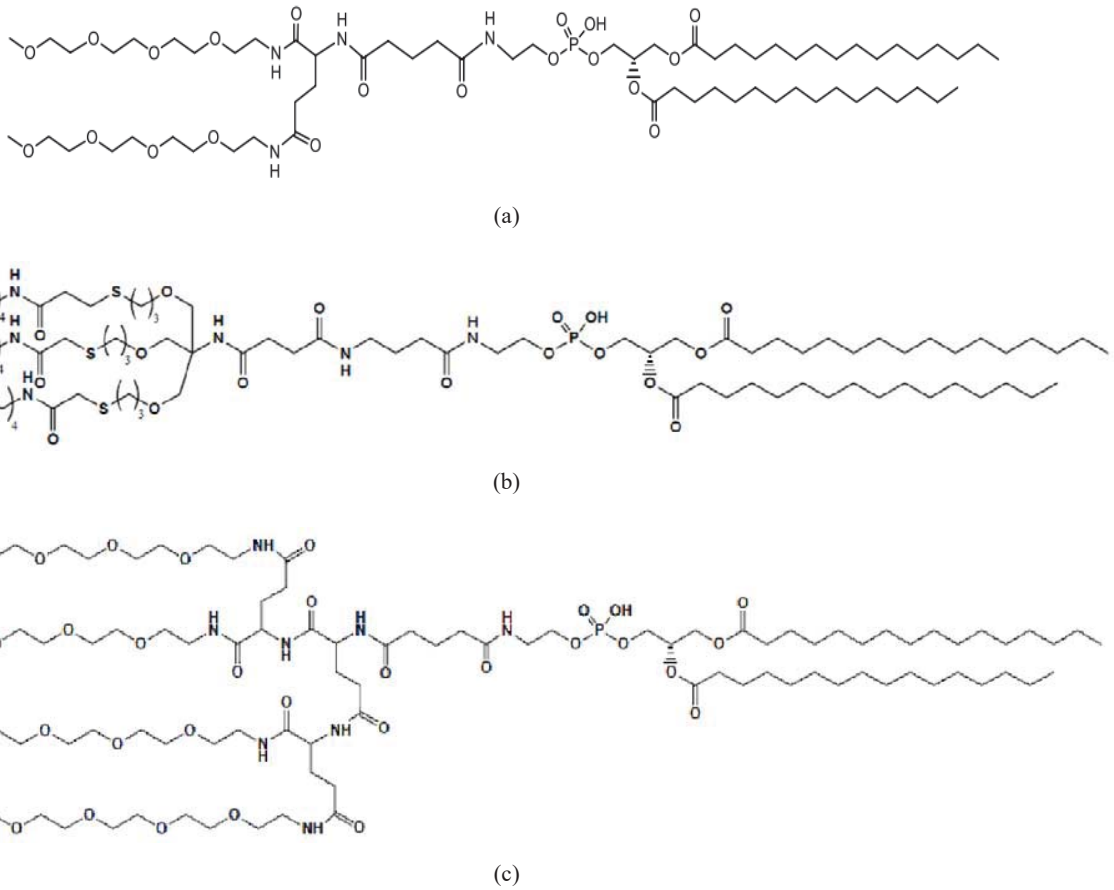


Fig. 2 Chemical structures of pegylated PLs that have been synthesized by our cooperation partner [3] Phosphatidyl ethanolamine (DPPE) with different chains (a) 2-chains-PEG-PL (b) 3-chains-PEG-PL (c) 4-chains-PEG-PL

The prepared liposomes were washed until no free resveratrol was observed in the washing water by using UV-VIS spectrometer. Then these liposomes were tested concerning release. Two calibration equations were used to calculate the resveratrol amount. The calibration equation of resveratrol in water is y = 0.1224 + 0.0029 (R^2 = 0.9999) and in ethanol:water (1:1) is y = 0.1322 + 0.0038 (R^2 = 0.9999). The Korsmeyer-Peppas equation was used to fit the release profile and is shown in (1):

$$\frac{M_t}{M_\infty} = k \cdot t^n \quad (1)$$

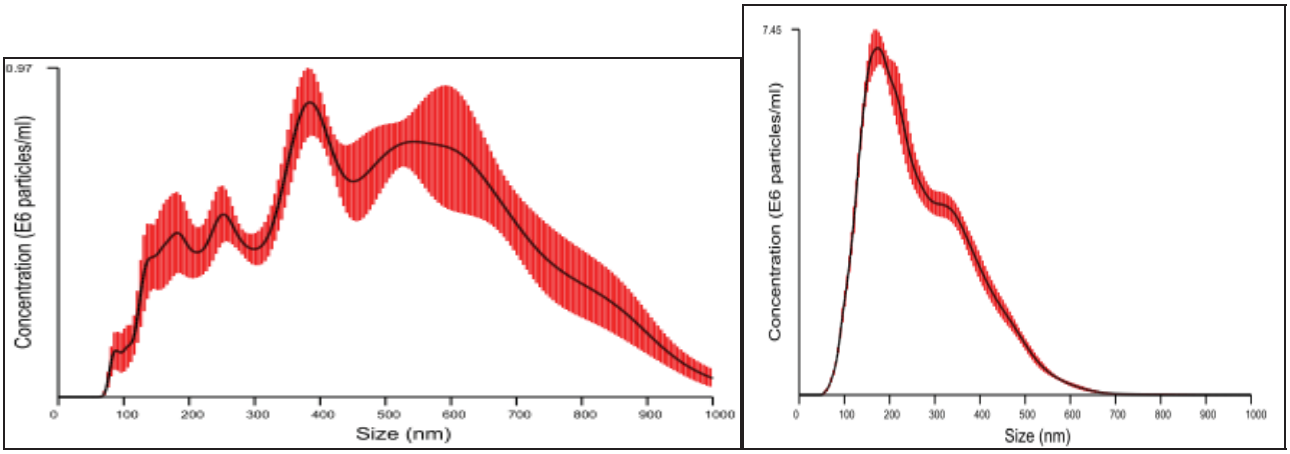
M_t/M_∞ = drug released fraction at release time t; k = constant corresponding the kinetic characteristic of drug/matrix system; n = release exponent, influenced by the drug release mechanism and shape of delivery system

If the value of n is ≤ 0.45 corresponds to a Fickian diffusion mechanism. Values of $0.45 < n < 0.89$ indicates a non-Fickian transport. If n = 0.89 a case II transport or zero order release with constant drug release during time and if n is higher 0.89 a super case II transport occurs [8].

III. Results and Discussions

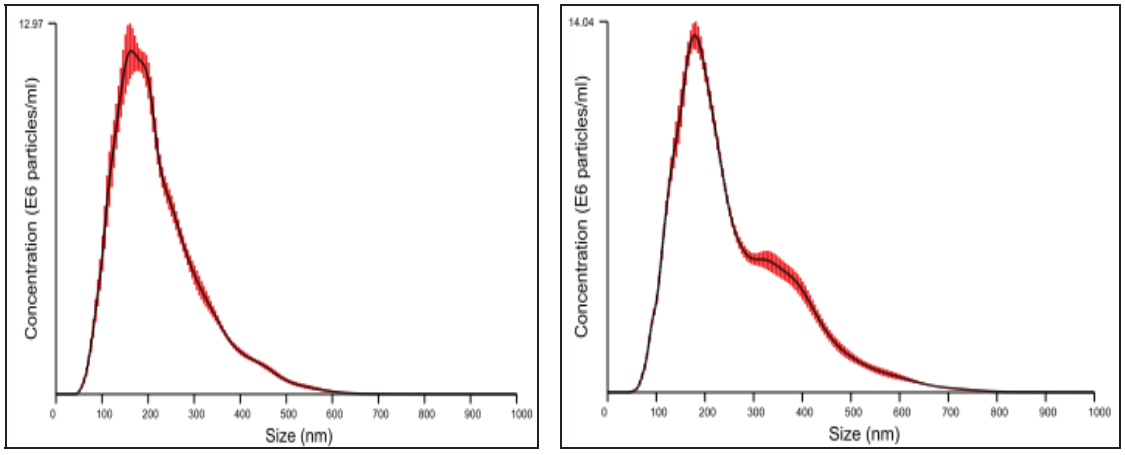
Fig. 3 shows the particle sizes of sample no. 1 before the samples were filtered through the membrane 0.22 μm. After the filtration all samples were smaller and the results were shown in Table III. Some examples of the particle size distribution were shown in Fig. 4. Samples from pegylated liposomes showed multiple peaks which might come from the bound PEG. Fig. 5 shows the mean particle size of all samples (Table III). Effects of PL types, resveratrol, cholesterol and PEG were shown.

Fig. 6 shows that neither cholesterol nor resveratrol has effect on the particles size of the liposomes from PL 90G. On the other hand, cholesterol and resveratrol have effect on the particle size of liposomes prepared from PL 90NG and PL 85G. (Figs. 7, 8).

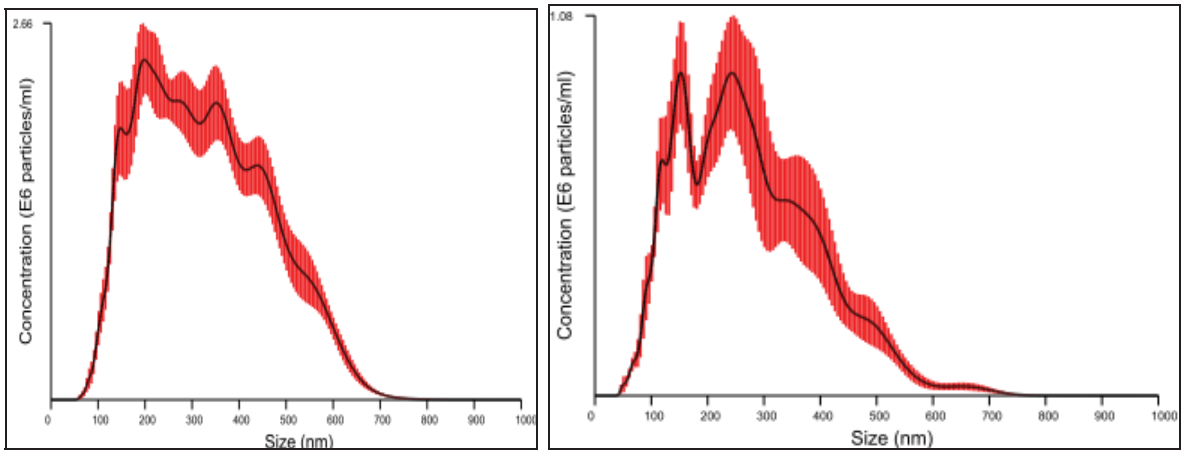


(a) (b)

Fig. 3 NTA-results showing particle size of the sample no. 1 (a) before filtration. (b) after filtration



(a) (b)



(c) (d)

Fig. 4 NTA-results showing particle size of different samples after filtration (a) sample no. 2 (b) no. 3 (c) no. 12 (d) no. 15

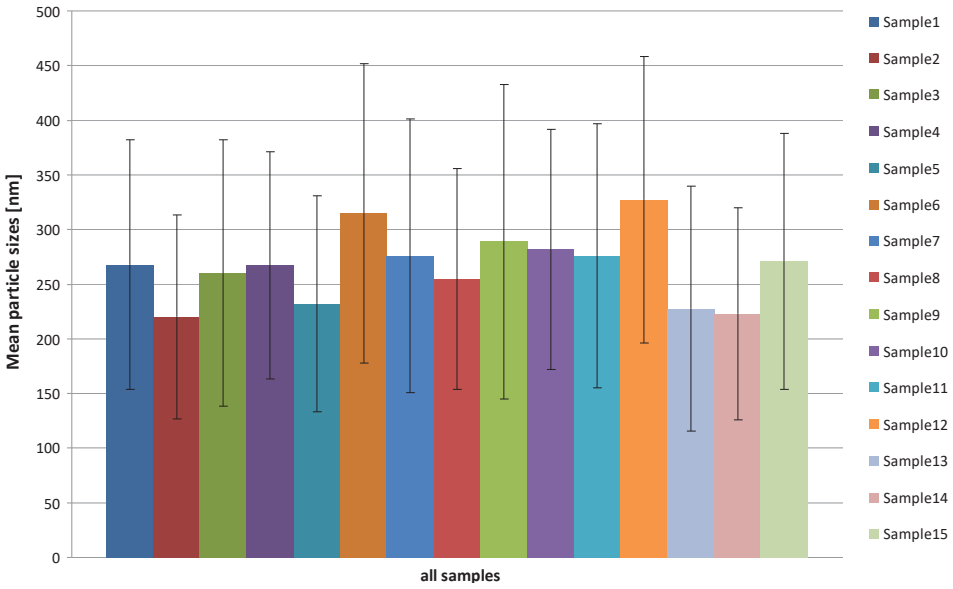


Fig. 5 NTA results showing the mean particle size of all samples

TABLE III
PARTICLE SIZE OF DIFFERENT SAMPLES MEASURED BY NTA

Sample	Mean Size [nm]	SD [nm]	D_{10} [nm]	D_{50} [nm]	D_{90} [nm]	Concentration [Particle/ml]	Tracks
1	268	114	138 +/- 1.7	244 +/- 4.6	431 +/- 6.7	16.63 +/- 0.53 E8	4196
2	220	93	119 +/- 2.6	198 +/- 4.5	353 +/- 6.3	21.89 +/- 0.72 E8	6401
3	260	122	133 +/- 1.2	225 +/- 4.5	432 +/- 7.8	26.70 +/- 0.33 E8	7180
4	267	104	145 +/- 2.2	251 +/- 4.5	411 +/-10.6	15.26 +/- 0.37 E8	3605
5	232	99	124 +/- 1.4	209 +/- 2.5	373 +/- 4.5	20.00 +/- 0.69 E8	5620
6	315	137	156 +/- 3.7	289 +/-11.3	513 +/-12.1	16.21 +/- 0.55 E8	3709
7	276	125	139 +/- 3.7	247 +/- 9.2	454 +/-15.0	14.76 +/- 0.48 E8	3620
8	255	101	138 +/- 3.5	239 +/- 7.0	399 +/-12.7	10.29 +/- 0.54 E8	2419
9	289	144	143 +/- 1.4	241 +/- 7.7	494 +/-16.7	15.03 +/- 0.71 E8	4214
10	282	110	152 +/- 5.5	268 +/-11.7	423 +/- 9.8	8.26 +/- 0.44 E8	1847
11	276	121	136 +/- 2.0	254 +/- 4.7	447 +/-10.0	26.96 +/- 0.14 E8	5500
12	327	131	165 +/- 9.5	310 +/-15.6	519 +/-11.0	8.44 +/- 0.23 E8	1732
13	228	112	104 +/- 2.6	194 +/- 8.9	396 +/-23.6	2.59 +/- 0.15 E8	804
14	223	97	115 +/- 3.8	201 +/- 5.8	362 +/-14.0	4.68 +/- 0.12 E8	1384
15	271	117	133 +/-10.5	251 +/-15.9	440 +/-29.2	2.56 +/- 0.23 E8	652

SD = Standard deviation

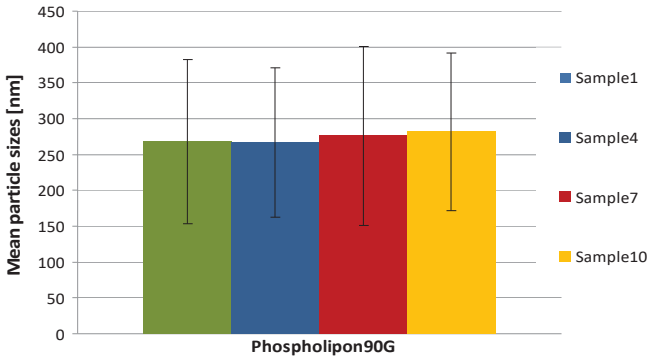


Fig. 6 NTA results showing the mean particle size of different liposomes prepared from PL 90G

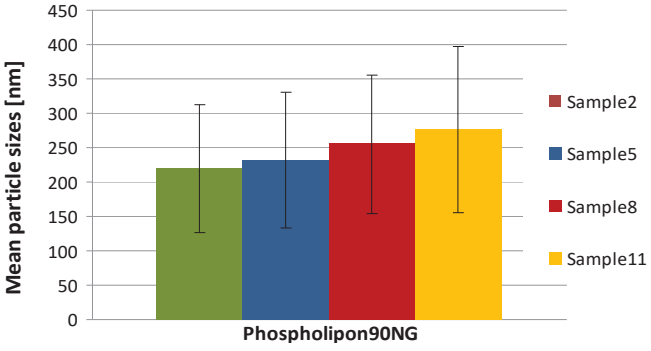


Fig. 7 NTA results showing the mean particle size of different liposomes prepared from PL 90NG

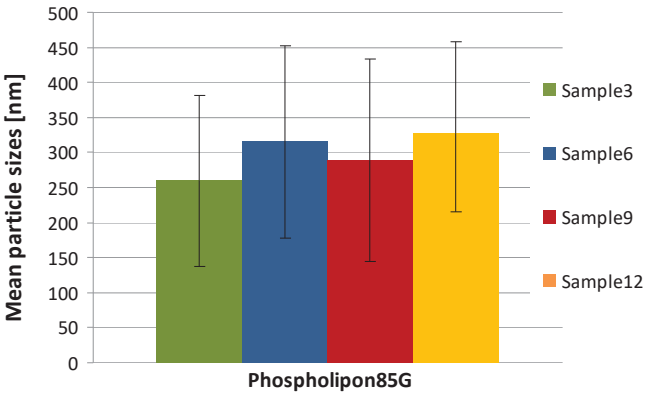


Fig. 8. NTA results showing the mean particle size of different liposomes prepared from PL 85G.

Fig. 9 shows the effect of PEG on the liposome from PL 90G. The 2- and 3-chains-PEG-PL caused in smaller particle sizes than the 4-chains-PEG-PL. The NTA technique shows that no micelles were formed during the liposomes preparation. The results of micell formation were compared with Tween 80, which can form micelles and NTA can detect this occurrence.

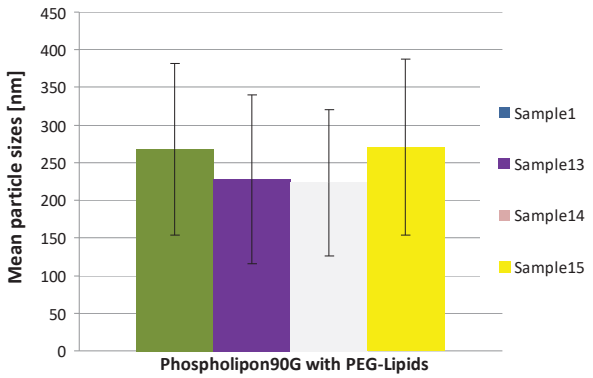


Fig. 9 NTA results showing the mean particle size of different liposomes prepared from pegylated PL

The prepared liposomes were washed with water many times until the washing water showed no absorption. Afterward the liposomes were tested to determine the release of resveratrol by putting the dialysis tube into the dissolution tester with paddle at 42 °C, 60 rpm, 150 ml water and the absorption were determined at the wavelength of 305.5 nm. All liposomes showed the delayed release during measuring time of 270 min. The maximum release amount of 16-20 % was detected with liposomes from 2- and 3-chains-PEG-PL. The other liposomes gave max. release amount of resveratrol only of 10%.

The stability of the prepared liposomes can be seen in Table IV. The sample no. 10 seems to be most stable during the storage (56 days) because the mean particle size was not significantly changed compare to other samples. The leakage of resveratrol from liposome sample no. 10 was also very low (~ 0.46 %) after 28 days.

TABLE IV
THE MEAN PARTICLE SIZE OF LIPOSOMES FRESHLY PREPARED AND AFTER STORAGE MEASURED BY PCS (ZETASIZER 3000)

Sample	Freshly prepared	Storage time	After storage
	(nm)	(days)	(nm)
7	1472.9	49	34.2
8	1575.8	49	214.1
9	531.5	35	328.8
10	1427.6	56	1418.5
11	1670.1	28	3030.5
12	560.3	35	208.8

Because during the release tests, some water was evaporated, the correction of this was done and the corrected release results were shown in Fig. 10. Samples 3a, 4a, 5a, and 6a give the highest release compared to the other samples. This means that the 2 and 3-chains-PEG-PL induce the release whereas 4-chains-PEG-PL did not change the release amount. Moreover, different temperatures (in our case room temperature (RT) and 42 °C) caused different releases. The comparison is shown in Table V. Higher temperatures caused higher release. This means the liposomes were thermosensitive.

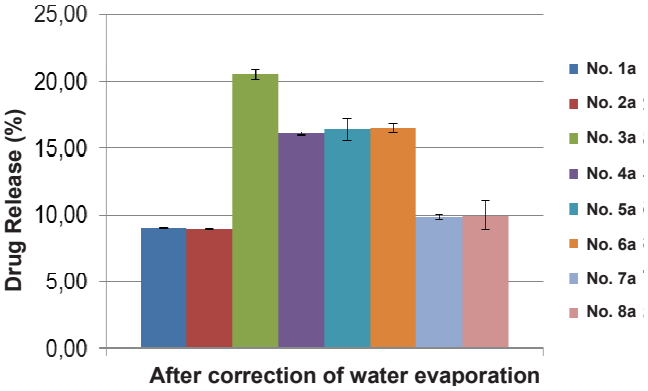


Fig. 10 Release of drug after correction of the water loss via evaporation

TABLE V
COMPARISON OF % RELEASE AT TWO TEMPERATURES (RT AND 42°C)

Time (Min)	% Release	
	RT	42 °C
0	0	0
30	18.77	32.31
60	29.11	37.87
90	36.22	42.55
120	37.89	43.33
195	43.30	-
270	-	46.57

The release results were shown in Figs. 11-18. The release curves were fitted with Korsmeyer-Peppas which showed that the releases were non-Fickian transport. The results are shown in Table VI.

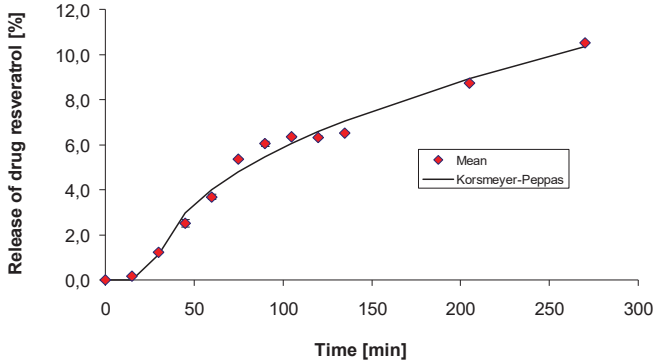


Fig. 11 Release of drug from PL 90G

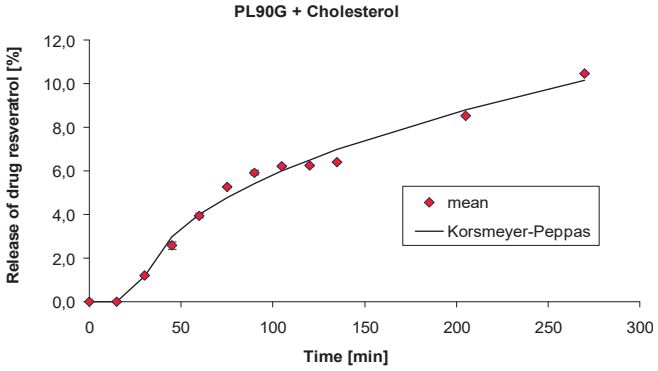


Fig. 12 Release of drug from PL 90G + Cholesterol

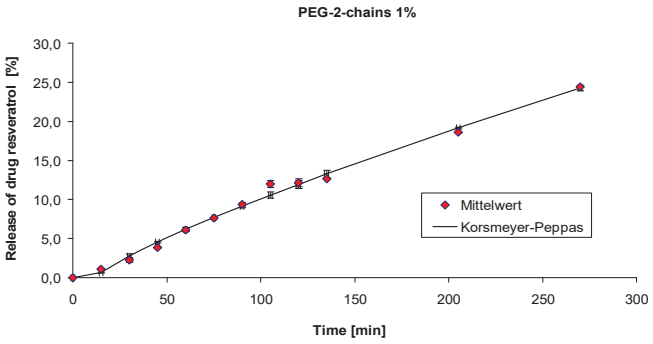


Fig. 13 Release of drug from 1% pegylated PL 2-chains

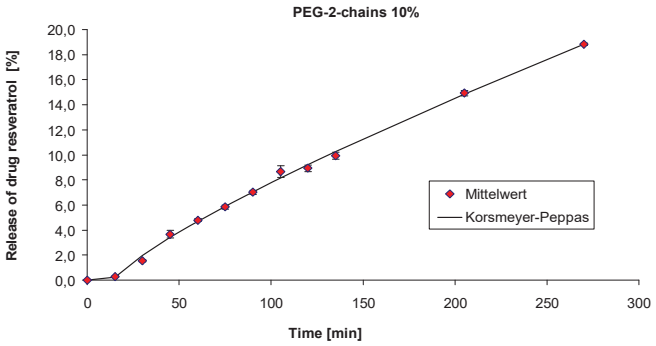


Fig. 14 Release of drug from 10% pegylated PL 2-chains

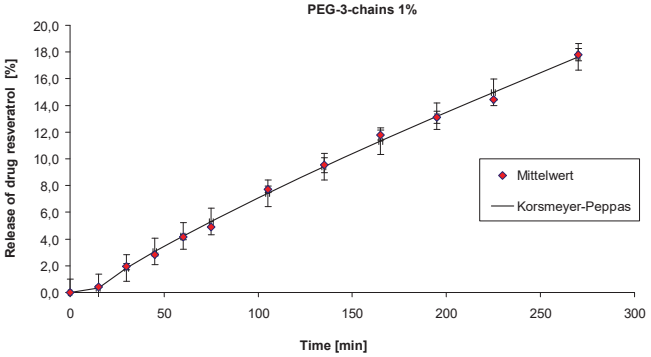


Fig. 15 Release of drug from 1% pegylated PL 3-chains

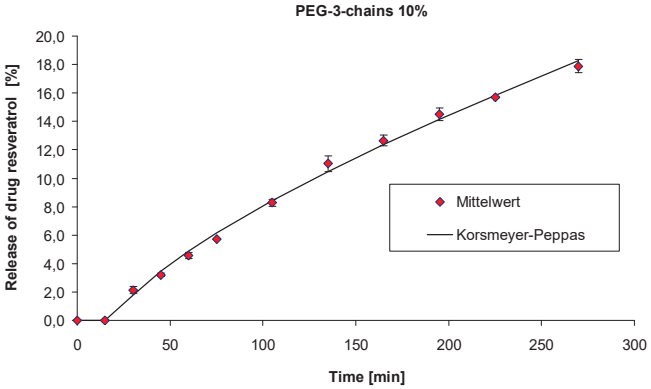


Fig. 16 Release of drug from 10% pegylated PL 3-chains

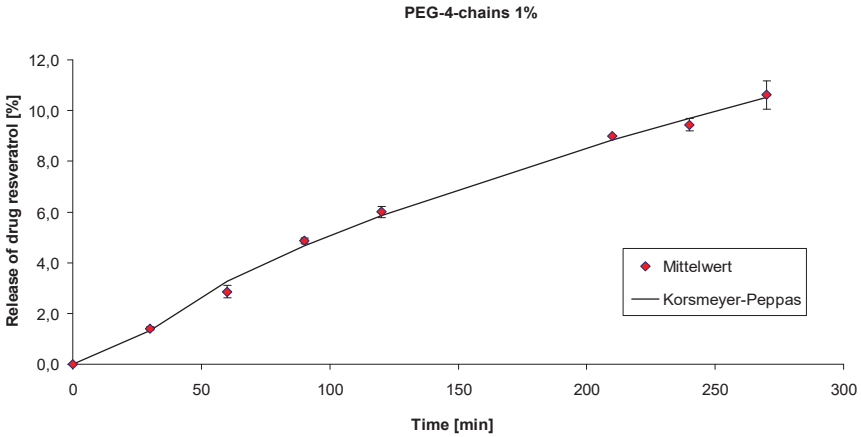


Fig. 17 Release of drug from 1% pegylated PL 4-chains

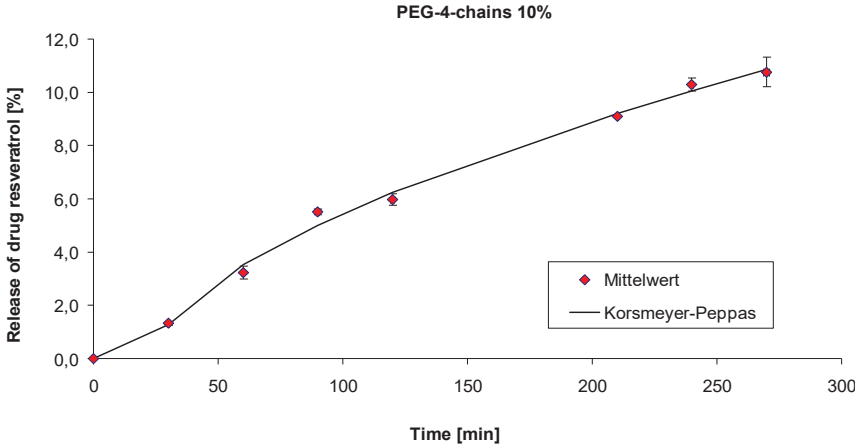


Fig. 18 Release of drug from 10% pegylated PL 4-chains

TABLE VI
KINETIC OF DRUG RELEASE OF DIFFERENT SAMPLES

Sample	k	t_{lag}	n	R^2	Particle size (nm)
1a	0.7831	27.83	0.4700	0.9775	1701.33
2a	0.8046	27.79	0.4618	0.9824	1819.48
3a	0.2631	11.90	0.8145	0.9849	549.16
4a	0.2095	13.98	0.8112	0.9932	497.48
5a	0.1600	12.47	0.8473	0.9977	511.64
6a	0.3518	20.48	0.7155	0.9972	445.17
7a	0.3145	20.52	0.6358	0.9965	442.93
8a	0.4172	23.29	0.5917	0.9959	432.08

IV. SUMMARY

The results reveal that the particle size of liposomes after the size reduction by membrane filtration can be well characterized by NTA technique. No micelles appear during this process. Liposomes from PL 90G did not change their particle size after the incorporation of cholesterol or of the drug resveratrol. On the other hand, cholesterol and resveratrol has an effect on particles size of liposomes from PL 90NG and PL 85G. Pegylation has an effect on the particle size of liposomes from PL 90G. The 2- and 3-chains-PEG-PL caused in smaller particle size than the 4-chains-PEG-PL. Liposomes from PL 90G and cholesterol were stable during storage at 8 °C of 56 days because the particle sizes measured by PCS were almost not changed. There was almost no leakage of resveratrol from liposomes PL 90G with cholesterol after diffusion test in dialysis tube for 28 days. All liposomes showed the delayed release during measuring time of 270 min. The maximum release amount of 16-20 % was detected with liposomes from 2- and 3-chains-PEG-PL. The other liposomes gave max. release amount of resveratrol only of 10%. The release kinetic can be explained by Korsmeyer-Peppas equation.

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