

## Electronic Supplementary information (ESI)

### Tunable stability of monodisperse secondary O/W nano-emulsions

R. Vecchione,<sup>\*a,b</sup> U. Ciotola,<sup>b</sup> A. Sagliano,<sup>b</sup> P. Bianchini,<sup>c</sup> A. Diaspro,<sup>c</sup> and P. A. Netti<sup>a, b</sup>

<sup>a</sup> *Center for Advanced Biomaterials for Health Care@CRIB - Istituto Italiano di Tecnologia, Via Largo Barsanti e Matteucci 53, 80125, Naples, Italy;*

<sup>b</sup> *Interdisciplinary Research Center of Biomaterials, CRIB - University Federico II, P.le Tecchio, 80, 80125, Naples, Italy.*

<sup>c</sup> *Nanophysics, - Istituto Italiano di Tecnologia (IIT), Via Morego 30, 16163, Genova, Italy.*

\*[raffaele.vecchione@iit.it](mailto:raffaele.vecchione@iit.it)

#### **Methods:**

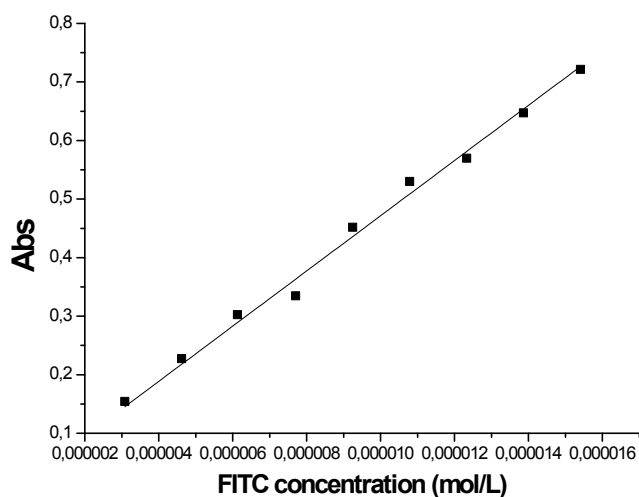
##### Chemical treatments on Chitosan

###### *Chitosan purification:*

A 1 wt% solution of chitosan in a solution of acetic acid and water at pH 2.5 (0.5 M acetic acid) was prepared. Chitosan is readily soluble in acidic conditions pH ~ 2.5, due to the protonation of its amine moieties. After complete dissolution under magnetic stirring, the solution was filtered; then the pH of the solution was changed to basic, adding a solution of NaOH 6M, thus allowing polymer precipitation. Chitosan was collected by centrifugation (15 min, 4 °C, 9000 rpm), re-dispersed in MilliQ water and re-collected by centrifugation (5 min, 4 °C, 9000 rpm) several times, until all NaCH<sub>3</sub>COO was removed and the pH of the chitosan suspension became neutral. Then, the sample was freeze-dried for 48 h and further dried in oven for 3 h at 80 °C to maximize removal of water, since chitosan is strongly hygroscopic.

*Synthesis of FITC-labeled chitosan:*

500 mg of purified and dried chitosan were dissolved in 50 ml of aqueous acetic acid solution (0.10 M). After complete dissolution, 50 ml of methanol were added. Then, 25 ml of a 1 mg/ml solution of FITC (fluoresceine isothiocyanate) in anhydrous methanol was prepared. This solution was slowly added to the chitosan solution and the reaction was carried out in the dark under magnetic stirring for 18 h at room temperature. Finally, chitosan was precipitated with a solution of NaOH and the precipitate was collected by centrifugation (9000 rpm; 4 °C; 15 min). The obtained FITC-labeled chitosan was dispersed in MilliQ water washed and centrifuged 6 times until pH reached a neutral value and no traces of FITC were visible in the waste washing solutions. As additional evidence that all the FITC was removed, a small amount of the functionalized sample was dispersed in a basic water solution (pH 10) (FITC, is pH sensitive and the fluorescence is very strong in these conditions) and then centrifuged. Negligible fluorescence was revealed when measuring the solution separated from the dispersed polymer at the Spectrofluorimeter (PerkinElmer EnSpire 2300 Multimode Plate Reader); therefore, the labeled polymer was considered purified. After reaction, the degree of functionalization was determined by means of UV-Vis analysis, comparing the absorbance with a FITC calibration line. The calibration line (Figure S.1) was calculated for FITC concentration from 6µg/ml to 1.2 µg/ml in a solution 8:2 water/DMSO with water phase at pH 6.13 (HAc/Ac- buffer). CT-FITC absorbance was recorded under the same conditions. At this pH, FITC's  $\epsilon$  was  $4.71 \cdot 10^4 \text{ M}^{-1}\text{cm}^{-1}$ . The calculated degree of functionalization was 1.3%.



**Figure S.1.** FITC calibration line for determination of chitosan degree of functionalization with FITC.

## Results:

### Reproducibility tests on primary emulsions

Emulsion size was proved to be quite reproducible at different processing conditions. As an example, we report the case of a L4 formulation (20 wt% of oil in water) processed under different conditions, Table S1.

Processing conditions		Size (nm)	PDI
Chamber 1	No cargo	81.92	0.092
Chamber 1	Nile Red (~0.06 mg/ml oil)	83.19	0.088
Chamber 2	No cargo	86.52	0.098
Chamber 2	No cargo	87.16	0.084
Chamber 2	Piperine (~20 mg/ml oil)	84.02	0.102

**Table S.1.** DLS size and PDI for L4 based primary emulsions at 20 wt% of oil in water after 3 cycles and 200 steps of processing at 200 bar.

As it is possible to see from the table, the emulsion preparation is highly reproducible by using the same processing chamber (less than 2% of variation) and slightly depending on the processing chamber (less than 5% of variation).

## DLS results on primary emulsions

Process conditions	L1			L2			L3			L4			L5		
	Size (nm)	PDI	Z-pot (mV)	Size (nm)	PDI	Z-pot (mV)	Size (nm)	PDI	Z-pot (mV)	Size (nm)	PDI	Z-pot (mV)	Size (nm)	PDI	Z-pot (mV)
Cycle 0	437	0.44	-42.7	317	0.38	-43.8	299	0.41	-42.9	261	0.37	-46.3	265	0.39	-42.9
Cycle 1	239	0.14	-48.9	201	0.17	-43.8	181	0.13	-40.9	156	0.14	-40.1	151	0.15	-43
Cycle 2	212	0.09	-40	180	0.12	-43.1	167	0.09	-40.9	143	0.13	-43.6	141	0.14	-45.6
Cycle 3	206	0.08	-40.3	172	0.09	-41.9	161	0.10	-34.1	134	0.11	-43.4	134	0.13	-43.1
Cycle 3 + 50 steps	181	0.06	-43.9	150	0.07	-41.4	132	0.08	-40.2	109	0.12	-43.4	107	0.12	-37.9
Cycle 3 + 100 steps	168	0.04	-42.5	133	0.07	-39.5	115	0.07	-37.4	97	0.11	-44.2	94	0.13	-28.5
Cycle 3 + 150 steps	164	0.05	-40.3	127	0.06	-35.9	107	0.06	-37.2	88	0.10	-52.1	83	0.11	-42.9
Cycle 3 + 200 steps	159	0.03	-42.1	119	0.06	-37.6	100	0.06	-36.2	82	0.09	-36.3	77	0.10	-38

**Table S.2.** Primary emulsions at 20 wt% of oil for the five different concentrations of lecithin (L1÷L5) at the different process conditions.

Emulsion Composition	Stability tests								
		0 time	1 day	3 days	1 week	2 weeks	1 month	2.5 months	2.5 months 4C
1,2 g lecithin / 20 ml soyoil	Size[nm]	158,3	162	169,5	162,6	160,7	153,5	1010	211
	PDI	0,042	0,028	0,153	0,066	0,052	0,057	0.330	0.259
2,4 g lecithin / 20 ml soyoil	Size[nm]	119	124,5	126,5	126,6	131,1	130,1	149.8	129
	PDI	0,073	0,051	0,05	0,055	0,078	0,055	0.063	0.05
3,6 g lecithin / 20 ml soyoil	Size[nm]	100,8	102,3	105,6	111,9	113,8	131,9	125.6	100.7
	PDI	0,073	0,085	0,097	0,128	0,122	0,137	0.2	0.085
4,8 g lecithin / 20 ml soyoil	Size[nm]	82,33	84	84,92	84,77	83,95	85,34	87.41	86.76
	PDI	0,103	0,107	0,093	0,104	0,089	0,102	0.1	0.106
6 g lecithin / 20 ml soyoil	Size[nm]	77,87	78,58	81,01	80,12	78,51	82,2	82.99	82.1
	PDI	0,1	0,113	0,124	0,116	0,126	0,097	0.099	0.1

**Table S.3.** Stability tests on primary emulsions over 2.5 months.

DLS results on secondary emulsions

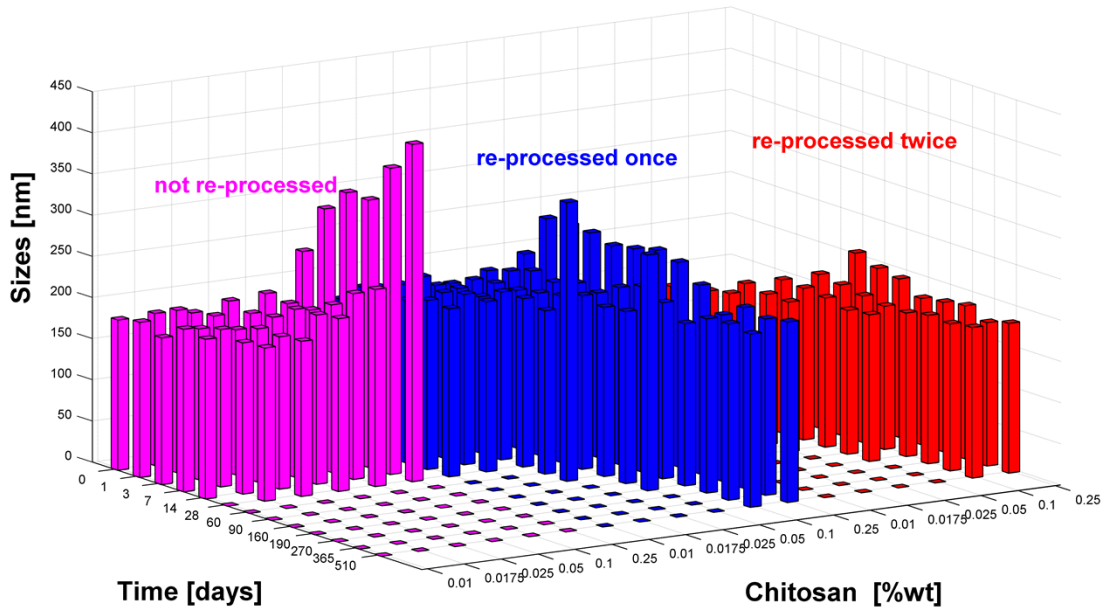


Figure S.2. L1 secondary emulsion size at different chitosan polymer concentrations over time, processed twice (red), once (blue) and not at all (pink).

L1 re-dispersed once through high-pressure homogenizer (A)																
Chitosan concentration [wt%]	0.005		0.01		0.0175		0.025		0.05		0.1		0.175		0.25	
TIME (days)	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI
0	188.4	0.07	172.4	0.06	180.7	0.05	174.2	0.04	173.2	0.05	182.9	0.10	199.3	0.11	197.6	0.11
1	215.9	0.12	195.6	0.08	192.7	0.08	185.8	0.09	186.7	0.10	191.2	0.13	220.4	0.12	249.1	0.16
3	199.2	0.09	193.1	0.1	210.8	0.20	190.8	0.08	191.1	0.09	200.0	0.10	237.2	0.13	250.0	0.14
7	208.6	0.08	197.1	0.09	200.2	0.08	189.4	0.07	191.3	0.09	195.2	0.12			249.2	0.17
14	209.5	0.11	205.6	0.08	207.1	0.10	203.8	0.09	196.7	0.10	202.5	0.15			242.2	0.11
28	215.1	0.10	204.1	0.06	206.8	0.07	204.6	0.07	202.1	0.09	199.5	0.10			247.2	0.12
75					<u>638.8</u>	<u>0.41</u>	198.6	0.08	211.0	0.08	214.8	0.08			254.2	0.08
90							<u>338.7</u>	<u>0.35</u>	205.5	0.04	224.8	0.06			248.3	0.07
160									208.9	0.12	214.0	0.09			229.0	0.12
190									<u>286.4</u>	<u>0.32</u>	197.1	0.10			201.7	0.10
270											211.6	0.04			219.3	0.06
360											214.3	0.1			214.2	0.07
510											210.7	0.09			219.4	0.10
L1 re-dispersed twice through high-pressure homogenizer after 1 month (B)																
28	214.4	0.10	195.6	0.08	183.9	0.06	187.0	0.06	185.7	0.08	186.5	0.07	179.7	0.09	206.4	0.07
31	<u>247.4</u>	<u>0.11</u>	189.4	0.09	194.1	0.08	190.1	0.08	181.9	0.07	184.4	0.07	182.6	0.08	200.7	0.06
42			193.7	0.09	191.2	0.07	190.8	0.07	193.0	0.15	178.8	0.08	181.4	0.08	206.0	0.12
60					189.7	0.06	183.0	0.10	184.5	0.07	182.3	0.08	181.9	0.07	196.7	0.08
95							182.2	0.08	181.2	0.09	177.7	0.08	170.5	0.08	192.5	0.12
160									175.0	0.11	174.0	0.09	167.8	0.09	177.5	0.09
190									178.2	0.07	174.4	0.12	168.0	0.07	181.5	0.12
270											180.2	0.08	170.8	0.08	185.9	0.09
360											178.7	0.12	165.0	0.06	174.2	0.07
510											182.8	0.10	169.0	0.05	182.0	0.06

L2 re-dispersed once through high-pressure homogenizer (C)																
Chitosan concentration [wt%]	0.005		0.01		0.0175		0.025		0.05		0.1		0.175		0.25	
TIME (days)	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI
0	134.8	0.11	128.4	0.08	128.7	0.06	127.0	0.06	125.1	0.10	139.5	0.12	162.0	0.17	196.8	0.17
1	134.4	0.10	130.3	0.09	131.3	0.06	132.2	0.07	128.7	0.09	138.3	0.11	167.6	0.16	189.1	0.17
3	143.3	0.07	138.1	0.06	139.1	0.09	139.1	0.06	136.8	0.09	148.8	0.12	179.7	0.16	206.6	0.16
7	148.2	0.11	135.7	0.09	131.9	0.07	141.7	0.08	143.0	0.09	155.1	0.11	185.4	0.14	203.8	0.15
14	<u>165.6</u>	<u>0.07</u>	139.3	0.08	149.8	0.12	142.7	0.07	142.8	0.08	148.7	0.13	172.9	0.14	198.0	0.16
28			134.9	0.05	137.4	0.07	145.5	0.07	150.1	0.09	154.7	0.08	171.9	0.12	193.6	0.15
75							<u>190.1</u>	<u>0.10</u>	148.8	0.08	165.8	0.07	179.5	0.07	194.3	0.13
90									144.7	0.06	166.8	0.06	177.0	0.09	187.7	0.13
160									<u>262.3</u>	<u>0.36</u>	163.3	0.12	160.5	0.07	197.9	0.22
190											142.4	0.13	146.0	0.09	151.6	0.11
270											159.5	0.08	163.7	0.07	165.2	0.09
360											160.4	0.10	152.9	0.07	162.8	0.17
510													158.6	0.08	159.3	0.09
L2 re-dispersed twice through high-pressure homogenizer after 1 month (D)																
28			134.1	0.06	134.4	0.08	132.3	0.03	127.0	0.07	128.3	0.08	145.3	0.09	156.7	0.09
31			134.1	0.07	135.0	0.06	136.1	0.04	135.7	0.07	134.6	0.08	147.6	0.09	158.2	0.09
42					132.0	0.06	130.6	0.05	131.2	0.08	135.5	0.11	141.5	0.09	153.4	0.09
60							128.9	0.06	127.9	0.04	131.0	0.06	137.6	0.09	145.8	0.09
95									129.9	0.11	131.3	0.12	136.5	0.14	142.9	0.14
160									121.6	0.07	122.5	0.04	124.8	0.09	133.3	0.15
190									128.7	0.06	125.0	0.09	125.1	0.06	136.4	0.16
270											127.8	0.06	117.8	0.17	137.7	0.14
360											129.7	0.09	126.3	0.12	131.2	0.14
510											131.1	0.08	129.5	0.09	131.1	0.10

L3 re-dispersed once through high-pressure homogenizer (E)																
Chitosan concentration [wt%]	0.005		0.01		0.0175		0.025		0.05		0.1		0.175		0.25	
TIME (days)	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI
0	102.5	0.08	101.9	0.08	103.4	0.10	102.6	0.10	108.7	0.12	115.4	0.13	126.4	0.18	142.0	0.20
1	103.2	0.10	105.4	0.09	106.1	0.09	101.6	0.08	111.4	0.12	122	0.15	134.1	0.18	156.0	0.19
3	108.6	0.09	107.0	0.09	110.2	0.08	111.2	0.13	110.2	0.11	125.4	0.14	144.0	0.17	167.3	0.12
7	117.4	0.06	107.9	0.08	114.0	0.09	111.7	0.01	111.7	0.11	123.9	0.15	147.1	0.17	170.2	0.17
14	<u>226.3</u>	<u>0.32</u>	111.2	0.07	111.5	0.06	109.5	0.07	106.4	0.08	123.2	0.13	128.4	0.15	127.9	0.14
28			126.3	0.07	115.8	0.08	120.6	0.06	125.0	0.08	132	0.12	149.4	0.14	169.5	0.15
L3 re-dispersed twice through high pressure homogenizer after 1 month (F)																
28			139.0	0.10	111.0	0.07	109.9	0.07	111.3	0.08	113.6	0.10	121.0	0.11	131.7	0.12
31			218.9	0.12	108.5	0.10	106.5	0.07	108.2	0.07	110.8	0.09	121.0	0.13	131.5	0.10
42					280.7	0.17	104.2	0.07	109.2	0.07	109.3	0.09	114.1	0.11	124.2	0.10
60							155.3	0.05	107.8	0.08	107.4	0.10	111.7	0.11	115.2	0.10
95									106.0	0.10	105.9	0.09	110.3	0.11	123.3	0.18
160									101.7	0.07	99.1	0.06	103.9	0.01	104.6	0.08
190									113.9	0.07	100.2	0.074	102.1	0.07	106.3	0.10
270											105.3	0.09	102.0	0.07	101.8	0.06
360											115.7	0.14	106.2	0.09	100.7	0.07
510											<u>120.2</u>	<u>0.23</u>	105.3	0.08	104.9	0.07

L4 re-dispersed once through high-pressure homogenizer (G)																
Chitosan concentration [wt%]	0.005		0.01		0.0175		0.025		0.05		0.1		0.175		0.25	
TIME (days)	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI
0	87.8	0.01	88.3	0.12	89.8	0.13	89.3	0.11	91.6	0.15	95.8	0.15	104.8	0.19	110.3	0.20
1	89.8	0.09	91.1	0.11	92.0	0.11	91.8	0.12	94.7	0.14	107.4	0.20	111.9	0.19	131.2	0.22
3	106.3	0.09	94.0	0.10	95.7	0.11	96.2	0.11	103.0	0.17	110.0	0.17	131.9	0.1	149.6	0.19
7	172.8	0.13	104.5	0.09	101.4	0.10	93.6	0.11	104.7	0.17	119.1	0.21	146.3	0.23	193.8	0.35
14			113.1	0.08	100.1	0.11	101.9	0.10	108.9	0.15	111.4	0.16	132.3	0.17	151.9	0.18
28					102.3	0.08	103.2	0.09	110.9	0.11	117.3	0.13	131.5	0.16	142.9	0.16
75									110.8	0.15	115.0	0.09	135.6	0.14	144.9	0.11
90									167.4	0.13	117.2	0.11	126.1	0.1	138.5	0.10
160											119.3	0.07	118.7	0.08	122.0	0.08
190											137.6	0.09	123.5	0.08	127.7	0.06
270											219.6	0.26	128.6	0.06	127.4	0.06
360													134.5	0.10	122.4	0.09
510													<u>161.5</u>	<u>0.13</u>	131.3	0.07
L4 re-dispersed twice through high-pressure homogenizer after 1 month (H)																
28			252.9	0.05	97.5	0.10	95.6	0.08	96.8	0.10	98.1	0.11	105.3	0.13	113.9	0.13
31					95.5	0.07	94.4	0.08	96.5	0.08	96.8	0.12	104.0	0.14	114.0	0.14
42							91.6	0.07	90.5	0.09	92.9	0.10	97.7	0.11	106.8	0.12
60									91.2	0.11	91.3	0.11	99.1	0.13	104.6	0.14
95									90.3	0.14	91.3	0.12	85.7	0.12	105.4	0.19
160									101.4	0.06	84.9	0.08	87.6	0.01	91.2	0.01
190									175.5	0.10	89.9	0.15	88.3	0.11	92.6	0.13
270											91.7	0.12	89.0	0.10	89.5	0.10
360											98.6	0.15	92.5	0.10	91.1	0.10
510											<u>120.7</u>	<u>0.30</u>	98.1	0.13	96.0	0.10

L5 re-dispersed once through high-pressure homogenizer (I)																
Chitosan concentration [wt%]	0.005		0.01		0.0175		0.025		0.05		0.1		0.175		0.25	
TIME	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI
TIME (days)	84.6	0.10	83.2	0.12	83.6	0.13	82.3	0.12	81.9	0.12	88.4	0.17	95.4	0.18	108.2	0.21
0	85.4	0.12	84.8	0.11	83.2	0.11	85.0	0.11	86.3	0.13	97.0	0.19	107.4	0.22	122.8	0.21
1	103.4	0.18	91.3	0.14	90.2	0.14	84.8	0.11	93.4	0.15	101.0	0.19	117.8	0.21	147.0	0.22
3	160.5	0.14	98.6	0.10	89.2	0.10	92.5	0.10	92.9	0.13	92.0	0.15	112.5	0.2	127.9	0.20
7			107.7	0.07	96.1	0.11	97.7	0.10	99.6	0.13	106.6	0.15	137.1	0.23	133.9	0.17
14					101.3	0.07	97.5	0.10	97.2	0.09	100.9	0.13	104.2	0.14	131.6	0.16
28									142.4	0.09	116.0	0.13	125.8	0.12	132.2	0.12
75											114.2	0.14	126.1	0.15	129.0	0.11
90											138.9	0.14	115.3	0.09	117.5	0.10
160											219.7	0.25	124.7	0.10	124.4	0.15
190													133.3	0.08	123.5	0.06
270													<u>155.4</u>	<u>0.10</u>		
360													<u>240.1</u>	<u>0.34</u>		
L5 re-dispersed twice through high-pressure homogenizer after 1 month (L)																
28					97.0	0.10	90.4	0.09	88.3	0.11	92.1	0.13	98.2	0.13	107.6	0.15
31					98.5	0.12	92.1	0.13	91.3	0.09	93.0	0.13	98.5	0.13	112.7	0.16
42							125.0	0.06	87.1	0.08	85.5	0.10	93.9	0.11	101.1	0.14
60									86.5	0.08	88.1	0.09	90.9	0.11	101.8	0.13
95									82.7	0.11	83.5	0.12	89.1	0.13	94.0	0.11
160											79.1	0.09	81.1	0.07	84.6	0.09
190											82.1	0.11	89.0	0.19	84.9	0.09
270											92.6	0.15	88.1	0.12	85.9	0.09
360											<u>104.7</u>	<u>0.19</u>	<u>96.6</u>	<u>0.18</u>	91.6	0.12
510											<u>166.6</u>	<u>0.50</u>	<u>108.6</u>	<u>0.28</u>	<u>101.8</u>	<u>0.19</u>

L1 no re-dispersion process (M)																
Chitosan concentration [wt%]	0.005		0.01		0.0175		0.025		0.05		0.1		0.175		0.25	
TIME (days)	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI	Size [nm]	PDI
0	<i>192.0</i>	<i>0.22</i>	182.7	0.08	184.5	0.11	179.3	0.08	187.7	0.12	190.9	0.13	203.0	0.15	236.1	0.22
1	<i>210.9</i>	<i>0.26</i>	188.6	0.12	197.2	0.11	184.4	0.1	181.3	0.11	187.3	0.12	253.9	0.2	<i>296.5</i>	<i>0.4</i>
3			178.4	0.05	183.2	0.09	176.2	0.07	185.5	0.1	188.2	0.1	232.6	0.19	<i>324.9</i>	<i>0.29</i>
7			197.5	0.13	191.1	0.15	186.2	0.11	203.8	0.11	203.3	0.11	254.2	0.23	<i>324.8</i>	<i>0.4</i>
14			194.0	0.15	183.8	0.1	185.2	0.06	205.6	0.12	225.6	0.14	<i>275.5</i>	<i>0.17</i>	<i>372.1</i>	<i>0.3</i>
28					186.2	0.14	188.4	0.07	210.0	0.13	<i>239.4</i>	<i>0.14</i>	<i>291.0</i>	<i>0.19</i>	<i>409.8</i>	<i>0.4</i>

**Table S.4.** Stability tests on secondary emulsions reprocessed once (A, C, E, G, I), reprocessed twice (B, D, F, H, L) and not re-processed (M). Some Notes: The data missing in A for the 0.175 wt% of CT are due to a mistake for which the sample was completely re-processed for the second time after three days; The sample in B corresponding to 0.175 wt% of chitosan was re-processed after three days instead of 1 month, as said. As it is possible to see in this case the size is slightly smaller than the two samples just above and below; maybe the faster processing avoided some aggregation ending up with a smaller average size; all the samples L3 in E were reprocessed after 1 month, as planned, but they were completely processed by mistake without keeping the sample for further stability tests; The times for measurements are rounded of a few days. The italic underlined values are the ones corresponding to samples not demixed, but with evident destabilization (PDI around/above 0.2 and/or size increased more than 20-30%).



### Emulsion coated with polylysine and further bilayer with heparin

For the first layer, we started from an oil concentration of 1 wt% and a polylysine concentration of 0.02 wt% in one case and 0.0125 wt% in a second case with no adjustment of the pH. L4 based secondary emulsion was reported as an example for this test. After one day from preparation, the secondary emulsion was processed through the high-pressure homogenizer for 100 continuous steps at a pressure of 700 bars and again under the same conditions after few days. A second layer was deposited after one day by preparing a solution of heparin with the concentration of 0.03 wt% and using this solution to dilute the dispersion 1:2.

	<b>No re-dispersion</b>	<b>One re-dispersion</b>	<b>Double re-dispersion</b>	<b>Bilayer</b>
Size (nm)	128.2 ± 0.6	118.7 ± 1.5	103.3 ± 0.3	
PDI	0.191 ± 0.003	0.124 ± 0.014	0.067 ± 0.003	
Z-Pot (mV)	30.8 ± 1.0	33.5 ± 0.6	36.8 ± 1.1	
Size (nm)	162.9 ± 1.206	96.11 ± 1.553	95.63 ± 0.743	121.4 ± 2.113
PDI	0.364 ± 0.011	0.089 ± 0.016	0.088 ± 0.014	0.11 ± 0.003
Z-Pot (mV)	23.8 ± 0.231	31.2 ± 0.493	36.5 ± 1.59	-35 ± 0.361

**Table S.5** Summary of size, PDI and z-potential of secondary emulsions (ratio polylysine/oil = 0.02 wt/wt) with no-re-dispersion, one re-dispersion and double re-dispersion and a bilayer with heparin (ratio heparin/polylysine=1.5 wt/wt) on L4.

### Emulsion coated with chitosan and further bilayer with heparin

For the first layer, we started from an oil concentration of 1 wt% and a chitosan concentration of 0.01 wt% in a solution at pH 4 as described in the experimental section. L4 based secondary emulsion was reported as an example for this test. After one day from preparation, the secondary emulsion was processed through the high-pressure homogenizer for 100 continuous steps at a pressure of 700 bars and again under the same conditions after 3 days. A second layer was deposited after one day by preparing a solution of heparin with the concentration of 0.0276 wt% and using this solution to dilute the dispersion 1:2.

	<b>No re-dispersion</b>	<b>One re-dispersion</b>	<b>Double re-dispersion</b>	<b>Bilayer</b>
Size (nm)	128.2 ± 0.6	118.7 ± 1.5	103.3 ± 0.3	130.4 ± 0.6
PDI	0.191 ± 0.003	0.124 ± 0.014	0.067 ± 0.003	0.109 ± 0.007
Z-Pot (mV)	30.8 ± 1.0	33.5 ± 0.6	36.8 ± 1.1	-38.2 ± 2.7

**Table S.6.** Summary of size, PDI and z-potential of secondary emulsions (ratio chitosan/oil = 0.01 wt/wt) with no-re-dispersion, one re-dispersion and double re-dispersion and a bilayer with heparin (ratio heparin/chitosan=2.76 wt/wt) on L4.

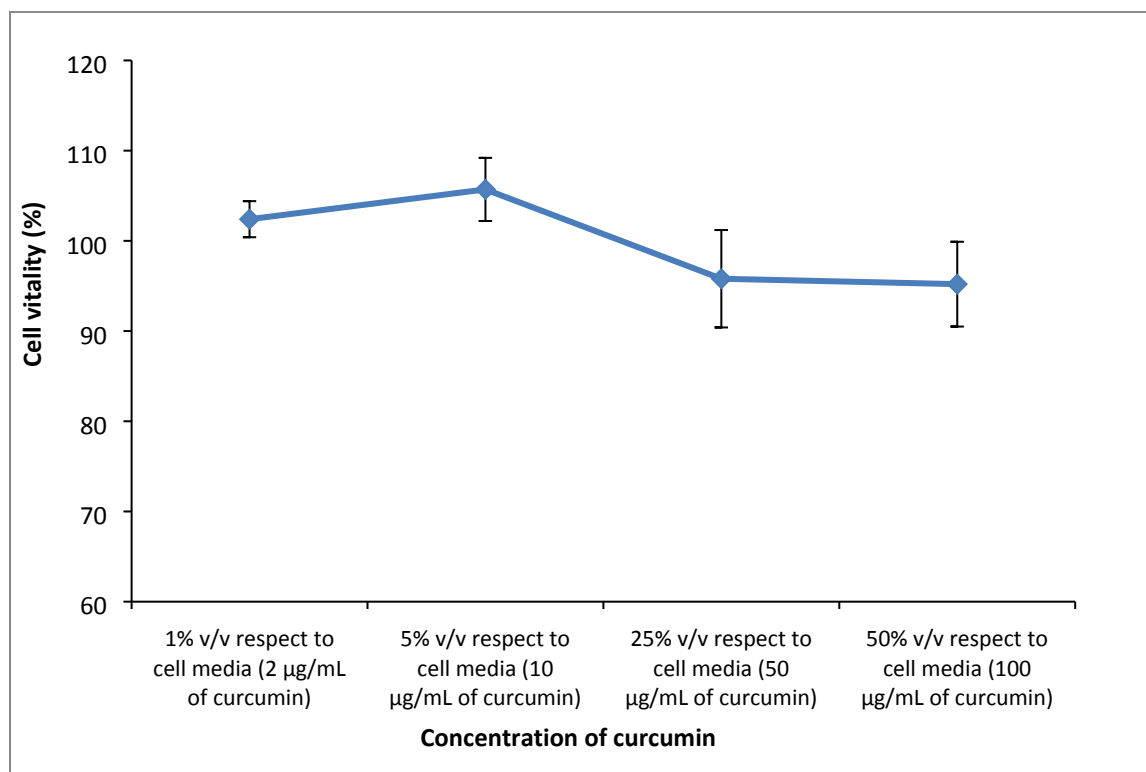
## Cytotoxicity analysis

### *Cell culture*

Mouse fibroblast (NIH/3T3, ATCC® CRL-1658™ ) cells were seeded (density 3000 cells/well) in a 96-well plate, cultured in Dulbecco's Modified Eagle's Medium containing 10% FBS, 1% Pen-strep solution (full medium) and incubated for 24h under standard sterile conditions for cell culture (5% CO<sub>2</sub>, 37 °C).

### *Cell cytotoxicity analysis*

The cytotoxicity of an emulsion stabilized with chitosan (chitosan/oil ratio = 0.1 wt/wt) and containing curcumin (20 wt% in oil) was assessed on fibroblast cell line (NIH/3T3). In particular, the secondary emulsion was diluted at different percentage (ranging from 1% to 50%) with cell media and added to the cells after washing them with PBS. Fibroblasts were then incubated for 24h under standard sterile conditions (5% CO<sub>2</sub>, 37 °C). At the completion of the incubation, cells were washed and incubated further for 2 hours in plain medium containing WST-8. The cytotoxicity was measured using a colorimetric method based on the bio-reduction of tetrazolium salt (water soluble) WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono sodium salt], into a colored formazan, which is soluble in full medium. The amount of formazan produced as measured by the absorbance at 450 nm is proportional to both the number and the metabolic activity of living cells in culture. 10 µL of WST-8 mixture were added to each well and mixed gently for 1 min on an orbital shaker. The cells were incubated for another 2 hours at 37 °C in a CO<sub>2</sub> incubator. Before reading, the plate was shaken for 1 min to ensure homogeneous distribution of the color. The absorbance readings were acquired at a wavelength of 450nm with the Tecan infinite M200 plate-reader using i-control software. The relative cell viability (%) was calculated by the formula  $[A]_{\text{test}}/[A]_{\text{control}} \times 100$ , where  $[A]_{\text{test}}$  is the absorbance of the test sample, and  $[A]_{\text{control}}$  is the absorbance of the control cells incubated solely with culture medium. Obtained values were, then, normalized by the total protein content. Quantification of total protein content was measured by using the Micro BCA protein assay kit (Pierce) in cell lysates.



**Figure S.3.** Cell viability after 24h of contact of emulsion with fibroblast cell line

This analysis shows no significant cytotoxicity of the secondary emulsion based on a chitosan layer surrounding the soybean oil emulsion tested on healthy cells. In the meantime, other in vitro tests on cancer cells show instead an evident cytotoxic effect on the cells due to the selective action of the curcumin.