Microfluidic-enabled magnetic labelling of nanovesicles for bioanalytical applications

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Electronic Supplementary Information

1. Estimation of diffusion coefficients for liposomes versus dye molecules

For the estimation of the diffusion coefficient of liposomes, the Stokes-Einstein equation (1) was used and the following approximations were made: liposomes were treated as rigid spheres with a diameter d_L of 200 nm and the viscosity η of pure water ($8.94 \cdot 10^{-4}$ Pa·s) was used.[1]

$$D_L = \frac{k_B \cdot T}{3 \cdot \pi \cdot \eta \cdot d_L} \quad (1)$$

where k_B is the Boltzmann constant and T is the temperature (25 °C). A diffusion constant D_L of $2.8 \cdot 10^{-8}$ $cm^2 \cdot s^{-1}$ was calculated. Compared to a mean diffusion constant for several Rhodamine dyes of around $4.2 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$,[2] liposomes have an around 150 times smaller diffusion constant.

2. Experimental

2.1. Chemicals and Instruments

Phospholipids, including 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (sodium salt) (N-glutaryl-DPPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (sodium salt) (biotin-DPPE), were obtained from Avanti Polar Lipids, Inc. (www.avantilipids.com), n-octyl-β-D-glucopyranoside (OG) was bought from Roth (www.carlroth.com) and cholesterol and sulforhodamine B were purchased from Sigma Aldrich (www.sigmaaldrich.com).

Iron oxide (II,III) magnetic nanoparticle solution (30 nm diameter, amine functionalized, 1 mg·mL-1 in H2O) and magnetic bead solution (approx. 1 µm in diameter, amine functionalized, 50 mg·mL-1 in H2O) were both obtained from Sigma Aldrich (www.sigmaaldrich.com).

N-Hydroxysulfosuccinimide sodium salt (sNHS) was purchased from Sigma Aldrich (www.sigmaaldrich.com). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Thermo Fisher Scientific (www.thermofisher.com).

Chloroform, cyclohexane and methanol were purchased from Fisher Scientific (www.fishersci.com).

BSA (albumin fraction V from bovine serum), di-potassium hydrogen phosphate trihydrate, di-sodium hydrogen phosphate dihydrate, formamide, potassium chloride, potassium dihydrogen phosphate and tri-sodium citrate dihydrate were bought from Merck (www.merckmillipore.com). Sodium azide and hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were obtained from Sigma Aldrich (www.sigmaaldrich.com). Ficoll 400 and sodium chloride were purchased from Roth (www.carlroth.com) and sucrose was purchased from VWR (de.vwr.com).

All other chemicals were of analytical grade and purchased from either Roth (www.carlroth.com), Merck (www.merckmillipore.com), VWR (de.vwr.com) or Sigma Aldrich (www.sigmaaldrich.com).

HEPES buffer was prepared from 10 mM HEPES, 200 mM NaCl and 0.01% (w/v) NaN3 with a pH of 7.5. For production of washing buffer, 0.05% (v/v) Tween 20 and 0.01% (w/w) bovine serum albumin were added to Phosphate buffered saline (PBS), which consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 and was adjusted to pH 7.4.

An extruder equipped with syringes, filter supports and membranes for extrusion of liposomes was obtained from Avanti Polar Lipids, Inc. (www.avantilipids.com). For column chromatography, sephadex G50 was obtained from Sigma Aldrich (www.sigmaaldrich.com).

For fluorescence measurements and determination of coupling efficiency, black or transparent MaxiSorp 96 well microtiterplates (MTPs) from Nunc purchased from Sigma Aldrich (ww.sigmaaldrich.com) or black 96 well half-area MTPs from VWR (de.vwr.com) were used. White streptavidin coated MTPs (KaiSA 96) with a biotin binding capacity of >14 pmol/well were obtained from Kaivogen Oy (kaivogen.com) for biotin-streptavidin assay. Fluorescence and absorbance measurements were performed with a BioTek SYNERGY neo2 (www.biotek.com).

2.2. Liposome Synthesis

For liposome synthesis, reverse phase evaporation according to an established procedure from Edwards et al.[3] was employed.

Therefore, 15 mg DPPC (20 µmol, 36%), 10 mg cholesterol (26 µmol, 46% of lipid composition) and 7.5 mg DPPG (10 µmol, 18%) are dissolved in 3 mL chloroform and 0.5 mL methanol. After sonication for 1 min, 2 mL of 10 mM SRB in 2 mM HEPES solution (pH 7.5) are added and the mixture is sonicated for another 4 min to form a microemulsion. Then, organic solvents are vaporized at a rotary evaporator at 60 °C under reduced pressure. After vortexing the mixture thoroughly, 2 mL SRB solution are added. The flask is positioned back at the rotary evaporator to eliminate any remaining organic solvent. The liposomes are extruded each 21 times at 60 °C through two polycarbonate membranes with 1.0 and 0.4 µm pores. Purification by size exclusion column chromatography (1.5 x 20 cm, Sephadex G50) is performed. Medium and high concentrated liposome containing fractions are collected and transferred to dialysis (MWCO 12 – 14 kDa, spectrumlab.com) against HEPES buffer.

To achieve DNA reporter probe modified liposomes, 25 µL of a 300 µM solution of DNA tagged with cholesterol (15 nmol) are added with the lipid ingredients. For carboxy or biotin modification, N-glutaryl-DPPE and biotin-DPPE are added to the lipid mixture, respectively. Biotin-DPPE is always added as 2% of total lipids (1.5 µmol), but liposomes with DPPE contents of more than 8% tend to be not formed, N-glutaryl-DPPE is added as 6 (3.5 µmol) or 8% (5 µmol), depending on the presence or absence of biotin-DPPE.

The concentration of liposome solutions is determined with a Spectroflame-EOP inductively coupled plasma optical emission spectrometer (ICP-OES) from Spectro (www.spectro.com) or an ELAN 9000 (ICP-MS) from Perkin Elmer (www.perkinelmer.com), whereas hydrodynamic diameters and ζ-potentials were measured by dynamic light scattering (DLS) at 20 °C with a Malvern Zetasizer Nano-ZS (www.malvern.com) in disposable PMMA cuvettes (semi-micro) and disposable PMMA capillary cells, respectively. Hydrodynamic diameters were determined 12 to 15 times for each sample and averaged, zeta potential 25 to 37 times.

2.3. Microfluidic setup and coupling procedure

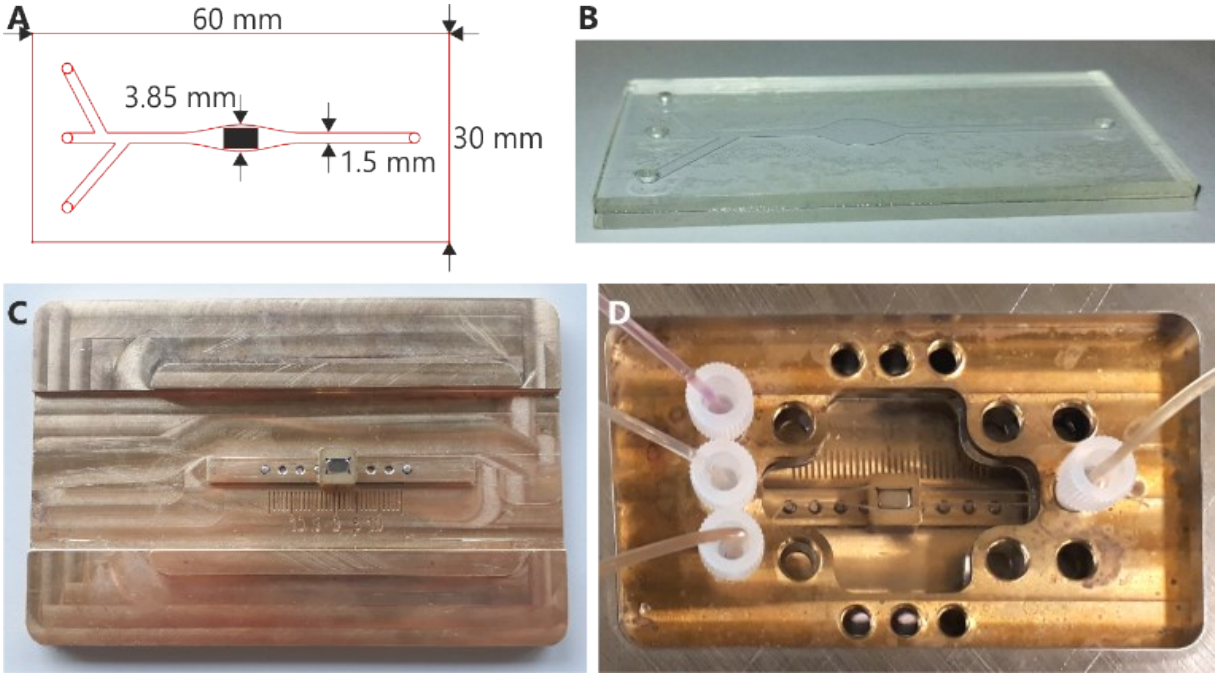

Figure S1: A: Shape and dimensions of the preliminary microfluidic chip with simple channel design. The black box represents the underlying magnet with a size of 5 x 3 mm. The final design is shown in Figure 2B in the main manuscript.. B: Image of the microfluidic chip made of two PMMA slides glued together by a double-sided adhesive tape in which the channel is laser cut. Inlet and outlet holes are also cut by a laser scriber into the top PMMA slide. C: Magnet holder plate. D: Microfluidic chip holder with magnet holder plate underneath and attached tubing.

2.4. Concentration determination

Concentration determination was performed with three different methods:

For florescence measurements, black 96 well half-area MTPs were used. For each calibration data point (2.5 to 50 µM total lipid) three wells are measured and for the sample 2 wells due to high material consumption. The solutions are first measured in 25 µL solution in liposome outer buffer for background signal, than 2.7 µL 300 mM OG solution are added for liposome lysis and the fluorescence intensity is measured again.

For ICP-MS and ICP-OES measurements, a Spectroflame-EOP inductively coupled plasma optical emission spectrometer (ICP OES) from Spectro (www.spectro.com) or an ELAN 9000 (ICP-MS) from Perkin Elmer (www.perkinelmer.com) were employed. Samples are diluted in 0.5 M HNO_3 to an end volume of 3 ML and the average of three measurements is used. A calibration curve from 1 µM to 50 µM phosphor was used.

Bartlett Assay was performed according to a protocol by Edwards et al.[3] Briefly, 20 µL liposome and calibrations samples (prepared from K_2HPO_4 from 1.25 to 20 mM), respectively, or 400 µL microfluidic product are heated to 180 °C to complete dryness. Then, 15 mL 1.67 M H_2SO_4 are added and heated to 180 °C for 2 h. 100 µL 30% H_2O_2 is added and heated to 180 °C for 1.5 h. After each step, the solutions are cooled to room temperature and mixed thoroughly. 4.6 mL 0.22% ammonium molybdate and 0.2 mL Fiske-Subbarow reagent are added, heated in boiling water for 7 min and then quickly cooled in an ice bath. The absorbance of the solutions is measured in transparent MaxiSorp 96 well MTPs at 830 nm.

3. Liposome characterization

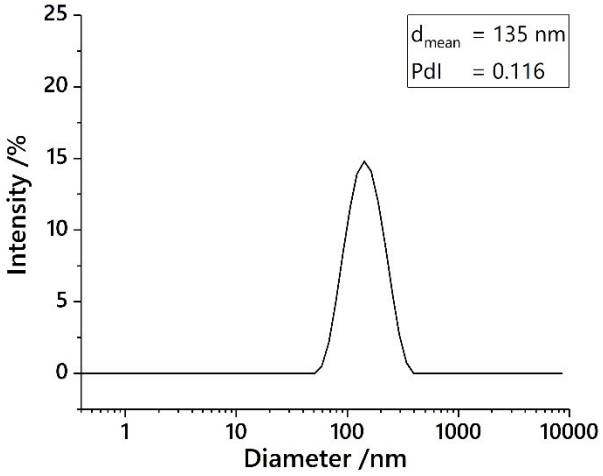

Figure S2: Hydrodynamic diameter of liposomes with 2% biotin-DPPE and 6% N-glutaryl-DPPE as determined by DLS.

4. Bulk phase coupling

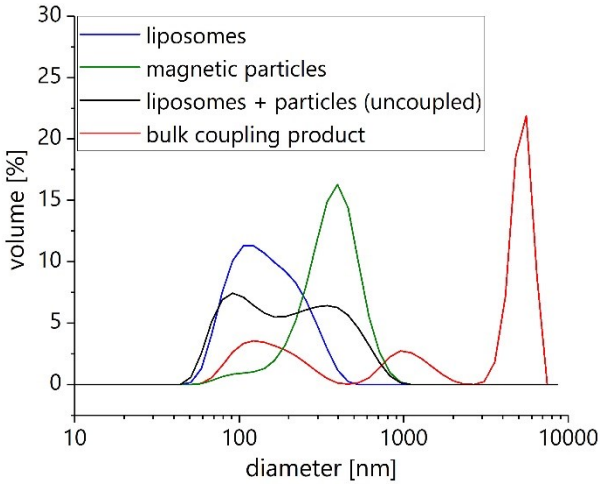

Figure S3: DLS measurement of pure liposomes, pure magnetic particles, a mixture of these two, and the product of a bulk coupling experiment.

5. Observation of hydrodynamic diameter evolution during microfluidics

To observe possible aggregation of magnetic particles due to magnetic capture inside the microfluidic channel, particles were captured and washed with buffer. The hydrodynamic diameter of these particles was compared to the diameter of pure particles from the stock solution, as well as with the microfluidic product after coupling with activated liposomes inside the microfluidic channel. MBs (~1 µm in diameter according to producer) show a (1.51±0.26)-fold increase in diameter after microfluidic treatment, which is too low for possible aggregation of particles, indicating that the particles are stable during magnetic capture and microfluidic treatment. After microfluidic coupling with liposomes, the diameter increases by (1.54±0.24) times and the distribution is much broader than for the other measurements.

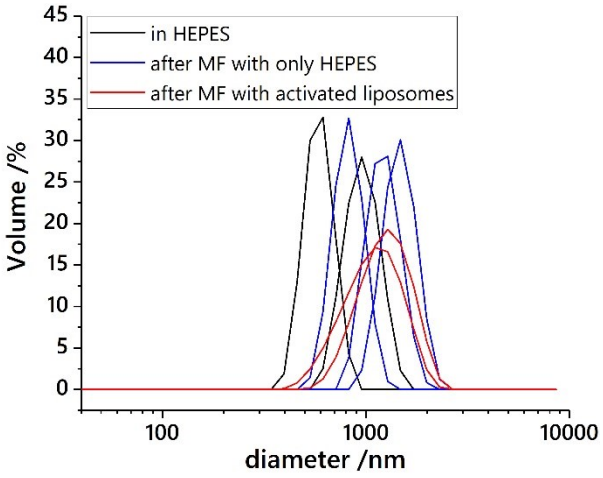

Figure S4: Hydrodynamic diameter of MBs (~1 µm according to producer) dispersed in HEPES buffer, pure (black), after microfluidic treatment with HEPES buffer (no coupling reaction with liposomes) (blue) and after coupling reaction with activated liposomes inside a microfluidic channel (red). (MF = microfluidics)

Table S1: Hydrodynamic diameter of MBs (~1 µm diameter according to producer) dispersed in HEPES buffer, pure, after microfluidic treatment with HEPES buffer (no coupling reaction with liposomes) and after microfluidic coupling with activated liposomes. The diameter ranges between ~950 and ~1100 nm with no clear trend visible.

	MBs in HEPES	MBs after MF with only HEPES	MBs after MF with activated liposomes
diameter (measurement 1)	(592±96) nm	(830±144) nm	(1121±351) nm
diameter (measurement 2)	(972±190) nm	(1236±233) nm	(1285±359) nm
diameter (measurement 3)		(1485±274) nm	
Z-Average (Average)	(782±106) nm	(1184±129) nm	(1203±251) nm

MF = microfluidics;

MNPs (30 nm in diameter according to producer) in pure stock solution show three different peaks. The peak at ~200 nm is most likely generated either by single particles with a thick water shell due to surface coating and measurement in buffer with high ionic strength, or by small aggregates like dimers or trimers. The other two peaks at ~1000 and ~5000 nm can be assigned to bigger aggregates. This assumption is fortified by the fact that sonication reduces the diameter of particles to a broad peak at ~350 nm, most likely produced by single particles as well as small aggregates, while the peak at ~5000 nm decreases, which indicates that still some huge aggregates are left, but less than in not sonicated solution. After microfluidic treatment with only HEPES buffer, only a peak at ~1000 nm remains. An explanation could be that the particles aggregate under the influence of the magnetic field, leaving no smaller aggregates or single particles in solution, while huge aggregates are caught in the channel or tubing, not finding their way into the measuring cuvette in the end. After microfluidic coupling with activated liposomes, also only one peak is present, but at a slightly elevated diameter of ~1700 nm, which might contain nanoparticle aggregates in the same size as after microfluidics with only buffer but now coupled to liposomes, as well

as larger nanoparticle aggregates. However, it is assumed that the presence of these aggregates would not negatively influence their coupling to liposomes.

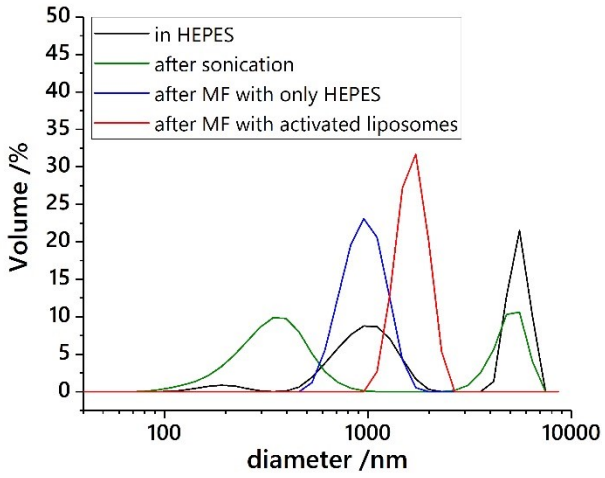

Figure S5: Hydrodynamic diameter of MNPs (30 nm according to producer) dispersed in HEPES buffer, pure (black), after sonication (green), after microfluidic treatment with HEPES buffer (no coupling reaction with liposomes) (blue) and after coupling reaction with activated liposomes inside a microfluidic channel (red). (MF = microfluidics)

Table S2: Hydrodynamic diameter of MNPs (30 nm diameter according to producer) dispersed in HEPES buffer; pure, after microfluidic treatment with HEPES buffer (no coupling reaction with liposomes) and after microfluidic coupling with activated liposomes;

	MNPs in HEPES	MNPs after sonication	MNPs after MF with only HEPES	MNPs after MF with liposomes
diameter (Peak1)	(192±42) nm	(354±139) nm	(974±228) nm	(1668±284) nm
diameter (Peak2)	(1008±307) nm	(4977±862) nm		
diameter (Peak3)	(5498±622) nm			

MF = microfluidics;

6. Development of magnetic setup

Different flow speeds were tested in a straight channel. It was observed that the optimum flow velocity for capturing magnetic beads is 10 µL·min^{-1} (Figure S6), which translates into 3.3 mm·s^{-1} linear velocity. When using a slower flow rate of 5 µL·min^{-1}, less beads were trapped on the magnet, because the particles already settled in the channel before reaching the magnet. With higher velocity, the beads flushed over the magnet, which is not strong enough anymore to accumulate them. These conditions obviously depend on the magnet used (here Ø2 x 1 mm, 130 g adhesive force, obtained from www.supermagnete.de) and the distance between magnet and bottom of the microfluidic channel, which is 100 µm here.

Figure S6: Pictures of three microfluidic channels loaded with the same amount of magnetic beads, but with different flow velocities: 5 µL·min^{-1} (left), 10 µL·min^{-1} (middle) and 15 µL·min^{-1} (right). (Top view, flow from left to right)

A small neodymium magnet (NdFeB, 5 x 5 x 3 mm, ~1 kg adhesive force, obtained from www.supermagnete.de) was employed for further experiments and the optimum orientation regarding the channel was investigated. As can be observed in Figure S7, the magnet captured the highest amount of magnetic beads in vertical arrangement (right image). Therefore, this orientation was used to create a magnet holder, which enables a simple and reproducible arrangement of the experimental setup for further experiments.

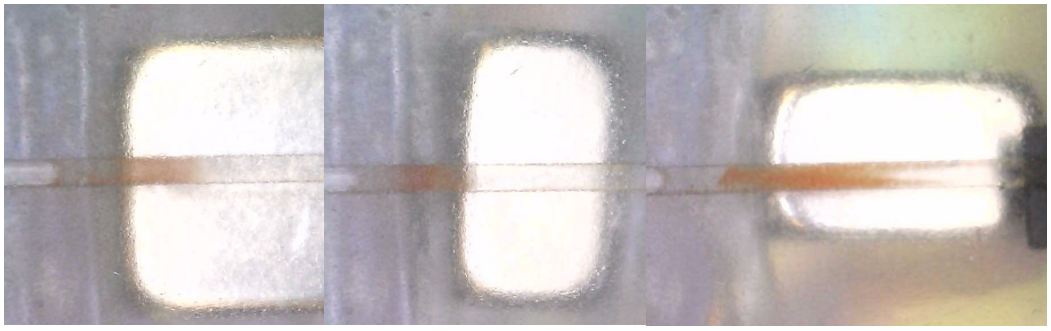

Figure S7: Pictures of three microfluidic channels loaded with the same amount of magnetic beads with same flow velocity, captured on a 5 x 5 x 3 mm magnet in three different orientations. (Top view, flow from left to right)

The magnetic field lines of the magnet were investigated with ferrum powder and are depicted in Figure S8. Their arrangement explains the broad band of particles captured at the front edge of the magnet, as there the particles enter the magnetic field and are stopped.

Figure S8: Images of ferrum powder with underlying magnet, top view as presented in the channel (**left**) and side view perpendicular to the channel (**middle)**. **right**: Scheme of the magnet (grey, top view) with channel (red) and magnetic field lines (black).

7. Concentration determination

For concentration determination of the microfluidic product, first the routinely used ICP-MS and –OES detection was employed, but as the microfluidic product is very low concentrated, a huge amount of material (~200 µL for MS, ~500 µL for OES) is necessary to end up in the measureable range. In addition, the measured concentrations (Table S3) are quite inconsistent, especially for magnetically separated samples. Therefore, other methods for determining the vesicle concentration in the microfluidic product were investigated.

Table S3: Measured total lipid concentrations from ICP. Mean concentration without magnetic separation is (41±9) µM, with magnetic separation (23±3) µM.

ICP-MS pure	ICP-OES pure	ICP-MS magnetic separation	ICP-OES magnetic separation
48 µM	40 µM	35 µM	6 µM
71 µM	35 µM	45 µM	
52 µM	28 µM		
21 µM			
(48±18) µM	(34±5) µM	(40±6) µM	6 µM

Fluorescence measurements in half-area MTPs were conducted, where each well is filled with 25 µL solution, which reduces the consumed material by 8 to 20 times. A concentration of (36±1) µM was determined, which deviates from the ICP-determined mean value by 13%. Another drawback of this method is that for the calibration curve pure liposomes are used, while the sample contains magnetic particles, which absorb and scatter light. This effect cannot be controlled totally, as the concentration of particles in the solution is as unknown as the lipid concentration.

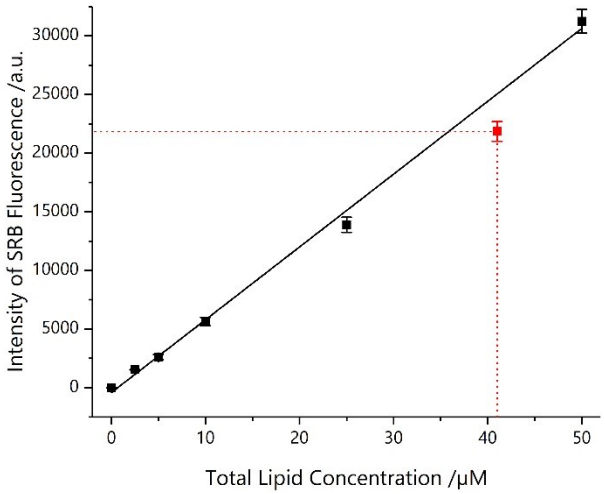

Figure S9: black: Calibration curve of pure liposomes (lipid concentration determined by ICP-OES) and red: total lipid concentration of microfluidic product as determined by ICP-MS/-OES. Fluorescence-determined concentration is (36±1) µM (13% deviation from mean ICP-MS/-OES value).

A third methods examined, the Bartlett assay, unfortunately did not produce reproducible results.

Therefore, a mean concentration of (40±6) µM total lipid, calculated from ICP-MS, ICP-OES and fluorescence measurements, is assumed for the microfluidic product used for the biotin-streptavidin binding-assays, as concentration determination of each individual sample affords a too high material consumption. For the proof-of-principle studies shown here, the assessment suffices. However, in the future, with an upscaled microfluidic setup, ICP-OES can be used for accurate concentration determination as larger volumes and amounts of magnetized nanovesicles are produced.

8. References

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