“Off-the-shelf” microfluidic devices for the production of liposomes for drug delivery

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1. Introduction

Lab-on-a-Chip (LOC) devices have been largely proposed as miniaturized bioanalytical systems for chemical/biological applications being able to perform multiple tasks associated with many laboratory procedures [1]. LOC devices offer indeed many advantages over standard (i.e. macroscopic) systems, including reduced sample and reagent consumption, faster analysis and higher levels of throughput and automation.

In spite of these advantages, the production and use of microfluidic chips still remain largely confined to academics. The costs for their development and fabrication remain indeed rather high; commercial standard chips are expensive (e.g. the price of a flow focusing chip ranges between 150 and 200 US dollars) and the price to pay for a tailored chip with particular channel geometry can dramatically increase up to thousands of pounds. In addition, the fabrication of a complete chips (with ports and connecting tubing) requires specific knowledge, facilities and equipment, therefore, only marginally applicable for protocols requiring rapid evaluation of prototypes [2]. Moreover the life span of a microfluidic chip can be dramatically short, especially if the channel width is below 100 μm, due to always possible channel clogging.

In order to reduce the high cost of microfluidic chips, some authors have proposed the use of "off-the-shelf" devices as alternative to the generally used PDMS, COC or glass 2D chips [3–5].

The concept of "off-the-shelf" microchip was first described in 2010 by Alex Terray and Sean J. Hart that described the construction and operation of a device assembled using only standard parts, available in the market as HPLC component. The authors demonstrated that, using this device, they were able to obtain a precise hydrodynamically focusing of particles in suspension; particularly, a flow focusing of particles was observed at the exit of the nozzle and within a connected microfluidic exit tubing [3].

Later, other authors described the use of "off-the-shelf" as droplet generator devices for the preparation of O/W or W/O emulsions [4]·[5]. Unfortunately, these manuscripts described the preparation of very simple emulsions, constituted of pure water, mineral oil and Span 80, representing a formulation very far from "real" emulsions, suitable for food, cosmetic or pharmaceutical applications. Commercial formulations are indeed normally constituted of many excipients (i.e. especially constituting the oil phase) and at least a drug.

Following the “off-the-shelf” microfluidics concept, in the current paper the use of 2 alternative devices, constituted of ready available commercial products, was described and compared.

Both devices were tested for the production of supramolecular colloidal system (i.e. liposomes) by a mechanism described as “self-assembly” in a controlled diffusion process. Attention was payed to the production of liposomes characterized by a lipid content/composition and drug content strictly resembling commercially available medicines based on liposomal drug-delivery system [6].

As a model drug, ivermectin was selected since recent studies have demonstrated that this drug could be used for the treatment of some RNA viruses [7]. Ivermectin is the semisynthetic derivative of avermectin B1 (a natural compound belonging to the macrocyclic lactone family) that is used in domestic animals and livestock, for the control of internal and external parasites.

Ivermectin is largely used in humans, millions of people are indeed treated with ivermectin for many diseases including onchocerciasis, lymphatic filariasis, and scabies. Administration of ivermectin in polyparasitised poor communities around the world is increasingly recognized as a mechanism to effectively improve overall quality of life and health for everyone [8],[9]. Importantly, ivermectin has recently proved to be a potent inhibitor towards both HIV-1 and dengue virus [10], therefore the drug has potential in the clinical setting as a dengue antiviral [11].

In order to apply this drug to virus treatment, it is mandatory to develop a convenient drug formulation able to improve the cellular internalization of ivermectin, reducing at the same time the unfavorable effect of the drug. In this respect, in the current paper the preparation of liposomal formulations by “off-the-shelf” microfluidic devices are described.

2. Materials and methods

2.1.Chemicals

Highly pure phosphatidylcholine (PC) 90% from soybean (Phospolipon 90G Lipoid, Germany); cholesterol 97% (Fluka, Germany); dimethyldioactdecylammoniumbromide (DDAB). Ivermectin (Sigma, UK) has the following specifications: molecular formula: $C_{48}H_{74}O_{14}$; molecular weight: 875.09; CAS registry number; 71827-03-7; solubility in water: sparingly soluble (0.080 g/l) (25 ºc); density: 1.23±0.1 g/cm^3 (20 ºc 760 torr); xlogp3: 4.1; hydrogen bond donor count: 3; hydrogen bond acceptor count: 14; heavy atom count: 62; formal charge: 0; defined atom stereocenter count: 18; appearance (color): white to off-white; appearance (form): powder; solubility (color): colorless to faint yellow; solubility (turbidity): clear, 50 mg/ml, methyl ethyl ketone; purity (hplc): ≥ 90 %. For the determination of drug entrapment efficiency into liposomes, size exclusion chromatography was conducted with Sepharose 4B (Pharmacia, Uppsala, Sweden) and Isotonic Palitzsch buffer pH 7.44, for 100 mL of buffer solution, 0.05 m sodium tetraborate (10 mL) were mixed with 0.2 m boric acid (90 mL); NaCl (270 mg) was added to adjust the tonicity of the buffer to 0.9 at 37°C [12]. All the other regents and solvents were from Sigma–Aldrich and were analytical grade.

2.2. Microfluidic chips

Two different ”off-the-shelf” chips were employed (see Fig. 1), namely #chip1-OFF-TJ (characterized by a T-junction geometry and 2 inlets) and #chip2-OFF-CF (characterized by a cross-flow geometry and 3 inlets). The devices were assembled using standard parts available on the market (IDEX Health & Science, USA); #chip1-OFF-TJ device is constituted of P-890 - Micro Tee PEEK - 1/16" with fittings to effectively join 1/16" capillary tubing together. The Micro Tee is made from polyether ether ketone (PEEK) and it is specifically designed with a 0.006" thruhole; it includes F-132 and P-416 fittings. #chip2-OFF-CF device is constituted by P-887-MicroCross PEEK 1/32" with fittings. The MicroCross is made from PEEK and it is specifically designed with a 0.006" thruhole that delivers a low swept volume. It and includes F-112 and P-416 fittings. To connect the ”off-the-shelf” devices to the syringes, teflon tubes with an inner diameter of 750 μm and 1/32” PEEK tubings, for #chip1-OFF-TJ and #chip2-OFF-CF, were respectively employed. KDS syringe pumps (KD scientific, New Era pump System, USA) were used to control the flow rate of liquids pumped through the devices.

The hydrodynamic flow focusing (HFF) #chip3-HFF, constituted of polydimethylsiloxane (PDMS), was produced with soft lithography technique. Briefly, an SU-8 mold with the designed microchannel pattern, consisting of three inlets and one main channel, was prepared following the standard procedure. Subsequently, the mold was covered with a layer of a 10:1 (w/w) PDMS precursor and curing agent mixture and heated for 1 h at 80°C for the polymer to cure. The PDMS sheet with the microchannel architecture fabricated on the surface was then removed from the mold and permanently bonded to a glass slide after oxidizing its surface with a plasma treatment.

2.3. Liposome preparation by "off-the-shelf" chips

Liposomes were prepared by injecting a lipid mixture (PC 90 mM, DDAB 10 mM) dissolved in ethanol into one of the inlets of #chip1-OFF-TJ or in the center inlet of #chip2-OFF-CF. Water is injected into the other inlet of #chip1-OFF-TJ or into the 2 side inlets of #chip2-OFF-CF. The flow rate ratio (FRR) was varied from 10 to 50, where FRR is defined as the water to ethanol volumetric flow rate. Liposome formation was investigated at different shear forces by changing the total flow rate (TFR), varying it from 18.75 to 75.00 μl/min. Ivermectin-loaded liposomes were prepared similarly to empty liposomes, by adding ivermectin (at 1.0 or 10.0 mM) into the ethanolic lipid solution.

Liposome preparation by ethanol injection method and hydrodynamic flow focusing chip

Lipids (PC 90 mM, DDAB 10 mM) were dissolved in ethanol. In the case of ethanol injection, lipids were injected using a micropipette into an appropriate volume of water under vortexing. In the case of microfluidic, a hydrodynamic flow focusing (HFF) chip was employed (#chip3-HFF), whose geometrical characteristics are reported in Fig. 1 B. The lipid solution was pumped into the center channel of the microfluidic network, while water was injected into two oblique side channels intersecting with the center channel. TFR and FRR were the same as for "off-the-shelf" chip experiments. All experiment were conducted at 20°C.

2.4. Liposome characterization

The dimensional analysis of liposomes was performed using a DLS Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK) with a backscattering detection angle of 173°, a He/Ne laser that emits at 633 nm, and a 4.0 mW power source. Dimensions of liposomes were indicated as Z-average (Z-ave), that represents the intensity-weighted mean diameter derived from the cumulants analysis and dispersity index (DI). The ethanol content present in the liposome samples was considered during

each determination of liposome size by DLS Zetasizer Nano-ZS. DLS values were calculated as average of 3 independent samples, measured in quadruplicate ±SD. The cryogenic transmission electron microscopy (cryo-TEM) analysis of liposomes was performed as previously described [12].

3. Results and discussion

To establish if "off-the-shelf" devices can be conveniently employed for liposome preparation, 3 different microfluidic devices were considered and compared; in addition a conventional "bulk" method (i.e. ethanol injection), was also employed as reference.

Particularly, 2 alternative "off-the-shelf" devices were build up and employed, namely: #chip1-OFF-TJ and #chip2-OFF-CF; the first one is a "T-junction" device, whereas, the second is a "cross-flow" geometry device. For details on the geometry and dimensions of the "off-the-shelf" chips refers to Fig. 1 A. As microfluidic control, a hydrodynamic flow focusing chip was selected, since it represents the most typical chip geometry adopted for liposome production [13],[14]. For instance, the #chip3-HFF, constituted of PDMS/glass sandwich is schematized in Fig. 1 B.

Initially, different liposome preparation experiments were performed, to investigate the dependence of liposome size, expressed as Z-average, on microfluidic device geometry and microfluidic flow conditions. The performances of #chip1-OFF-TJ and #chip2-OFF-CF were compared in experiments conducted varying both the TFR (from 18.75 to 75 μl/min) and FRR (from 10 to 50). The range of variation for the TFR was selected in reason of the following considarations: (a) taking into consideration the current "state of the art" for similar preparation procedures described in the literaure [1], (b) using a minimal TFR allowing the preparation of an amount of sample needed for the liposome characterization (about 1 ml for DLS size determination) in a reasonable time (i.e. about 55 min for 1 ml) and (c) using a maximal TFR not causing eccessive backpressure inside the chip, causing the connecting tube leakage.

Data reported in Fig. 2 indicate that chip geometry (i.e. T-junction Vs. cross-flow) had a marginal effect on the liposome size; being the liposomes produced by #chip2-OFF-CF only slightly larger (≈ 15 nm) than those obtained by #chip1-OFF-TJ. TFR has a very limited effect on liposome size (Fig. 2 A), whereas an increase of FRR causes a progressive decrease of the vesicles dimension (Fig. 2 B). These data are well in agreement with data previously reported in recent articles describing the production of liposomes by conventional microfluidics with chips characterized by a HFF geometry [15].

In this respect, the liposomes produced by the "off-the-shelf" #chip1-OFF-TJ device were compared with those obtained by a conventional "bulk" (i.e. ethanol injection) and microfluidic (i.e. HFF) methods. Fig. 3, reports, indeed the Z-ave and DI of liposomes produced by the three different

methods; the results indicated that #chip1-OFF-TJ produced liposomes with a slightly larger Z-ave compared to #chip3-HFF and similar to those obtained by ethanol injection. Notably, the dispersity index of #chip1-OFF-TJ liposomes was largely smaller than those of other methods, being always ≤ 0.3 for all FRR tested. This result appear to be particularly important in perspective of liposome production for medical applications. The size uniformity of disperse systems is indeed an important feature of pharmaceutical formulations, assuring batch to batch reproducibility and uniform drug release profiles.

As further demonstration of the applicability of "off-the-shelf" devices, drug containing liposomes were produced, namely liposomes containing the antiviral drug ivermectin. Fig. 4 shows that the presence of ivermectin (at 0.1 or 1.0 mM) does not greatly influences the size of liposomes, confirming that "off-the-shelf" devices can be conveniently employed to the production of liposomes intended for drug delivery. Moreover, the determination of drug encapsulation yield, performed by size exclusion chromatography, demonstrated that liposomes produced by "off-the-shelf" devices, presented a very high yield, always > 95%.

Finally, in order to determine the architecture of liposomes produced by "off-the-shelf" devices and to study their morphology and lamellar shapes, together with an estimation of the size, a cryo-TEM analysis was performed. The images reported in Fig. 4 B demonstrate that both empty and ivermectin-loaded liposomes were spherical in shape. The majority of the empty liposomes had a size smaller than 150 nm in diameter, in agreement with the PCS analysis. Liposomes containing ivermectin were similar to the empty ones, only slightly larger in size.

In conclusion, this study reports that off-the-shelf microfluidic devices, can be conveniently designed and assembled with low-cost, commercially available components. We demonstrated that off-the-shelf devices allowed the rapid production of narrowly dispersed and very reproducible unilamellar liposomes for drug delivery. Liposomes in the size range of 100-150 nm with high encapsulation efficiency were indeed obtained, in addition by varying the microfluidic experimental conditions (mainly FRR), the size of the produced liposomes can be finely tuned.

These results suggest that off-the-shelf microfluidic devices can hold great promises for the efficient preparation of different lipid based colloidal systems for biomedical applications.

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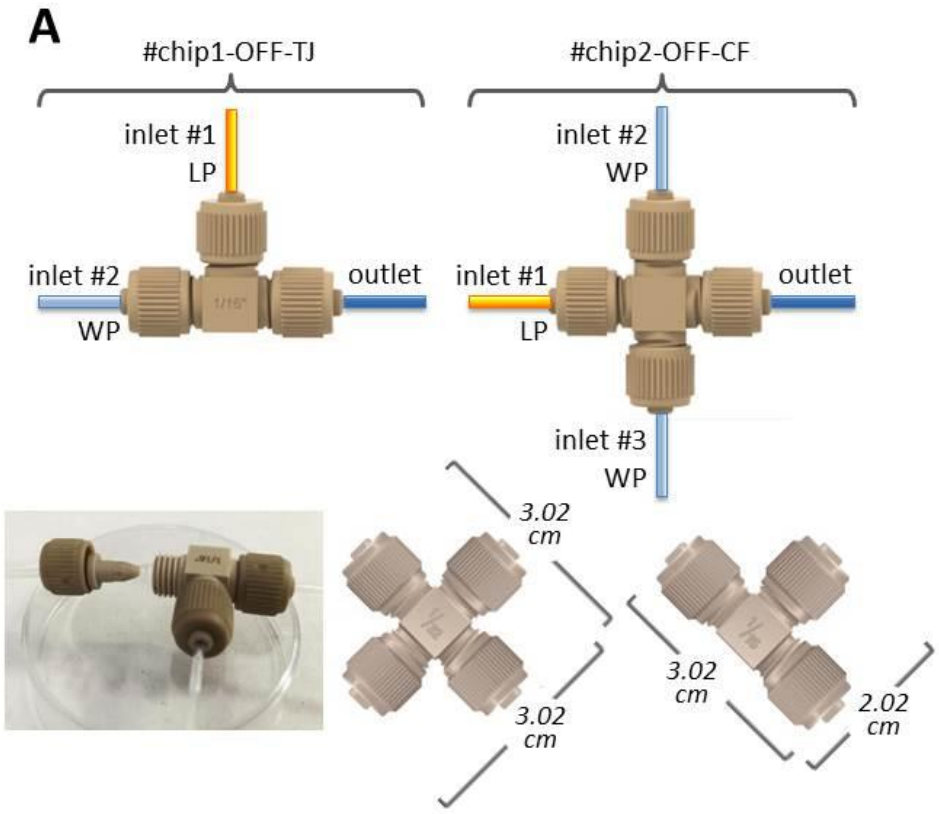
Figure legends

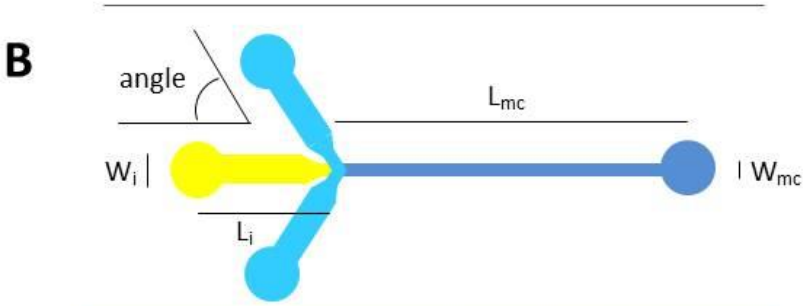
Fig. 1 Scheme showing the geometrical characteristics of “off-the-shelf” (A) and conventional cross-flow (B) microfluidic devices. Two different “off-the-shelf” devices were employed, namely: #chip1-OFF-TJ, characterized by 2 inlets and a T-junction geometry and #chip2-OFF-CF characterized by 3 inlets and a cross-flow geometry.

Fig. 2 Effect of total flow rate (A) and flow rate ratio (B) on the dimension of liposomes produced by “off-the-shelf” microfluidic devices. #chip1-OFF-TJ (triangles), #chip2-OFF-CF (squares). Data represent the average ± SD of 3 independent samples measured in quadruplicate.

Fig. 3 Comparative analysis of the dimensional characteristics of liposomes produced by ethanol injection (red lines), conventional hydrodynamic flow focusing microchip (#chip3-HFF) (blue lines) and “off-the-shelf” devices (#chip1-OFF-TJ) (black lines). Data are presented as Z-ave (A) and dispersity index (B) and represent the average ± SD of 3 independent samples measured in quadruplicate.

Fig. 4 Effect of drug-loading on the dimensional (A) and morphological (B) characteristics of liposomes. Liposomes produced by #chip1-OFF-TJ in the presence of 0.1 (closed circles) or 1.0 mM (closed squares) ivermectin. In B1 and B2 panels, cryo-TEM microphotograph of empty and 1.0 mM ivermectin liposomes, are respectively reported. Bar corresponds to 200 nm.





chip code	material	geometry	channel dimensions (width/depth. µm); (length. mm)	channel angle
#chip3-HFF	PDMS/glass	HFF	150/100; 30	40°

Figure 1

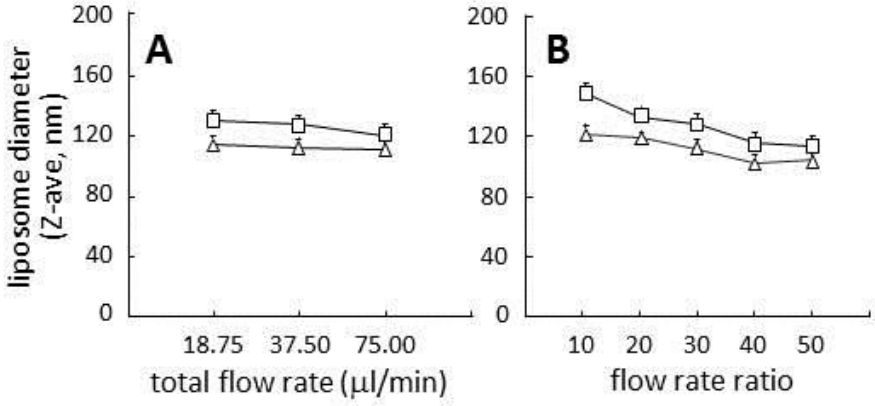

A
B
liposome diameter (Z-ave, nm)
200
160
120
80
40
0
18.75
37.50
75.00
total flow rate (µl/min)
10
20
30
40
50
flow rate ratio

Figure 2

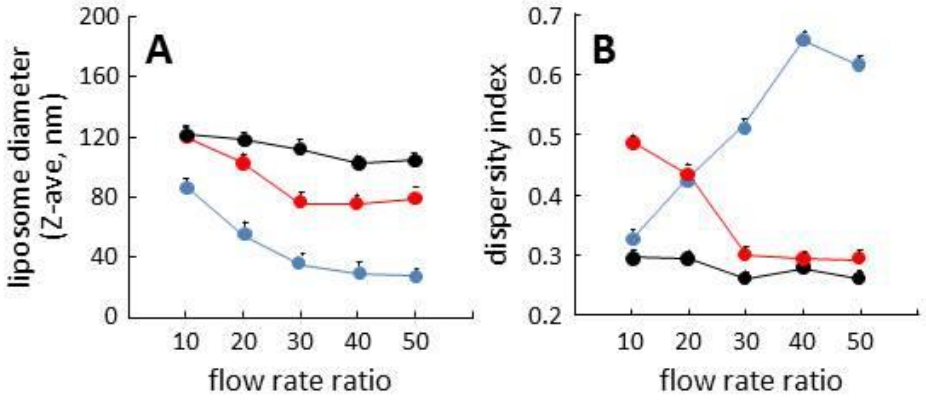

A
liposome diameter (Z-ave, nm)
200
160
120
80
40
0
10
20
30
40
50
flow rate ratio
B
dispersity index
0.7
0.6
0.5
0.4
0.3
0.2
10
20
30
40
50
flow rate ratio

Figure 3

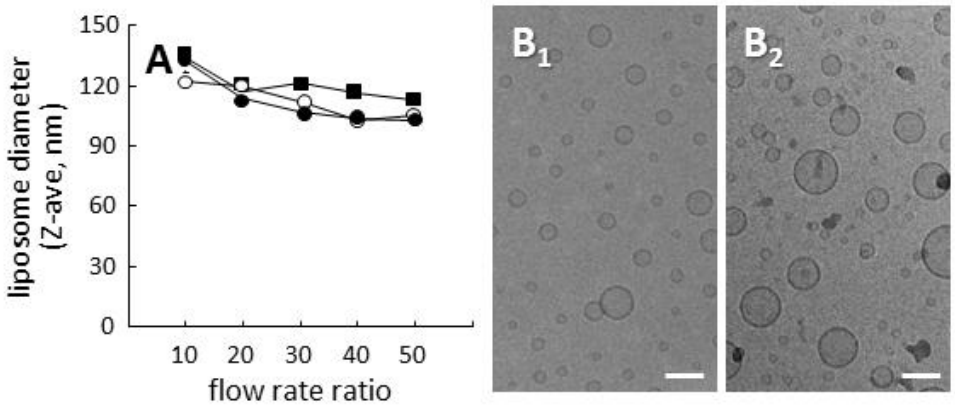

A
liposome diameter
(Z-ave, nm)
150
120
90
60
30
0
10
20
30
40
50
flow rate ratio
B1
B2

Figure 4