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Droplet-based Microfluidic Systems for Production and Transfection *In Vitro* of Non-Viral Vectors for Gene Delivery

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ABSTRACT

Nowadays, many researchers in the field of gene delivery are focused on develop methods to produce nanoparticles with physicochemical characteristics in reproducible, continuous and scalable process without requiring post processing steps. Another key factor is to transpose conditions from *in vivo* gene delivery into cells and tissue to *in vitro*. In this context, microfluidics technology is emerging to replace the traditional methods to produce nanoparticles, incorporate nucleic acids into them and transfect cells *in vitro*. Within microfluidics, droplet-based systems have been highlighted by some special parameters provided by picoliter compartments created using two immiscible liquids. Several micro/nanoparticles used in gene delivery can be produced through droplet-based systems with low polydispersity index, such as liposomes, bio/polymeric nanoparticles, metal nanoparticles, polymersomes, microgels. In the case of *in vitro* transfection, it is known that conventional procedure in wells lead to a diffusive micro/nanoparticles transport to cells, however in droplet microfluidic platforms there is also the convective contribution that facilitates and enhances the control of transfection. Moreover, hydrogel droplets can provide a 3D environment for cells similar to living tissues, achieving cell behavior *in vitro* more similar to *in vivo*. Thus, the purpose of this review is to summarize new trends in microfluidic droplet systems developed to gene delivery studies, since micro/nanoparticles production to transfection *in vitro*. This review brings new insights for future challenges and shows that with theoretical principles we can develop robust microfluidic systems to gene delivery studies

INTRODUCTION

Microfluidics is a technology that has been exploited in many areas, with emphasis on cell biology [1]. The advantages are countless, such as the use of small quantities of samples and reactants, minimum devices size, control over mass and heat transfer, laminar flow, low cost and power dissipation and low cost of devices production [2,3]. In the field of nanoparticles production for gene delivery, many efforts are being made in order to develop scalable processes with reproducible physicochemical characteristics [average diameter, zeta potential and polydispersity index] [4]. In this context, microfluidics appears like a powerful strategy for continuous, reproducible, and scalable production of micro and nanoparticles highly monodispersed [5]. Moreover, microfluidic droplets platform can provide a high encapsulation efficiency, since it allows self-assembly processes in which nanoparticles are formed and actives inserted in only one-step, using low quantity of reagents [6].

Besides than nanoparticles production, droplet-based microfluidic systems can be also used to transfect cells *in vitro*. The most commonly is to use physic methods, like electroporation and microinjection, or chemical methods by using lipid and polymeric nanoparticles [7]. Since microfluidics generates extremely uniform droplets, they are appropriate for single-cell encapsulation or for *in vitro* expression of single genes [8]. Molecules secreted by cells are fast detected due to the low volume

surrounding each cell, allowing investigation of transfection parameters in culture real time ^[9,40]. Microfluidics can also provide a three dimension [3D] environment when using hydrogel droplets, which is very important for attachment cells cultivation and proliferation ^[41]. Microenvironment more similar to *in vivo* opens new approaches in transfection field, like how mechanic stimulus [matrix stiffness] can increase pathway of internalization in order to facilitate non-viral vectors insertion in cells and, consequently, enhance efficiency transfection ^[42].

In this review, we present the state-of-the-art of current micro engineering methods for the synthesis of micro/nanoparticles and cell transfection methods in droplet-based microfluidics platforms. First we present an overview about gene therapy and the role of non-viral vectors in this context, after we present some techniques to produce micro/nanoparticles in microfluidic droplets systems considering also key factors to produce controllable and stable droplets, and at last we show methods of transfection inside droplets comparing with the conventional transfection in wells.

Nanoparticles as non-viral vectors for gene delivery

Gene therapy refers to the transfer of genetic material encoding a therapeutic gene of interest into a cell, tissue, or whole organ with consequent expression of the transgene in order to treat a disease. However, for the success of gene therapy, the development of sophisticated and efficient delivery systems capable of transferring genes is a key factor ^[13,14]. In brief, we can describe two classes of efficient nucleic acid carriers: viral and non-viral vectors; which are able to protect nucleic acids from degradation, have an intracellular delivery and a relative safety ^[15]. Over a decade ago, patients with immunodeficiency-X1, which blocks T and NK lymphocyte differentiation, were treated with gene therapy trial for X-linked severe combined immunodeficiency [SCID-X1] based on the use of cDNA in a retrovirus ^[16,17]. After, French patients have had their T cell leukemia treated with retrovirus vector integration near to the LMO2 proto-oncogene promoter, producing aberrant transcription and expression of LMO2 ^[18]. These facts have led to optimism in the gene therapy research. Nowadays, gene therapy clinical trials are present in worldwide, mainly in United States of America [62.6%], United Kingdom [10.3%] and in Germany [4.1%] and are generally used in the treatment of cancer [63.8%], monogenic diseases [8.9%] and infectious diseases [8.2%] ^[19]. The most commonly vectors used in gene therapy are still virus vectors, highlighting adenovirus [22.5%] and retrovirus [18.8%], but also lipofection [5.2%] and naked pDNA [17.5%] are raising their use ^[19]. Notwithstanding the viral vectors represent the majority of vectors used in gene therapy due to their high efficient transfection, they can invoke immune responses or protooncogene activations. In this context, the non-viral vectors have a promise and potential future, taking into account their reproducibility and safety of use ^[20].

Cationic liposomes are non-viral vectors mainly composed of cationic lipids that guarantee their positive superficial charge, in order to interact electrostatically with negatively charged nucleic acids until forming complexes with still positive liquid charge ^[21]. Hence, these positive complexes enter easily inside cells, whose surface membrane is negatively charged ^[22]. Once into cells, the complexes release nucleic acids in cytosol, in case of RNA, for rapid synthesis of a target protein when is important to achieve a fast response against a disease like cancer ^[23]; or in cell nucleus, in case of DNA, to control gene expression for a long-term ^[24]. Felgner^[22] were pioneer in the development of a cationic liposome for nucleic acid delivery composed of 1:1 ratio of DOTMA [N-[1-[2,3-dioleoyloxy] propyl]-N,N,N-trimethylammonium chloride] and DOPE [dioleoyl- α -phosphatidylethanolamine], which was then commercialized as Lipofectin. For example, this cationic liposome was used *in vitro* to carrier efficiently hepatitis C virus proteins into a human hepatocyte cell line [HUH7] ^[24] and *in vivo* to delivery linamarase gene for the treatment of brain tumors in animals or humans ^[25]. Recently, other commercial cationic liposomes are being used in gene therapy, which due to their protocols well-established; provide high efficiency of transfection in some specific cells and with some specific nucleic acids, such as Lipofectamine, DMRIE-C, Oligofectamine, Ambion, 293 fectin, Optifecta, Invivofectamine, FuGENE, TransFast, TransFfection and CLONfectin. Our research group ^[26] also showed the feasibility of dehydrated-rehydrated liposomes composed of EPC [egg phosphatidylcholine], DOTAP [1,2-dioleoyl-3-trimethylammonium propane], DOPE [50/25/25% molar, respectively] carrying polynucleotides encoding HSP65, for prevention and treatment of tuberculosis. And more recently, we obtained the same cationic liposomes produced in a large scale by ethanol injection method to delivery nucleic acid into dendritic cells as a potential tool for cancer immunotherapy ^[27]. Furthermore, other structures of nanoparticles composed of lipids, like solid lipid nanoparticles, can also be used for gene delivery. For example, Del Pozo-Rodríguez ^[28] showed lyophilized solid lipid nanoparticles as a stable structure to delivery pDNA encoding green fluorescent protein in HEK293 culture cells. Rudolph ^[29] showed solid lipid nanoparticles applied to delivery dimeric HIV-1 TAT peptide into bronchial epithelial cells *in vitro* and application to the lungs of mice *in vivo*.

In addition to cationic lipids, cationic polymers, such as chitosan, form also efficient complexes with nucleic acid ^[30]. Chitosan, copolymer composed of D-glucosamine and N-acetyl-D-glucosamine residues, is a unique poly[aminosaccharide] obtained by chemical N-deacetylation of chitin ^[31,32]. Chitin, in turn, is the structural component of mollusks ^[33], arthropods ^[34] like insects ^[35] and crustaceans ^[36], nematodes ^[35] and is in cell walls of fungi ^[37], yeasts and hyphae ^[38]. Chitosan at pH below 6.5, its pKa value, shows deacetylated subunits with primary amine groups positively charged. Likewise cationic liposomes, these cationic polymers are able to link electrostatically with nucleic acids, providing a net positive charged polyplexes that allows their interaction with cells ^[39]. Chitosan has been considered to be a good gene carrier candidate, since it is known as a biocompatible, biodegradable, and low toxic material with high cationic potential ^[40]. For instance, Dass^[41] incorporated a plasmid expressing pigment epithelium-derived factor into chitosan micro particles and the results *in vitro* showed an anti-invasion and increased adhesion of the osteosarcoma cell line SaOS-2 and *in vivo* this complex was confirmed as promise for gene therapy

of osteosarcoma. Additionally, the mucoadhesive property of chitosan allows a sustained gene delivery of therapeutic genes *via* gastrointestinal mucosa administration, like showed by Zheng [42] that used pDNA encoding green fluorescent protein [GFP] to quantify the transfection efficiency in the mucosa of the stomach, duodenum, jejunum, ileum and large intestine. Although chitosan is the most commonly polysaccharide used for nanoparticles fabrication, there are others biopolymer nanoparticles for gene delivery whether composed by synthetic polymers like polylactide and [D,L-lactide-co-glycolide] [43] or composed by another natural polymers, like albumin, gelatin, alginate, collagen and silk fibroin [44].

Moreover, metal nanoparticles also show as good potential candidates for gene delivery, since they can be functionalized to bind covalently or non-covalently with nucleic acids [45]. McIntosh *et al.* [46] showed gold nanoparticles functionalized with tetraalkylammonium ligands to interact electrostatically with DNA by a non-covalent bond, in order to inhibit the transcription by T7 RNA polymerase *in vitro*. On the other hand, Oishi [47], by a covalent bond, immobilized siRNA in PEGylated gold nanoparticles containing thiol groups to form SH-siRNA binds, that when applied *in vitro*, enhanced gene silencing activity in HuH-7 cells. Additionally, semiconductor nanoparticles, e.g. carbon nanotubes, quantum dots and magnetic nanoparticles, can be modified to be used as nanocarriers in gene delivery. For instance, single-walled carbon nanotubes functionalized with lipopolymers allow siRNA link, so that it can be delivered inside human cells decreasing or silencing expression of HIV-specific cell surface receptors. Likewise, DNA could be encapsulated spontaneously into carbon nanotubes *via* van der Waals or hydrophobic forces when exposed to a water solute environment [48], or if functionalized with ammonium derivatives, nucleic acids can interact electrostatically with carbon nanotubes [49]. Yau [50] presented quantum dots functionalized with amino groups or streptavidin conjugated with linear nucleic acid cassette molecule to treat or prevent disease in plants. In addition, Kami *et al.* [51] demonstrated the feasibility of using magnetic nanoparticles with modified surface derived from iron oxides, such as magnetite [Fe₃O₄] and maghemite [γ -Fe₂O₃], for gene delivery. Basically, the mechanism of magnetofection [transfection by magnetic nanoparticles] is similar to using lipidic nanoparticles, however after adding magnetic nanoparticles/nucleic acids complexes to cell culture media, it is applied a magnetic force to approach complexes to cell surface, thereby achieving greater transfection efficiencies [51]. However, equally important to nanoparticles role as non-viral vectors in gene delivery field, is the method of nanoparticles production, in order to guarantee their reproducibility and nonhazardous for *in vitro* and *in vivo* applications. In this context, microfluidic droplet systems came up as a hopeful bottom up method to produce these nanoparticles.

Microfluidic droplet technologies in nanoparticles production

The general concept of microfluidics is in the manipulation of small amounts of reactants inside micro channels with the capability of controls and manipulates molecules in space and time [52]. In microfluidics it is possible to work with small amounts of reactants, the period of reactions are short, it is possible to work with parallel operation [53], large surface to volume ratio and the diffusion of the compounds and heat transfer are fast [54]. The advantages can be related to the flow in microfluidic devices which is laminar and corresponds to a low Reynolds number [52,55], as a consequence the mixture between two flowing streams occurs by mainly diffusion [53]. The origin in microfluidic is in the 90's for Micro Electro Mechanical Systems area [MEMS] [54]. Nowadays microfluidics has been exploited in numerous areas as:

Biological analyses - detection of biomolecules [56,57], manipulation and amplification [58] and separation of DNA by capillary electrophoresis [59].

Microbial growth - Screening of variables and kinetic parameters [60,61].

Nanoparticles production – polymeric particles [62,63], liposomes and lipid vesicles [64-66], metallic nanoparticles [67].

Gene Delivery/Transfection – electroporation [68], hydrodynamic force and optical energy [69].

Different materials can be used for the construction of micro channels and for biological application glass and polymers are detached [70]. Glass is considered to be biocompatible, impermeable to gases [70,71], has physic and chemical stability and it is hydrophilic [70]. The techniques to prepare microdevices in glass are laser ablation and wet etching [70]. Microdevices can also be made by polymers which are not expensive, there is the possibility to change the chemical formulation [71], are stable [52] and hydrophobic [70]. The elastomer which has been used extensively for microdevices construction is poly[dimethyl syloxane] well-known as PDMS, moreover the technique employed is soft lithography [72].

In addition, it is important to consider the wettability related to the microchannel material and the droplet system. Since the continuous phase wets the walls faster than the disperse phase and forms a thin film between droplet and walls [73]. The droplet breakup occurs when the continuous phase wets the device walls instead the disperse phase, thus the droplet morphology is a result of the interaction between the material which the devices is formed and the continuous phase [73].

Summarizing hydrophobic channels are required to prepare water in oil systems, inversely hydrophilic channels are necessary for forming oil in in water emulsions [74]. Considering the advantages of using PDMS for devices construction, in the literature different strategies are reported to change the wettability of the material, through chemical modifications [74,75].

The two main principles used in to operate microfluidic systems are hydrodynamic flow focusing and droplets methods. Some

advantages of droplet processes can be described here in comparison with laminar flow processes. In laminar flow, which solute are all distributed over the solvent, the efficiency of chemical reactions and the detection of some molecules inside the channels can be decreased, this phenomenon is called Taylor-Aris dispersion [76,77]. The use of droplets processes also cut out the contact with solid walls reducing the probability of reagents adsorption into the channels walls [77]. Droplets with the samples inside can be seen as micro-reactors which allows the manipulation of small volumes [15,78]. In addition, in droplets microfluidics it is possible to carry out many reactions without increasing the number and the channels size [13]. Furthermore, at micro scale range, considering the relation between the surface area and volume, the reactions are faster because the heat and mass transfer times and also diffusion distances are shorter [3,13]. The use of segmented flow which the reactants are separated in different picoliter/nanoliter droplets has been used in cases when miniaturized systems has to be achieved [79]. The segmented flow is the principle of emulsions which are a metastable colloidal systems [80] with two immiscible liquids. In this case there is one continuous phase and one disperse phase in droplets formats [73,79].

In order to form stable droplets, the use of surfactants is important. Surfactants are known as amphiphilic molecules with different groups and having affinity for different phases which are immiscible. Due to different groups in the structure, surfactant molecules go to interface and as a result the surface tension between the phases decreases [81]. An essential requirement to use droplets as microreactors is to avoid the coalescence of them. Thus the surfactant addition provides stabilization in the metastable state [80,81].

Furthermore choose the surfactant is the rule to the success of stable droplets and for biological applications there are appropriate molecules which biocompatible to the systems [81]. Fluorinated oils are promising to be used in biotechnology area; however few surfactants are available to stabilize water in oil interfaces emulsions [81]. Different molecules are being studied and developed to microfluidic applications. Nowadays, block copolymer of perfluoropolyether and polyethylenoxide are the most interesting molecules existent. It is known that these molecules reduce protein adsorption or the interactions with cell membranes [81].

Another important molecule is a triblock copolymer surfactant composed of perfluoropolyether [PFPE] and polyethylene glycol [PEG] blocks [82]. However the problem is the limitation of surfactant modification which can be just by varying the molecular weight or chain-end functionalization. In this way, Wagner et al proposed to synthesize and characterize polyglycerol-based triblock surfactants, and exemplified in droplet-based microfluidics their application in cell encapsulation and *in vitro* gene expression studies [82]. The uniformity and the little volume of these droplets allow them to be used for quantitative assays requiring reduced volumes of reagents, and as a result, providing a low cost for this technique [2]. Moreover, droplets compartment design can furnish combined information about molecules function [activity or inhibitory functions], molecules identity and also to evaluate the molecules ability to carry out its function by measuring, for example, a fluorescence product. In addition, droplets inside microfluidic platforms give the possibility to make several unit operations in a device, like droplets can be divided, fused, incubated, analyzed, sorted and broken up [83]. The use of droplets technique provides better mixing efficiency and control over the reactants concentration [3].

Two different categories are used in order to achieve efficient mixing and droplet generation: passive and active methods [13,15,30]. The passive methods are based on the micro channels geometry and/or liquid flow rate [70]. Passive methods can be divided into categories according to types of geometries used to promote the droplet formation. The main types are: Co-flowing, cross-flowing, and elongational streams [73].

On the other hand, active methods relies on the use of external energy to accomplish desired mixture [30]. The external energy can be electrical fields [84], acoustic fluid shaking [85], ultrasonic vibrations, etc. The use of microfluidic droplet technologies for particles production is still a challenge in terms of size since the works in the literature reports microparticles and reports of nanoparticles are scarce. In terms of droplets size the majority works are in few to hundred micrometers in a uniform and continuous stream and the polydispersity can be very small [73].

One comparison to the *traditional methods* can be made in order to visualize the difference with microfluidic droplets techniques. One principle to produce polymeric particles by the droplets methodology is in the preparation of aqueous droplets in oil phase and then the solidification of the droplets occurs through the addition of a cross linker according in **Figure 1**, this method is called emulsion crosslinking and it has been extensively used to prepare chitosan micro particles [86].

In the microfluidic methodology the method is miniaturized and the nanoparticles production occurs inside the channels in continuous mode. One simple scheme is described in **Figure 2** and the configuration of the system can be changed according to the nature of the particle. In some cases, the addition of the crosslinker is made in a second region with inlets after the droplet formation [87].

Different methods to produce nanoparticles based on droplets techniques are being studied. Herein, we will summarize some methods. Basically to produce different particles the main step in droplet technology is based on the production of single emulsion or double emulsion. The following steps are inherited to the type of the particle that intent to produce. For example,

monodisperse biodegradable dextran-hydroxyethyl methacrylate [Dex-HEMA] microgels were prepared in in-line channel geometry by droplet process. These microparticles are generally used for controlled delivery of proteins. The continuous phase was mineral oil and the aqueous phase was aqueous Dex-HEMA solution [30% w/w]. Then, the authors used the nonionic surfactant, which was added to the oil phase in the concentration of 4% [v/v]. The droplets were formed when pressure were applied to the reactants reservoirs. Unlike, increasing the pressure in Dex-HEMA reservoir the rate of droplets production also increased [32]. Before the droplets preparation to the aqueous solution containing dex-HEMA it was also added a photoinitiator in order to polymerize the droplets when they left the device, still in oil by UV irradiation. In order to separate, the oil from the aqueous phase, centrifugation was carried out following by washing steps with deionized water. The mean average diameter of the prepared dex-HEMA monodisperse microgels was $D = 9.9 \mu\text{m} \pm 0.3 \mu\text{m}$. [32].

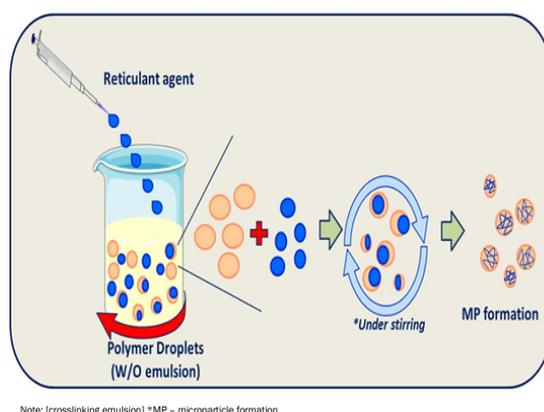


Figure 1. Schematic configuration of conventional method for production of polymeric particles.

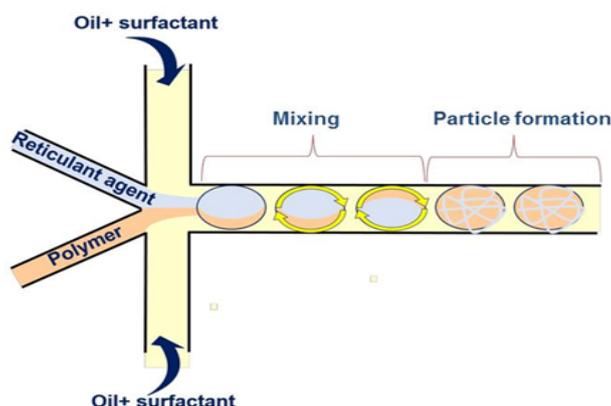


Figure 2. Schematic configuration of microfluidic method for production of polymeric particles.

Additionally, Hung [39] presented two different techniques to produce poly [lactide-co-glycolide] PLGA micro/nanospheres in microfluidic devices using droplet process. PLGA is a synthetic copolymer formed by glycolic acid and lactic acid and also it is approved by US Food and Drug Administration [FDA] to be used in therapeutics. The first technique was based in the solvent evaporation producing PLGA particles in the microscale. In this approach, PLGA was dissolved in the organic solvent dimethyl carbonate [DMC]. The second step was the production of the droplets since the organic solvent is immiscible in water. The microfluidic devices were made of PDMS, then it was necessary a surface treatment in the device in order to favor the formation of *oil in water* droplet generation process. The treatment was the application of poly [vinyl alcohol] [PVA] inside the micro channels. PLGA was studied in the concentration from 0.5% to 5% [w/w]. PVA was also used as the surfactant in aqueous phase. After de droplet production the organic solvent evaporated due to the high volatility and the particles are formed. The micro particles sizes are directly proportional to the PLGA concentration varied from 3 to 30 μm approximately. Moreover, the second microfluidic technique studied by the authors was called solvent extraction [39]. For this case the surface of the PDMS devices were not treated with PVA, because the principle of the droplet generation was *water in oil*. The devices were constructed with three inlets: for silicone oil with span 80 [1%], PLGA in dimethyl sulfoxide [DMSO] [PLGA was dissolved at 1% wt in DMSO] and water. PLGA-DMSO droplets and water droplets were produced separately, but they fused in sequence and DMSO was extracted into the water causing the PLGA precipitation. The formation of homogeneous PLGA Nano spheres occurs and the size was around 100 nm. A method to produce biopolymer hydrogels is described in the literature by Zhang [40]. The approach was used to prepare capsules of alginate, κ -carrageenan and carboxymethylcellulose [here we are focused in describe the alginate capsules only]. The hydrogels production was conducted in PDMS Y shaped planar devices. The disperse aqueous phase contained alginate, and the crosslinking agent $[\text{Ca}^{2+}]$ dissolved in the continuous oil phase [undecanol]. The formation of the droplets occurred by the immiscibility of the two reactants and the hydrogels were formed when the Ca^{2+} diffused from the organic solvent to the aqueous Phase containing the

alginate. The increasing in the flow rate caused the decreasing in the droplets size. This change can be seen when the Q_{aqueous} was changed from 0.3 to 0.7 mL/h and Q_{oil} from 0.2 to 15 mL/h and the diameter ranged from 230 to 30 μm . One interesting finding is the observation of the gelation occurrence in the alginate droplets by the droplet surface which changed with the time. The alginate microgels were stable in PBS solution and 5000 particles/ min were produced in the tested device.

Polymersomes can be easily described as polymer vesicles or a self-assembling shell of block copolymers which are amphiphiles^[88,89]. The general method to produce polymersomes is similar to liposomes production. The hydrophobic groups tend to associate in order to minimize the contact with water. The amphiphilic copolymers when hydrated tend to form vesicles^[88]. Usually for biomedical application the materials used to compose the amphiphilic block copolymers are biocompatible, biodegradable hydrophobic polymer [polyesters or polyamino acids] blocks covalently bonded to biocompatible hydrophilic polymers as polyethylene glycol [PEG]^[89]. Different authors describe microfluidics techniques to prepare polymersomes in the microscale range. Lorenceau^[90] explained the difficulty of conventional methods to obtain polymersomes with low polydispersity, hereupon the authors developed a droplet microfluidic technique to obtain polymersomes with low polydispersity from double emulsions. To guarantee the formation of double emulsion the devices were composed by 2 round capillary tubes which were wrapped for an outer square tube. The double emulsions structure were like water-in-oil-in-water and the diblock copolymers used were poly[normal-butyl acrylate]-poly[acrylic acid] [PBA-PAA] and the concentration varying from 0.1 –5 wt %. The organic phase which di block copolymers were dissolved was a mixture of tetrahydrofuran [THF] and toluene in the concentration from 50-50 wt % to 80-20 wt %. Double emulsion formation can be explained as inside the inner capillary tube flowed the aqueous phase [distillate water] and the organic phase flowed coaxially. The outer aqueous phase [mixture of 80 wt% of glycerol in water] flowed in the opposite direction from the outer square tube and hydrodynamically focusing the previous emulsion in a second capillary tube. Then the double emulsion was produced. According to the authors polymersomes formation occurs when the mixture of organic solvent evaporated from the middle phase of the double emulsion. The flow rate study showed that the droplets generation could assume two schemes like dripping when low outer flow rates were used or jetting when high outer flow rates were used. The polymersomes production were proven when quantum dots particles were added to the inner aqueous phase and no trace of fluorescent particles were found outside the polymer membranes^[90]. Similar microfluidic approach from the previously reported study was used to produce polymersomes from the diblock copolymers PEG-*b*-PLA [polylactic acid]^[91]. Recently polymersomes were prepared using the diblock copolymers PEG-*b*-PLA, however the difference between the previous work is that the polymersome core contained alginate which reacted with calcium ions by creating an osmotic pressure^[92].

Chitosan is a biopolymer, which has been used extensively in medical area^[93]. The main principle used to chitosan particles production occurs through ionic gelation between the amine groups from chitosan and opposite charge from the reticulante agent^[94]. In the literature, the methods described for chitosan particles production are in the majority, conventional processes. In microfluidic processes using hydrodynamic flow focusing few studies are reported^[62,95]. In droplet microfluidic system, Yang^[87] chitosan microparticles containing ampicillin were produced with copper sulfate as crosslinking agent [CuSO_4]^[87]. The material device was poly methyl acrylate [PMMA] and it was composed by 5 layers which were integrated by screws with 4 inlets [1 chitosan inlet, 2 oil inlet, 1 crosslinking agent inlet] and 1 cross- junction channel. The continuous phase was formed by sunflower seed oil, the droplets formation occurred when chitosan [3% w/v] with ampicillin [16.6% w/v] got in contact with oil phase through the cross-junction. Latter the droplets produced reacted by ionic gelation with CuSO_4 . Different flow rate ratio was tested in order to verify the influence in the microspheres size. The authors found that keeping the same aqueous phase flow rate and increasing the oil flow rate the droplets size decreased. The aqueous phase flow rate was set from 0.001 mL/min. – 0.1 mL/min. and the continuous phase from 0.1 mL/min. to 1 mL/min. The microspheres size varied from 100 to 800 μm changing the flow rates in the microchannel devices. In a similar approach, the same group investigated chitosan microparticles production^[96]. However, the nanoparticles formation occurred outside the microfluidic device because chitosan emulsion was dripped in a reactor containing the reticulante agent sodium triphosphate [TPP]. In this case, chitosan microparticles size varied from 180-680 μm ^[96].

Liposomes are well known vesicles formed by phospholipid bilayer. In the literature the continuous method for liposomes production using microfluidic channel was already reported^[64,65]. The use of droplets techniques to prepare liposomes still remains in the formation of giant vesicles in the microscale range. Sugiura^[97] used lipid coated ice droplet hydration method to prepare giant vesicles between 4-20 μm in three basic steps. The organic phase was hexane, 3% wt of sorbitan monooleate [Span 80] and stearylamine as emulsifiers. After the emulsion O/W was formed in hydrophobic channels, the droplets are frozen and subsequent separation from hexane solution. The temperature was kept in -10 °C and Span 80 was substituted through the addition of hexane with phosphatidylcholine, cholesterol and sterylamine in the molar ratio proportion of 5:5:1. The organic solvent was removed and water was added to the system to maintain the giant vesicles^[97].

Another approach to produce lipid vesicles was the formation of double emulsion [water in oil in water] in consecutive flow focusing on order to generate droplets with small variation of size^[66]. The subsequent step was the production of vesicles through the extraction of the solvent. The diameter of vesicles was between 20-110 μm . The lipid solution used was DOPC [1,2-dioleoyl-sn-glycero-3-phosphocholine] dissolved in oleic acid. The inner aqueous phase was water with Pluronic as a surfactant and the external aqueous phase was composed by glycerol, Pluronic, ethanol and water. The authors showed that the flow rates variation changed the vesicles diameter. The devices used produced vesicles with controlled diameter and also efficient encapsulation of bioactive reactants^[66]. Furthermore, gold and silver nanoparticles can be obtained using emulsion microfluidic techniques. The

microdevices prepared with PDMS were chemically treated with the fluoropolymer poly[1H,1H,2H,2H-perfluorodecyl acrylate-co-ethylene glycol diacrylate] to prevent that only the oil phase wets the channels. The microchannels geometry was like multiple T-injection. The configuration promoted mixing between the metal salts and ionic liquid reducing agent. It was used as continuous phase fluorocarbon oil. The aqueous inlets were: solutions of H₂AuCl₄/1-methylimidazole [first inlet] and 1-butyl-3-methylimidazolium borohydride as also denoted as BMIMBH₄ [third inlet] both dissolved in 1-butyl-3-methylimidazolium bis[trifluoromethylsulfonyl] imide [BMIMTf₂N]. Between these two inlets pure BMIMTf₂N buffer stream was injected to avoid the diffusion between the two other streams [second inlet]. The authors found optimal flow rate conditions being for the continuous phase 10 mL/h and for the disperse phase being 0.5 mL/h. Spherical gold nanoparticles was found to have \approx 4.28 nm, moreover the same approach was used to prepare silver nanoparticles with \approx 3.73 nm [98].

Nano/microparticles are used as non-viral vector for gene delivery [99]. In the literature few works report complexation between non-viral vectors and nucleic acid using droplets microfluidic techniques [7,99]. Picolitre microfluidic reactor and incubator system [99] was used for the controlled formation of lipoplexes with cationic lipid and DNA. The oil phase used was mineral oil and the surfactant was Sorbitan Monooleate at 0.4% [v/v]. Lipofectin, which was used as cationic lipid, and plasmid DNA were diluted separately in reduced serum medium. After that, cationic lipid and DNA were introduced in different inlets in the microchannel. The narrow region of two aqueous phases meets the oil phase was called by the authors as pinch-off point and the droplets were formed. In order to cause the complete mixture of the reactants, the droplets entered in a serpentine channel to cause chaotic advection. Chaotic advection is characterized as the stretching and folding of the fluids repeatedly in order to form thinner fluids layers to causes fast diffusion between the reactants [15]. After the serpentine region, the droplets flowed to self-splitting region in order to split the droplets and they were collected into an incubation region. The lipoplexes formed in microfluidic devices showed in terms of size narrower distribution if compared to the method prepared in bulk [formation of lipoplexes through vortexing or hand shaking] and transfection efficiency was also consistent [99].

The production of polyplexes formed between polymer and DNA was investigated by Grigsby and co-workers [7]. The authors compared the polyplexes prepared in traditional way with that prepared in microfluidic devices using droplet techniques. The polymer used was a poly [amido amine] and plasmid DNA or messenger RNA was used as nucleic acid. A microfluidic cross flow droplet generator was used with 4 inlets: 1 inlet with the oil phase containing FC-40 fluorocarbon oil and 2% of PEGylated fluorosurfactant, 1 inlet with the corresponding nucleic acid, 1 inlet [between nucleic acid and polymer] with buffer solution and the last inlet with the polymer. The droplets were produced in the junction channels. The flow rates were established as 7,5 ul/min in the oil phase and 2,5 ul/min in the aqueous phases. The droplets were collected were broken using properly reactants and the polyplexes were used straight from the aqueous supernatant. Experiments of physic chemical characterization and cellular investigation were conducted. The authors showed that through microfluidic technique size, zeta potential and polydispersity index decreased if compared with the bulk method. Furthermore colloidal and binding stability increased and transfection was improved in different cell types [7]. It is important to highlight that the formation of complexes between non-viral vectors and acid nucleic through microfluidic droplets techniques still must be extensively investigated.

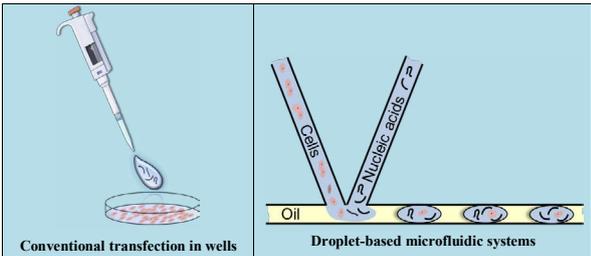
Droplet-based microfluidic platforms for *in vitro* transfection

Transfection, the procedure to introduce nucleic acids into cells, is used to study gene and protein function and regulation [100]. Thus, cells are commonly transfected in culture systems on well plates Then be inserted *in vivo* [101,102] or only be analyzed *in vitro* [103]. Briefly, there are three transfection methods: physical, biological and chemical, which are chosen according to cell type and purpose [100]. Physical-mediated methods are comprised of microinjection [104], biolistic particles delivery [105], electroporation [106], optical gene transfection [107], sonoporation [108] and magnetic nanoparticles [109]. Biological methods include those with virus as vector [110], chemical methods consist of lipid and polymeric vectors [111,112] and calcium phosphate [113]. Transfection method is chosen in order to have high transfection efficiency, with safety, reproducibility, low cytotoxicity and should be easy to use [100].

Although, when studying cellular uptake of nanoparticles for *in vitro* transfection, usually the physiologically relevant stimulus of fluid flow is overlooked and internalization assays are carried out under static conditions. But, both in theory [114] and practice [115], it has been shown that the mechanical stimulus of fluid flow is an important factor to achieve optimal transfection efficiency [116]. In conventional transfection methods performed in wells, there is only diffusive transport of nanoparticles to the cellular targets surface. In microfluidic systems, a convective contribution can be added to the process, increasing the collision rate between nanoparticles and cell surface, and consequently, showing more controllable and easier transfection [117].

Thus, droplet-based microfluidic devices can be used for *in vitro* transfection, in which one droplet defines a picoliter compartment that allows single cell encapsulation and *in vitro* expression of genes, using fewer reagents than conventional methods [8]. Microfluidic environment also offers unique possibility to mimic dynamic conditions, opening new possibilities to investigate. So, processes and techniques used in gene delivery, such as used in physical methods, can be better controlled and optimized in the micro-scaled environment [69]. To summarize, we made a comparative table showing differences between both, conventional transfection in wells and in microfluidic systems (**Table 1**).

Table 1. Comparative table summarizing differences between *in vitro* transfection by conventional transfection in wells and by droplet-based microfluidic systems.



	Conventional transfection in wells	Droplet-based microfluidic systems
Operation	Fast and easy	Slow (employ low flows) and require microfluidic knowledge
Reagents	Large quantity	Little quantity
Accuracy	Detect only large quantities of biomarkers	Able to detect little quantities of biomarkers
Transfection efficiency	Measured at the end	Real-time measurement
Nucleic acids transport to cells	Diffusion	Diffusion and chaotic advection
Analysis	Cell population	Single cell

Microfluidics, and more specifically, cell transfection inside microchannels for parameters analysis, are current topics. As a result, in the last decade, many patents involving these issues were filed. For example, cells can be merged in microfluidic devices developed for handle cell with electroporation and electrofusion ^[118,119] or they can be analyzed and sorted individually by measuring the signal of an optical-detectable molecule ^[120,121]. Thus, the integration of these steps can create a biological environment similar to those presented on macroscopic scale, but with advantageous engineering features of the microscopic format. Droplet microfluidics can provide a laminar flow behavior with heat and mass transfer phenomena improved, due to high surface area to volume ratios ^[10]. Furthermore, droplet-based microfluidic systems exhibit more sensibility for biomarker discovery than single-phase microfluidic systems or conventional methods, since they amplify the detection of extremely low levels of biomarker molecules, due to the very low volume around reagents inside droplets which restrain their dispersion ^[9,122]. Other advantages of droplet-based approach are the rapid mixing of reagents in which a chaotic advection is added to the mass transport by diffusion inside droplets ^[55,122], little or no cross-contamination between different compartmentalized samples. The compartmentalization reduce interaction of reagents from different droplets and let an easy parallelization of independent experiments without increasing device complexity or size, providing a very high throughput for these kind of devices ^[15].

From the point of view of the *in vitro* transfection, droplets in microfluidic systems can be applied as single cells compartment. Recently, some patents have been published in order to obtain inventions that can encapsulate cells one by one in droplets independent of cells densities ^[123]. Or also encapsulate cells in nested labeled matrices, which can be compared to cell tissues, enabling to track or identify different cell populations ^[124]. Thus, in a similar way to molecules assays, single cells analysis in this type of system allows a rapid detection of cell-secreted molecules, due to the low volume surrounding each encapsulated cell. Moreover, as the risk of cross-contamination decreases, cells can be analyzed by various techniques mainly by fluorescence detection ^[9]. Furthermore, fluorescence detection can be applied for both adherent and non-adherent single cells through using microfluidic devices in conjunction with optical systems, enabling the manipulation of isolated single cells and also the investigation of contents from disrupted single cells ^[125]. For example, Huebner ^[126] described the preparation of micro droplets containing few or individual cells, by detecting the expression of a fluorescent protein in the cell with simultaneous measurement of droplet size, fluorescence and cell occupancy. Equally important is the possibility to carry out spot-checks of single cells in the time courses, since the response of cell population at the end, not always describes the behavior of a single cell in certain times, which is often governed by an all-or-nothing principle ^[10]. This technology lets us to obtain more data about transfection characteristics, like the real point when transfection started and the ideal cell lines to be used in a transfection with a specific nucleic acid and transfection method. To verify the potential utility of this technique, Schmitz ^[127] used drop spots devices to monitor the levels of β -galactosidase in a population of single yeasts using the reporter enzyme fluorescence technology and also to monitor yeast growth rates. Boedicker ^[128] showed another important application for this technique: rapid detection and drug susceptibility screening of bacteria in samples without pre-incubation that can likely generate rapid and effective patient-specific treatment of bacterial infections. They determined the antibiogram of methicillin-resistant *Staphylococcus aureus* [MRSA], an antibiotic sensitivity strain, against many antibiotics and measured the minimal inhibitory concentration [MIC] of the drug cefoxitin against MRSA and also signalized sensitive and resistant strain of *S. aureus* in complex samples, like human blood plasma ^[128].

On the other hand, hydrogel droplets can be used to mimic *in vitro* multicellular organisms providing a 3D microenvironment for adherent and non-adherent mammalian cells. In contrast to vast majority of *in vitro* cell biology studies, which are performed using cell monolayers cultured on flat substrates, 3D microenvironment recapitulates the architecture of living tissues and involves a dynamic interplay between biochemical and mechanical signals provided by the extracellular matrix [ECM], cell-cell interactions and soluble factors ^[129,130]. These hydrogel droplets, composed by natural or synthetic polymers, should provide cells attachment

and proliferation ^[131]. Adherent cells need relatively resistant substrates, like collagen, to anchor and pull on their surroundings, since they are dependent on myosin-based contractility and cellular adhesion ^[11]. Moreover, hydrogels can be engineered to promote efficient gene transfer with non-viral vectors ^[12] and can be also used to investigate cellular pathways to internalize nanoparticles ^[132]. Dhaliwal ^[12] identified extracellular matrix [ECM] proteins and their combinations as a microenvironment that significantly enhances mouse mesenchymal stem cells [mMSCs] transgene expression. They showed that proteins that enhanced cell proliferation and spreading increased cell gene expression as well ^[12]. Additionally, studying transgene expression pathway, Dhaliwal ^[133] showed that RhoGTPases, which mediate the crosstalk between cell and fibronectin [Structural component of ECM], regulate internalization and effective intracellular processing of Nano vectors resulting in efficient gene transfer.

However, there are some drawbacks that have to be taken into account before encapsulate cells, particularly when using robust analysis over longer periods of time, like how to maintain viability of cells within the droplets, risks of coalescence, nutrient depletion or the accumulation of toxic metabolites ^[9]. Thus, to minimize these obstacles by preventing droplet coalescence, biocompatible surfactant-oil formulations that allow oxygen diffusion and prevent molecules leaking out into the oil phase have been developed ^[134-139]. Clausell-Tormos ^[134] demonstrated the feasibility of using this strategy for long periods by culturing adherent and non-adherent mammalian cells into aqueous micro drops of water-in-oil emulsion systems for up to two weeks, applying the technique of spot-checks to analyze cells viability in the device and also after controllably breaking the emulsions to recover cells after a couple of days.

In the case of microfluidic droplets, that involves immiscible phases, other important topics to be considered are the dominant physical mechanisms related with microfluidic scales, such as surface tension and diffusion. Some research groups ^[140] explore these parameters, coupled with quantitative measurements within droplets for the purpose of advance biological science and technology. Hence, in order to keep a droplet stationary for long term observation and to provide combinatorial measurements on a single image, Fradet ^[141] combined rails and anchors in microfluidic system with laser forcing, enabling the creation of highly controllable 2D droplet arrays. Additionally, in microfluidic droplet systems a more rapid mass transfer can be expected than in single-phase microfluidic systems, since the interfacial area ratio between different fluids per unit volume is larger ^[142]. Then, Abbyad ^[143] applied this rails and anchors design to control the transport oxygen for cell cultures or to induce a temporal or spatial variation of gas content in droplets, thus verifying the polymerization of intracellular hemoglobin by deoxygenating droplets that encapsulate red blood cells from patients suffering from sickle cell disease.

Furthermore, with microfluidic systems we can investigate how the shear stress induced by flow can affect the transfection efficiency. Shin ^[144] described a microfluidic device to investigate shear stress effect upon transfection efficiency of liposomes/DNA complexes in primary cultured neurons, achieving 45% of transfection. Particularly in droplet-based systems, the phenomenon of mass transfer diffusion is highly studied by computational ^[145-147] or experimental instruments ^[148-150], aiming to obtain an appropriate mixing [accelerate mass transport] inside droplets. For transfection applications, with Abbyad ^[143] system, where droplets can remain anchored even in the presence of a varied external flow, is possible to investigate the shear stress felt by cells according to the flow passed through the stationary droplets.

Droplet-based tools for single-cell analysis draw attention of many researchers for many different applications, especially *in vitro* transfection applications, mainly due to the potential of high-throughput screening and the benefits of enclosed individual microchambers ^[9]. Thus, we summarize in **Table 2** different methods to transfect using droplet-based microfluidic platforms, in which stationary single cells can be transfected in microfluidic devices.

Table 2. Description of different sources [electroporation, microinjection and nanoparticles] to provide single-cell transfection in droplet-based microfluidics platforms.

Transfection methods	Description
Electroporation	Delivery genes into cells by applying an external electric field, with lower voltages compared to Conventional electroporation ^[151,152] . ✓ Advantage: Higher transfection efficiency ✓ Disadvantage: Lower cell viability
Microinjection	Micro needles are used to inject nucleic acids and others bioactive compounds into the same target Single-cell ^[153] . ✓ Advantage: Quantitative introduction of multiple components into the same cell ✓ Disadvantage: Technical skills are required to prevent cell damage
Nanoparticles	Nanoparticles as non-viral vectors for safely and Reproducible gene delivery into cells ^[154-156] . ✓ Advantage: Higher cell viability ✓ Disadvantage: Lower transfection efficiency

In the specific case of using droplet-based microfluidic platforms for *in vitro* transfection, single cells are placed in contact

with nucleic acids, and physic stimulus or non-viral vectors are taken into account to increase transfection efficiency. Even though some systems use two immiscible phases [oil and aqueous phase] to generate droplets, the nonconductive property of oils delays physic stimulus action upon cells ^[157], that is because some research groups prefer to use geometric artifices, like chambers ^[158] and micro-holes ^[159-162], to trap single cell in certain points of device. In particular, optical stimulus uses a transient laser field that increases cell membrane permeability allowing the nucleic acid injection inside cells, in a process called optical transfection ^[163]. Nevertheless, this kind of optical apparatus allow optical injection and physical manipulation of cells with the same laser source; in other words, you can hold cell by laser force and transfect cells one by one even without a droplet compartment ^[163]. More generally, Chang ^[164] invented microfluidic droplet-based devices for biological applications, including operations involved in synthetic biology like cloning genes and transfecting cells.

The most commonly transfection in microfluidic devices is the electrotransfection, that is a method for delivering genes into cells by applying an external electric field, that, due to the compartmentalization, require much lower voltages compared to conventional electroporation, thus enabling a higher cell viability ^[69]. Zhan ^[151] demonstrate a simple microfluidic device that encapsulates cells into aqueous droplets in oil flow and then electroporates the encapsulated cells; the results show an enhancement in delivering green fluorescent protein [EGFP] plasmid into Chinese hamster ovary cells [CHO-K1], reaching approximately 11% of transfected cells. Thus, to demonstrate the potentiality of this transfection method also in other cells, Luo ^[152] showed that fluorescein could be introduced into yeast cells by applying a low alternating current voltage to a couple of Au microelectrodes.

Additionally, microinjection can be used to transfect cells in air-liquid droplet systems, enabling the introduction of multiple bioactive compounds, including nucleic acids, into the same target single-cells, which are difficult to handle in conventional methods ^[165]. Thus, Lee ^[153] showed the feasibility of a microinjector by engaging a microvalve in a microfluidic device to form a droplet at the microneedle tip allowing, for example, gene delivery in single-cells.

In a similar way, nanoparticles, generally composed by biopolymers or lipids, can be used as non-viral vectors for safely and reproducible gene delivery into cells. Oftentimes, hydrodynamic effects, governed by mechanical and geometrical variations around fluid, interfere in microfluidic droplets devices containing nanocomplexes to enhance gene transfer ^[69]. Hydrodynamics can use flow of fluids in curved paths and in long capillaries to improve mixing inside droplets, and if the geometrical dimensions are reduced, the fluids flow with high pressure, rising the shear stress ^[55,69,166]. Similarly to Shin ^[144], Chen and co-workers ^[154] transfected CHO-K1 cells with a plasmid encoding EGFP, but in this case, it was conducted with a chemical stimulus, PolyFect [activated-dendrimers] complexed with pDNA, reaching 25% of transfection efficiency. We highlighted two relevant points shown in the referred work, the first important point is the necessity to use fluorocarbon oils as continuous phase due to their biocompatibility with cells and smaller loss of nanoparticles from aqueous to oil phase, and the second point is that smaller droplets provide an increase in efficiency transfection probably because of a better interaction between cell and complexes ^[154]. Likewise, Hufnagel ^[155] transfected CHO-K1 cells with the same reporter gene, pEGFP, but using polyplexes as nanovectors, achieving 20% of transfection efficiency. Even though, this work approaches a modular microfluidic system that allows several operations with cells inside it, such as seeding, cultivation, manipulation, detachment, collection, encapsulation and transfection ^[155]. Finally, Lee and Hsieh ^[156] invented a microfluidic droplet device where is possible to complex nucleic acids with liposomes and transfect cells at the same time. They transfected pDNA encoding GFP into U2OS cells, reaching nearly 50% of transfection rate, which is much higher than transfection by bulk methods; thus demonstrating the potentiality of these systems in gene delivery applications ^[156].

Current and Future Developments

The miniaturization of systems allows better control in reactions, minimizing the amount of reactants used compared with traditional methods. The droplet methodology provides very small polydispersity, however particles and their complex (particles with nucleic acids) are produced in microscale range. In this way, many efforts must be made in order to transpose already reported traditional methods of nanoparticles production to microfluidic systems. The challenges of microfluidic techniques in general are in increasing scale.

Microdroplets systems for cells transfection is a promising future, since many parameters can be investigate by them in order to approximate to *in vivo* transfection conditions. For example, droplets can be submitted to a shear stress providing a convective contribution to be added to the diffusive transport of nano-vectors to cells, like occurs when complexes are inserted in certain tissue types or specific parts of the vasculature. Moreover, hydrogel droplets can provide a 3D environment for cells comparable to the architecture of living tissues, allowing to achieve cell behavior *in vitro* more similar to *in vivo*, considering biochemical and mechanical signals transferred.

Diseases with different causes, like cancer, generate huge difficulties in establishing a single formulation for all patients, thus requiring an individualized therapy. In this way, transfection through nanoparticles in microfluidic droplet systems seems

to be a more appropriate mechanism than using electroporation or microinjection, to insert nucleic acids into cells in order to investigate gene therapeutic strategies. Therefore, micro droplets systems are a powerful tool in preliminary assays to develop therapeutic vaccines for each individual, since they seem to lead to studies more similar to *in vivo* transfection and to producing monodisperse nanoparticles in large scale.

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