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Microfluidics-Directed Self-Assembly of DNA-Based Nanoparticles

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Abstract: The 'bottom-up' paradigm of nanofabrication mostly relies on molecular self-assembly, a process by which individual components spontaneously form ordered structures with emerging functions. Soft nanoparticles made up of therapeutic DNA condensed by cationic lipids or surfactants hold a great potential for nonviral gene delivery. Their self-assembly is driven by strong electrostatic interactions. As a consequence, nanoparticles formulated in bulk often exhibit broad size distributions not suitable for practical delivery applications. We will review the recent strategies we developed to control the self-assembly kinetics by using microfluidic devices. This combined approach may open attractive opportunities for the directed self-assembly of complex soft nanomaterials in particular for biomedical purposes.

Keywords: microfluidics; self-assembly; DNA; nanoparticle; nonviral gene delivery

Nadzorovano samourejanje DNA nanodelcev na osnovi mikrofluidike

Izvleček: Paradigma nanoizdelave „od spodaj navzgor" (angl. 'bottom-up') v glavnem temelji na molekularni samosestavljanju oziroma samourejanju, to je procesu, s katerim posamezne komponente spontano tvorijo urejene strukture s specifičnimi funkcijami. Mehki nanodelci, sestavljeni iz terapevtskih DNK, dobljenih z metodo kondenzacije kationskih lipidov ali površinsko aktivacijskih snovi (surfaktantov), predstavljajo velik potencial za nevirusno dostavo in vnos genov. Njihovo samourejanje je posledica močne elektrostatične interakcije. Posledica tega je, da imajo nanodelci, ki s samourejanjem tvorijo kompleksne strukture, pogosto široko porazdelitev velikosti, kar pa ni vedno primerno za praktične aplikacije. V članku je podan pregled razvoja novih strategij za nadzorovan proces kinetike samourejanja s pomočjo uvedbe mikrofluidnih pristopov, s katerimi lahko odpravimo zgornjo pomanjkljivost. Predstavljeni novi kombinirani pristopi omogočajo kontrolirano samo-sestavljanje kompleksnih mehkih nanomaterialov, zlasti primernih za biomedicinske namene.

Ključne besede: mikrofluidika; nanodelci; samourejanje; DNA; nevirusni vnos genov

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1 Introduction

Microfluidics is a developing field with applications covering tissue engineering [1-3], cell analysis [4-7], drug discovery [8-9], bioassays [10] and chemical synthesis [11-15]. Technology has arrived at a stage where it is now possible to handle and to shape the molecular constituents of matter with nanometer accuracy, whether they are inorganic or biological. As George M. Whitesides put it [16], "the physical sciences offer tools for synthesis and fabrication of devices for measuring the characteristics of cells and sub-cellular components, and of materials useful in cell and molecular biology; biology offers a window into the most sophisticated collection of functional nanostructures that exists." Two paradigms have emerged for the fabrication of nanometer-scaled materials: the 'top-down' approach – widely used in the microelectronics industry through lithography – enables to pattern bulk materials such as silicon with features size down to one nanometer and with high batch-to-batch repeatability. The technol-

ogy is limited so far to two-dimensional structures at the surface of a substrate. The 'bottom-up' approach in turn has been successfully exploited by nature to build up the most complex systems with high throughput, namely, living organisms. The limitation mostly arises from our inability to tune the interactions between constituents in such a way that they self-assemble into desired structures in a repeatable manner. Our present scientific knowledge gives us access to only a small set of architectures and functions, while nature has benefited from billion years of evolution to learn how to make the most elaborate devices such as the human brain with a low error rate. A third paradigm is subsequently emerging and consists of combining the two others. In other words, it aims at fabricating complex three-dimensional structures via self-assembly with high reproducibility.

DNA-based nanoparticles are such complex structures and hold a great potential in medicine. Their architecture and their function are inspired from virus in the sense that they carry genetic information encoded in compacted nucleic acids – either DNA or RNA – in view of its delivery into target cells [17]. As a matter of fact, a number of viruses have been engineered in such a way that they deliver therapeutic genes with the efficiency of a viral infection. Indeed, the regular function of a virus is to inject its genes into an infected cell, which will then express viral proteins and nucleic acids to make up new viruses. The strength of viruses is that they can circulate inside an organism while being not recognized by the immune system and targeting specific cells. However, they can induce inflammatory responses and provoke cancer through uncontrolled gene insertion. By contrast, nonviral vectors are safer and more versatile than engineered viruses, even though their efficacy is still insufficient. The objective of nonviral gene delivery [18] is therefore to devise nanometer-scaled synthetic particles containing nucleic acids to deliver into specific cells with high efficacy. The particles must be nontoxic, easy to fabricate, and with excellent batch-to-batch repeatability.

This article reviews our recent works on the self-assembly of DNA-based nanoparticles for use in nonviral gene delivery. It shows in particular how the third paradigm of nanofabrication can be used through different microfluidic strategies to produce surfactant-DNA nanoparticles with a good control on their morphological properties.

2 Supramolecular structure: the case of lipid-DNA nanoparticles

The architecture of simple viruses consists of the genome encoded in nucleic acids, which are compacted and protected inside a protein shell called the capsid. Remarkably, the capsid alone [19] or the capsid with genome [20] can self-assemble *in vitro* from purified components. Nonviral DNA-based nanoparticles try to mimic this architecture. Likewise, they result from a self-assembly process, which is driven by a delicate balance between weak (H bond, hydrophobicity, entropic effects) and strong (electrostatics, van der Waals forces) noncovalent interactions [21]. DNA is a negatively-charged polyelectrolyte and undergoes a coil-globule transition upon the addition of positively-charged agents, which can be synthetic polyelectrolytes, peptides, lipids or surfactants. This compaction process can be further enhanced by attractive interactions between positively-charged agents via hydrophobic forces as is the case with the alkyl chains of lipids and surfactants. Resultantly, the self-assembly of such DNA-based nanoparticles is driven both by electrostatics and by hydrophobic interactions, and it can give rise to a rich phase diagram.

Lipids have played an important role in nonviral gene delivery because they are the main constituents of cell membranes. A lipid-based vector has thereby the ability to fuse with the membranes of host cells and to release efficiently its DNA. Lipids are organic molecules made up of a hydrophilic charged head and a hydrophobic alkyl tail [22]. When dispersed in water, they self-assemble into 4~5 nm-thick bilayers in such a way that the alkyl tails are protected from the aqueous environment. At high volume fractions, the bilayers become stacked into a lamellar phase denoted L_a. More importantly, when cationic lipids are mixed with DNA, they form nanoparticles with local liquid-crystal order. Depending on the shape of the lipid molecule, i.e., cylindrical or conical, we mostly observe complexed lamellar L_a^C and complexed inverted hexagonal H_{II}^C phases [22]. The L_a^C phase consists of alternating monolayers of DNA rods and lipid bilayers. In the H_{II}^C phase, DNA rods are coated by a lipid monolayer and arranged on a two-dimensional hexagonal lattice. Very interestingly, lipid-DNA nanoparticles in H_{II}^C phase transfer their DNA to cells much more efficiently than those in L_a^C phase. However, cationic lipids are toxic to cells because they interact strongly with the negatively-charged membranes and disturb their biological functions. An alternative option is to use natural anionic lipids associated with DNA via multivalent cations [23]. The cations, in weak amounts, are intercalated between lipids and DNA [24], and the complexed lamellar and inverted hexagonal phases are recovered. The transfer efficiency of DNA is similar to that obtained with cationic lipids but the toxicity level is significantly lower.

At large scale, lipid-DNA nanoparticles exhibit a certain degree of disorder. When cationic lipids and DNA are

mixed manually in a test tube, the typical size of the resulting nanoparticles ranges from 30 to 500 nm and each nanoparticle contain plenty of DNA chains. Cryo-transmission electron microscopy images of H_{II}^{C} lipid-DNA nanoparticles revealed a local hexagonal packing of DNA [25]. However, we could also see striations, which were hexagonal bundles of DNA bent under the collapsing effect of hydrophobic interactions, and which suggested that DNA bundles took different orientations within the nanoparticles (Figure 1).

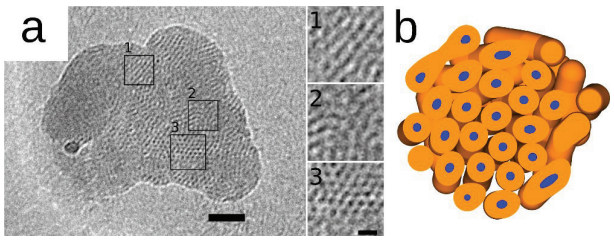

Figure 1: (a) Cryotransmission electron micrograph of a single lipid-DNA nanoparticle. The scale bar of the large view is 50 nm and that of the magnified views is 10 nm. (b) Cross-section of a coarse-grained model of a lipid-DNA nanoparticle calculated by Monte Carlo simulation. DNA is represented in blue and lipids in orange. Adapted with permission from [25] and [26]. Copyright 2011-2012 American Chemical Society.

The morphological properties of lipid-DNA nanoparticles affect their transfer efficiency. Large particles (>200 nm) cannot penetrate deeply into tissues and are less prone to be internalized into cells by endocytosis. Besides, high degree of local order is related to large internal energy and to thermodynamic state close to equilibrium. As a result, the nanoparticles are very stable and do not release their DNA readily inside the host cells. The transfer efficiency is therefore low. This trend is generic and was reported also with polyelectrolyte-DNA nanoparticles for which small size and internal disorder yielded high transfer efficiency [27].

3 Control of the mixing kinetics by hydrodynamic flow focusing

DNA-based nanoparticles with large size are not suitable for *in vivo* gene delivery for three reasons [28]: (i) they have poor circulation properties and are easily recognized by the immune system; (ii) the hydrodynamic and shear forces are greater and subsequently work against attachment to cell membrane; and (iii) they cannot penetrate deeply into tissues. Furthermore, high polydispersity of nanoparticle size gives rise to nonrepeatable results. Consequently, there is a need to develop methodologies enabling to control finely the morphology and the size distribution of DNA-based nanoparticles. Since the self-assembly process involves molecules interacting at the nanoscale, microfluidic devices are well suited for controlling the kinetics of mixing between DNA and condensing agents. Through the control of the mixing kinetics, the size distribution of the resulting nanoparticles can be tuned with a better flexibility than manually in bulk (Figure 2).

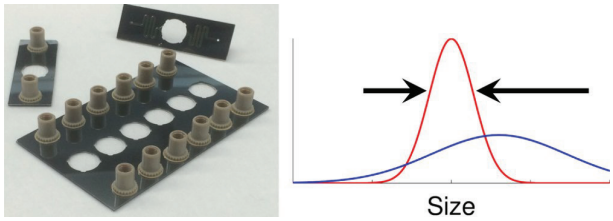

Figure 2: Illustration on the use of microfluidic devices (left) for the control of the size distribution of DNA-based nanoparticles (right).

In a seminal article, Johnson and Prud'homme [29] demonstrated that the time for a solution of copolymer in a good solvent to be mixed with a poor solvent, could control the diameter of the resulting micelles. More precisely, they reported that when the mixing time τ_{mix}, which is the typical timescale for homogenizing the solvents, was shorter than the aggregation time τ_{agg}, which is the average time for a copolymer molecule to diffuse and bind to another one, the diameter of micelles was minimal. Above τ_{agg}, the diameter increased as a power law of τ_{mix}. τ_{agg} was around 40 ms and to achieve mixing times smaller than this value, the investigators used a turbulent mixer. For applications involving DNA or other fragile macromolecules, turbulent mixer is not suitable because the applied shear stress is so strong that it tears apart the molecules and breaks them into small pieces. That is why Karnik and coworkers [30] used hydrodynamic flow focusing in a microfluidic device to achieve millisecond mixing times. The principle is depicted on Figure 3: a central stream containing copolymer is focused by two lateral streams of poor solvent. As a result, the poor solvent diffuses through the focused central stream within a timescale that can be tuned through the flow rates. Assuming that the fluids are incompressible and the flows laminar, the mixing time can be approximated by [31]

$$\tau_{mix} \approx \frac{w_o^2}{9(1+R)^2 D_s} \quad (1)$$

where $R=2Q_B/Q_A$ is the flow rate ratio and D_s the diffusion coefficient of the poor solvent or of the molecules to mix. In a microfluidic device, the width of the outlet stream w_o can be typically 60 µm or less, the flow rate ratio R is at least 10 for a good focusing effect and D_s, in the case of pure water, is 10^{-9} $m^2.s^{-1}$, which yields a mix-

ing time of 3.3 ms. As a rule of thumb, the aggregation time can be estimated from the diffusion-limited reaction rate between the associating molecules,

$$\tau_{agg}^{-1} \approx 16\pi\rho D R_H \quad (2)$$

where r denotes the density of the molecules, D their diffusion coefficient and R_H their hydrodynamic radius. The product of the two last quantities is given by the Stokes-Einstein relationship, i.e., $DR_H=k_BT/6\pi\eta$, with k_B the Boltzmann constant, T the temperature, and η the viscosity of the solvent. For molecules at a density of 10^{19} m^{-3} dispersed in pure water (η≈ 1 mPas at 20 °C), the aggregation time is around 9 ms.

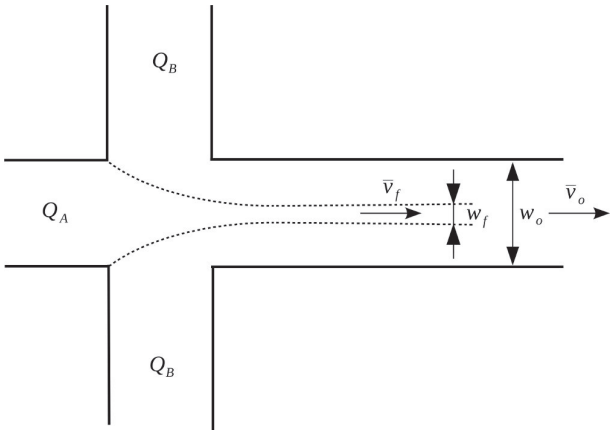

Figure 3: Schematic illustration of hydrodynamic flow focusing in a microfluidic device. Q_A and Q_B are the flow rates of the central and lateral streams respectively, w_f and w_o denote the width of the focused and outlet streams, and v_f and v_o are the average flow velocities in the focused and outlet streams. Adapted with permission from [31]. Copyright 2014 American Chemical Society.

We have exploited hydrodynamic flow focusing for the self-assembly of DNA-based nanoparticles. Unlike copolymers in poor solvent, the association of DNA with condensing agents is driven by strong electrostatic interactions, which, in bulk, lead to kinetic traps and metastable states with broad size distributions of nanoparticles. The microfluidic strategy ensured homogeneous electrostatic attractions at the mixing interface between DNA and condensing agents in addition to a good control over the mixing time. We designed and fabricated a series of microfluidic devices with different layouts in order to achieve either a rapid or a slow mixing. The device structure was generic and is depicted on Figure 4. We opted for a combination of glass and silicon rather than poly(dimethylsiloxane) (PDMS) because the channels were thus hydrophilic, which minimized the nonspecific interactions with the alkyl chains of condensing agents. The microfluidic structure was patterned in a silicon die by deep reactive ion etching and the channels were sealed by bonding a glass die on the top of the silicon die. Prior to sealing, a 150 nm-thick SiO_2 layer was thermally grown on the silicon so as to produce a hydrophilic surface. The flow rates were adjusted by a MFCS-FLEX pumping system (Fluigent, France) equipped with a mass flow controller for each channel.

The principle was validated on the self-assembly of cationic surfactants (dodecyl trimethylammonium bromide; DTAB) with semi-flexible anionic polyelectrolyte (sodium carboxylmethylcellulose; carboxyMC) [32]. Numerical calculations solving the Cauchy equation of motion in three-dimensional geometry confirmed that the width of the focused stream scaled as $(1+R)^2$ as predicted analytically. Instead of focusing the central stream from the two lateral sides, we also tried to focus it from only one side. In that case, the mixing time varies differently with the flow rate ratio and we can demonstrate that it scales as $\tau_{mix} \infty R^{-1}$. Therefore, we carried out microfluidic-directed self-assembly of DTAB-carboxyMC nanoparticles in the two configurations, with carboxyMC flowing in the central stream and DTAB flowing in the lateral streams. Remarkably, we observed that the nanoparticle sizes were systematically smaller when the central stream was focused from two lateral sides, which was in good agreement with the fact that the mixing time was much shorter for any given R. Unfortunately, this method failed to compact efficiently DNA and the nanoparticle sizes were always larger than 100 nm. This was due to the fact that the lin-

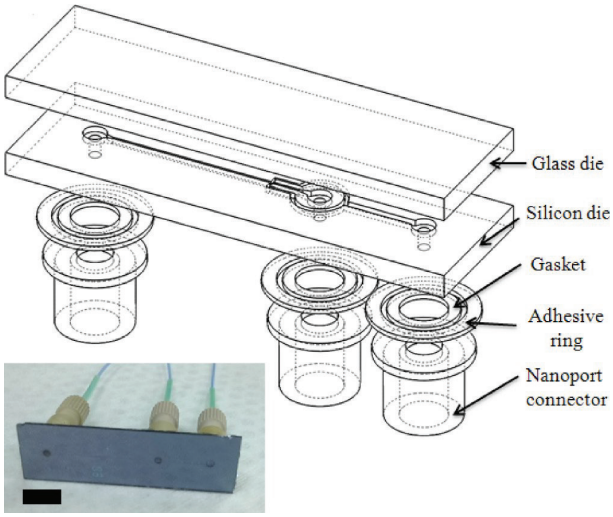

Figure 4: Microfluidic device for hydrodynamic flow focusing with an exploded view showing the various parts made in a combination of glass and silicon. The photograph shows the bottom of the device. The scale bar is 1 cm. Adapted with permission from [32]. Copyright 2013 American Chemical Society.

ear charge density of DNA is more than twice as large as that of carboxyMC. The surfactants were strongly attracted by DNA and the aggregation time was consequently shorter than in the case of carboxyMC. As a result, the process gave rise to large nanoparticles with uncontrolled size distribution.

4 Towards monomolecular DNA-based nanoparticles

Consequently, we adopted an alternative method: since the aggregation time was reduced with DNA, we had to find a way to shorten further the mixing time. The diffusion coefficient *Ds* appearing in Equation 1 is that of the solvent or of the molecules in the lateral streams. When DNA was compacted by surfactants in the lateral streams, τ_{mix} was a few tens of milliseconds because surfactants diffused slowly through the focused stream ($Ds \sim 10^{-10}$ m^2/s). We therefore pre-mixed DTAB and DNA in 35% ethanol in such a way that surfactants were loosely bound to DNA without compacting it. Indeed, 35% ethanol is a good solvent for DTAB, which does not form micelles at our working concentrations (~1-10 mM). By rapid mixing with pure water, surfactant-bound DNA molecules collapsed into globules due to the change of solvent quality, just like the copolymers mentioned before [30]. Since the diffusion coefficient of pure water was an order of magnitude higher ($Ds \sim 10^{-9}$ m^2/s) than that of surfactants, we could achieve a mixing time of a few milliseconds. The nanoparticle size was generally below 100 nm for a broad range of DNA concentrations [31]. The polydispersity index measured by dynamic light scattering was lower than 0.2 and sometimes below 0.1, which indicated a good monodispersity of the nanoparticles. However, a monomolecular DNA-based nanoparticle, that is, which contains only a single DNA chain of a few thousands of base pairs, should be around 30 nm in size. This method was therefore not efficient enough to produce the smallest nanoparticles permitted in theory.

In the last approach, we proceeded by increasing dramatically the aggregation time [33]. Instead of associating rapidly DNA and surfactants, the two reactants diffused slowly through a stream of pure water (Figure 5a). As a result, they encountered each other almost one molecule at a time, as if they were in a very dilute regime. Nanoparticle sizes as small as 30 nm and with a polydispersity index below 0.1 were obtained as shown on Figure 5b. By raising the surfactant flow rate from 20 µL/min to 35 µL/min – the water flow rate being fixed at 50 µL/min – the nanoparticle size increased in an exponential manner. Similarly, we observed a very strong effect of the surfactant concentration: below 5 mM of DTAB, the nanoparticle size was smaller than 80 nm but at 7 mM, the nanoparticle size was close to 600 nm. These findings emphasized the sensitivity of the assembled nanoparticles on the initial conditions: a small variation of concentration can have dramatic effects on the morphology. They fully justify the use of elaborate methods based on microfluidics.

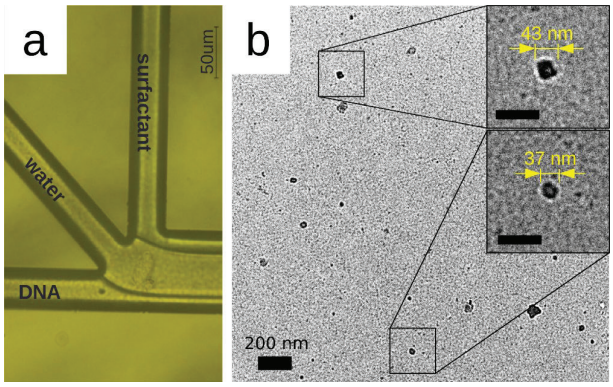

Figure 5: Assembly of DNA-based nanoparticles by slow diffusion. (a) Optical image of the microfluidic device. (b) Transmission electron microscopy images of DTAB-DNA nanoparticles. The scale bars of insets are 100 nm. Adapted with permission from [33]. Copyright 2015 American Chemical Society.

5 Conclusion

DNA-based nanoparticles play an important role in biomedical sciences as vectors for nonviral gene delivery. Their efficiency of gene transfer strongly depends on their morphological properties. In particular, small size allows them to diffuse deeply into tissues and not to be recognized by the immune system, while a narrow polydispersity ensures a good batch-to-batch reproducibility. Formulation in bulk does not respond satisfactorily to these criteria and elaborate strategies are therefore necessary to achieving a fine control over the size distribution.

If DNA-based nanoparticles result from a self-assembly process, further control can be obtained by using microfluidics, and accordingly, by taking advantage of the third paradigm of nanofabrication, which combines 'bottom-up' and 'top-down' approaches. Microfluidics enables to direct the self-assembly by tuning the convective-diffusive mixing of reactants at the nanoscale. The resulting objects are kinetically frozen and trapped in nonequilibrium state. They still evolve but over timescale sufficiently long (several days) with respect to the time required for a typical gene deliv-

ery experiment (several hours). Thereby, we devised a series of microfluidic devices based on hydrodynamic flow focusing, which allowed us to finely tune the mixing kinetics of DNA with surfactants. We managed to obtain surfactant-DNA nanoparticle size as small as 30 nm with a good monodispersity, which means that only one or two DNA molecules were packaged within each nanoparticle.

The microfluidics strategy is versatile and can presumably be applied to any complex soft nanomaterials. By following different kinetic pathways, we can access a wide range of states – albeit metastable – and produce nanomaterials with structures and functionalities that cannot be obtained solely at equilibrium. It also opens up the route to elaborate assembly schemes where multicomponent nanoparticles can be assembled sequentially within a microfluidic 'factory' on chip.

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