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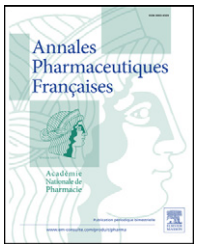

ORIGINAL ARTICLE

Interest of designed cyclodextrin-tools in gene delivery☆

Intérêt des outils cyclodextrines-modèles dans la délivrance de gènes

R.-E. Duval, I. Clarot, F. Dumarcay-Charbonnier, S. Fontanay, A. Marsura*

Laboratoire structure et réactivité des systèmes moléculaires, complexes UMR CNRS 7565, université de Lorraine, BP 70239, 54506 Vandœuvre-les-Nancy cedex, France

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SiRNA

Summary Cyclodextrins (CyDs) currently displays even today the image of a natural macrocyclic compound largely dominant in the formation of inclusion complexes with small hydrophobic molecules. During the past 10 years, advances in this field allowed to achieve more and more sophisticated CyDs derivatives opening a simple access in scale-up quantities to original and better CyD-based gene delivery systems. In addition, possibility to combine covalent and supramolecular approaches offers new venues for the design of tailor-made CyD-based nanovehicles to improve their transfection ability and gene transfer in cells. In this account, we describe our recent progress in the construction of a novel CyD-based G0 (generation number) core dendrimer, scalable to CyD oligomers by a strategy using protonable guanidine tethers and whose concept can be generalized for the assembly of CyD pre-coated dendrimers. The synthetic strategy based on an original Staudinger-Aza-Wittig tandem coupling reaction. We present an outline of the different analytical strategies to characterize CyD-ODN (cyclodextrin-oligodeoxynucleotide) complexes. Among them, Capillary electrophoresis (CE) was used to perfectly characterize our CyD-siRNA and CyD-DNA complexes and shown to be a very attractive method with advantages of low sample consumption, rapid analysis speed, and high efficiency that make this technology a major tool for association constant measurement. Finally, we present the different biological methods that can be used, in vitro, to study gene delivery, and more precisely ones we have performed to evaluate the capability of our original model bis-guanidinium-tetrakis-β-cyclodextrin dendrimeric tetrapod, to deliver efficiently DNA or siRNA in eukaryotic cells.

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* Corresponding author.
E-mail address: alain.marsura@pharma.uhp-nancy.fr (A. Marsura).

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MOTS CLÉS
Cyclodextrine ;
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Électrophorèse capillaire ;
Transfection ;
ARNsi

Résumé Les cyclodextrines (CyDs) présentent encore aujourd'hui l'image de composés macrocycliques naturels largement dominants dans la formation de complexes d'inclusion avec de petites molécules hydrophobes. La possibilité de combiner des approches covalentes et supramoléculaire offre de nouveaux espaces pour la conception de nanovéhicules sur base CyDs, taillés sur mesure visant à améliorer la capacité de transfection et de transfert de gène dans les cellules. Dans cet article, nous décrivons nos récents progrès dans la construction d'un nouveau dendrimère G0 (nombre de niveaux) à base CyD. Le concept choisi étant extensible à des analogues d'ordre supérieur pour l'ensemble des dendrimères de CyDs. La stratégie de synthèse est basée sur une réaction de couplage tandem *Staudinger-Aza-Wittig* originale. Par ailleurs, nous présenterons un aperçu des différentes stratégies analytiques pour caractériser les complexes supramoléculaires Cyclodextrines-OligoDesoxyNucléotides (CyD-ODN). Parmi elles, l'électrophorèse capillaire (EC), une méthode très attractive avec des nombreux avantages et utilisée pour la caractérisation de nos complexes CyD-siARN et Cyd-ADN. Enfin, nous présentons les différentes méthodes biologiques qui peuvent être utilisées, in vitro, pour étudier la transfection de gènes et plus précisément celles que nous avons utilisées afin d'évaluer la capacité de notre tétrapode dendrimérique à transférer efficacement l'ADN et un siRNA dans des cellules eucaryotes.

Introduction

Developing new, highly efficient, and nontoxic gene delivery carriers in cells remains even today an important challenge both in basic sciences and in clinical research fields. During the past 10 years, different nonviral approaches using macromolecular systems as carriers were conducted with more or less success [1]. There is currently an intensive effort to develop original and efficient macromolecular systems susceptible to achieve DNA or ODN (Oligonucleotide) high-level delivery and more recently siRNA in cells. To achieve this goal, two families of molecules are represented, on one hand nanoparticulate systems [2,3] and on the other hand polymeric systems [4,5]. Nonviral vectors (cationic polymers, lipids, dendritic polymers, polyplexes, etc.) have been studied as an alternative strategy to viral vectors for gene delivery because of their lower toxicity, non-immunogenicity, and convenient handling [6]. The fundamental disadvantage with them remains with their relatively lower transfection efficiency than viral vectors. This poor performance was also due to polyplex aggregation as a consequence of strong decreasing water solubility induced by charge neutralization arising in the association of a polyanion as DNA and the polycationic vector [7]. Some attempts to circumvent this problem have been proposed in the recent literature with the synthesis of polycation/DNA composite structures obtained by direct grafting of additional components as PEG (poly(ethylene glycol)) [8] or combination with adamantane-poly(ethylene glycol) (AD-PEG) conjugates [9]. Such modifications have partly succeeded in resolving the difficulties: stabilize particles in biological fluids at physiological salt concentrations, minimize the toxicity, enhance solubility of the polycationic polymers and increase transfection of polyplexes in cells.

Among the numerous and various approaches that have been used in the past decade to generate and improve gene delivery in cells, cyclodextrin-based gene transfer systems appeared as one of the most promising ones, this action is even recognized in the Biopharmaceutics Classification System (BCS) [10]. However, still, the design and synthesis of new cyclodextrin-based molecular systems remain a significant and ongoing challenge within gene delivery technology. This account focuses on up-to-date research on simple and rapid synthesis of cyclodextrins (CyDs) molecular systems and their application in the field of in vitro and in vivo gene delivery. The central theme is to highlight the important role played by CyDs, to structure the supramolecular architecture, to promote mild to strong host-guest interactions, to contribute to the global water solubility, to minimize as much as possible cytotoxicity of the vector. First, new achievements in developing different simple approaches for the synthesis of diverse cyclodextrin-based gene transfer supramolecular systems with a rich variety of shapes are summarized. Secondly, a key point also discussed here, is the characterization of the [CyD-based/DNA] supramolecular assemblies and determination of some major quantitative binding parameters as stoichiometry and dissociation constant between the biomolecular species and the CyD-based molecular system. The analytical strategy which leads to these parameters must be considered with great attention for allowing as much as possible an accurate estimation of the non-covalent interactions between biomolecule and CyD-based synthetic vector. Thirdly, considering the synthesis and the physicochemical characterization of the complexes are so many preliminary steps to design new vectors capable of better complex active principles, to increase the bioavailability, to improve the delivery; involve necessary stages for the implementation of a new gene therapy, but the most important results above all to confirm all the qualities of this vector are reached during in vitro experiments on eukaryotic cells followed by in vivo performance. Finally, we conclude with a look at the possible future challenges and prospects in the synthesis and application aspects of CyDs tools for gene delivery.

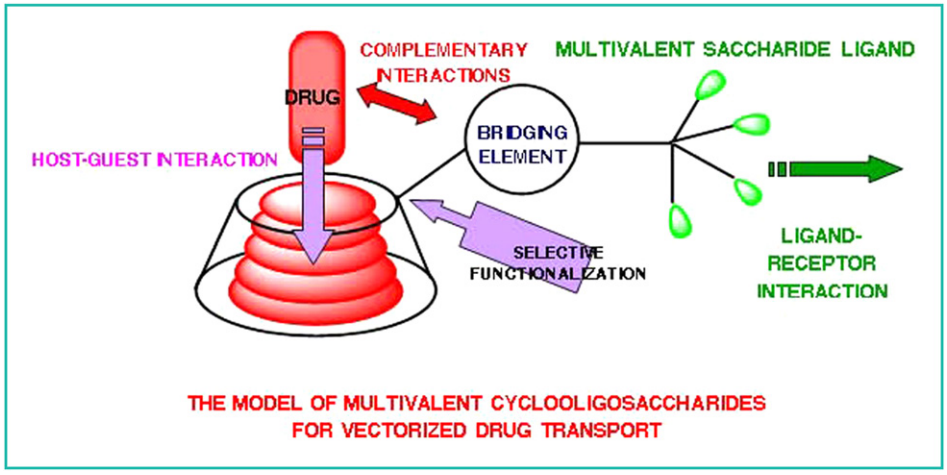

Figure 1. Supramolecular vector concept for drugs and gene delivery, A. Marsura, French Ministery project of research 1991.
Concept de vecteur supramoléculaire pour les médicaments et la délivrance de gènes, A. Marsura, Projet de recherche, Ministère de la Recherche 1991.

Strategies for nucleic-acids cell-transfer by cyclodextrins

Chemistry of cyclodextrins is known to play an important role in supramolecular chemistry field as earlier defined by J.-M. Lehn [11]. First discovered by Villiers in 1891, cyclodextrins (CyDs) are today industrially produced in tons from the biomass (starch) in pharmaceutical grade quality in low price. Cyclodextrins comprise a family of three well-known major cyclic oligosaccharides which are crystalline, homogeneous, nonhygroscopic substances. They are torus-like macrocycles built-up from 4C1 glucopyranose units (α-CyD = 6 units, β-CyD = 7 units and γ-CyD = 8 units) with a non-polar cavity and a polar surface. One fundamental property and advantage of the CyDs is that they readily form stable inclusion complexes with a wide range of small hydrophobic molecules. Above-mentioned properties enable the rational design and synthesis of CyDs molecular systems for nonviral carriers for nucleotides, ODN and DNA summarized in Fig. 1.

The pioneer work of Agrawal [12] in 1995 describes CyDs and their functionalized derivatives as potent carriers for phosphorothioates-ODN transferring. At this time, it was demonstrated that cellular uptake was concentration and time-dependent. Further, numerous contributions appear from that time in which native or modified CyDs were evaluated as efficient ligands for nucleotides, ODN and DNA [13—15]. Our first contribution on the subject in 1997 elects monothiogalactosyl and heptakis-thiogalactosyl-β-cyclodextrins to the status of novel nonpolymeric efficient carriers for small antisense-ODN [16] (Fig. 2).

In order to design valuable and superior CyDs derivatives to achieve or improve the transfer of nucleic-acids in cells, several ways have been explored. Considering the literature on the subject a review covering the most exciting reports regarding the use of cyclodextrins in nonviral gene vector design was published very recently [17]. On the light of literature results, we have chosen to investigate in 2007 a new concept of nonpolymeric CyD oligomer ''bottom-up'' synthesis (summarized in Fig. 3) having CyD cavities regularly distributed around a simple central backbone and including a pre-defined and adequate number of cationic guanidinium centres [18]. The structure was

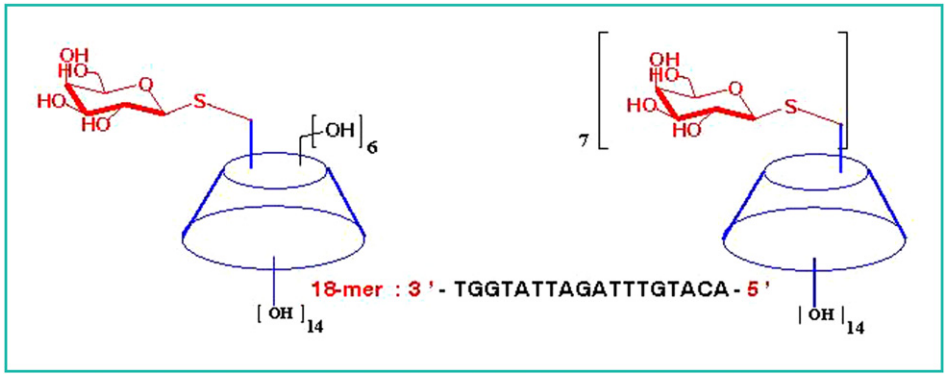

Figure 2. Synthesized monothiogalactosyl and heptakis-thiogalactosyl-β-cyclodextrins carriers for a bovine enteric coronavirus 18-mer intergenic concensus sequence.
Synthèse de transporteurs monothiogalactosyl et heptakis-thiogalactosyl-β-cyclodextrines pour une séquence concensus 18-mer du corona virus entérique bovin.

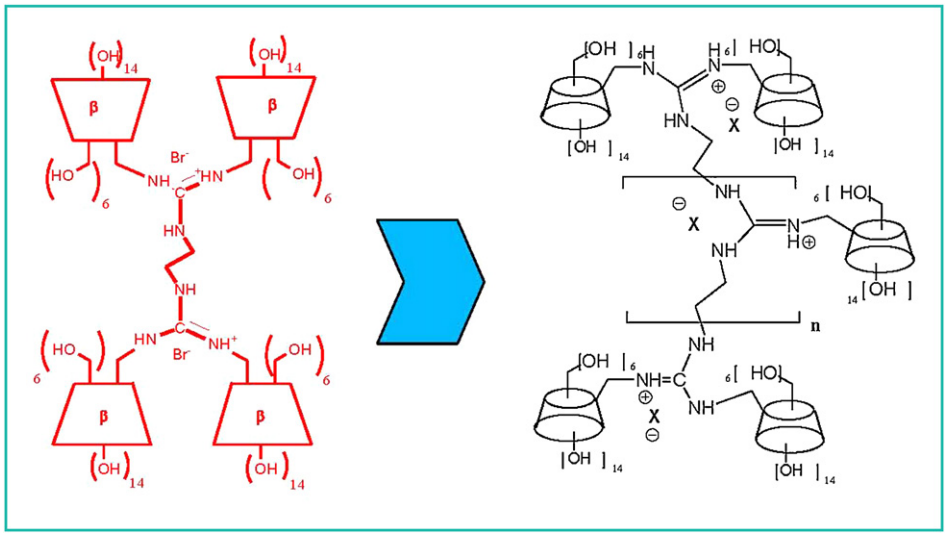

Figure 3. Model for nonpolymeric CyD oligomer development. In the following example presented, $n = 0$. The extra-cyclodextrin unit (n) was shown as the future devices possible extension.
Développement d'un oligimère de CyD non polymérique modèle. Dans l'exemple présenté, n = 0. Les unités cyclodextrines supplémentaires (n) sont indiquées comme possibilité future d'extention.

designed to induce three types of non-covalent interactions between the host (CyD) and the guest (nucleotide): electrostatic, hydrogen bonding and hydrophobic inclusion in CyD cavity. As a preliminary work was investigated in order to verify the three types of interaction reality are working with our model. Firstly, we find our ''phosphine imide'' strategy was an efficient easy way to obtain designed CyDs water soluble multipod in large scale, allowing a full control on the number of cationic centres and of CyDs cavities introduced in the final molecular skeleton [18]. Secondly, it was established that the tetrapod model was able to recognize the nucleotides AMP, ADP and ATP at a supramolecular level, combining host-guest hydrophobic inclusion of both ribose and nucleobase moieties into CyDs and strong electrostatic interactions between guanidinium sites and phosphate anions. At this occasion, an original complexation scheme was determined with the above-cited nucleotides as illustrated in Fig. 4. Toxicity studies clearly demonstrate the cellular harmlessness of the tetrapod at a high concentration [18].

The compound presented here is the first example of a new molecular family including future CyD linear oligomeric analogs (penta-, hexapod...), presently in progress. It could form 1:1 supramolecular water soluble complexes with single strands DNA and siRNA as shown by capillary electrophoresis (see chapter 2) and it was demonstrated a better association with siRNA instead DNA. Elsewhere, efficiency of siRNA and DNA transfection in cells was

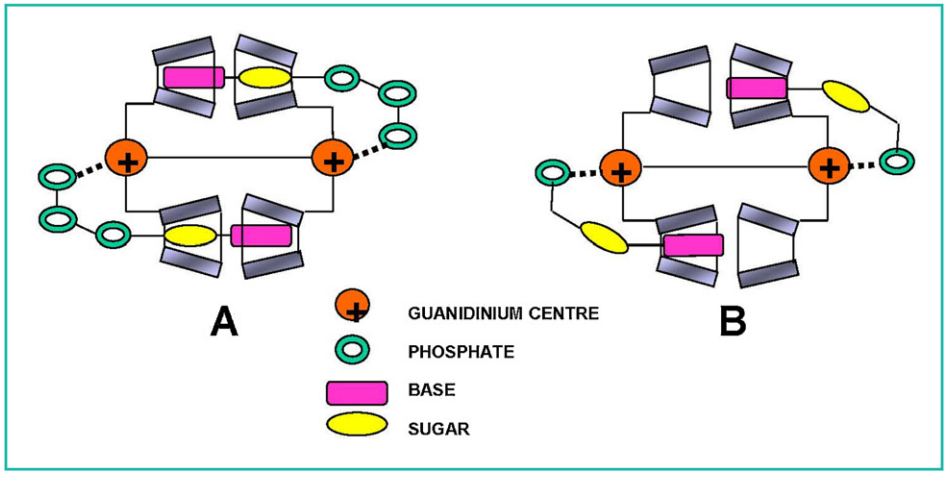

Figure 4. Graphic representation of the complexation scheme for the [2/1] complexes of nucleotides with the tetrapode: A, with 5′-ATP2Na; B, with 5′-AMPNa.
Représentation graphique du schéma de complexation pour les complexes [2/1] de nucléotides avec le tétrapode : A, avec 5′-ATP2Na ; B, avec 5′-AMPNa.

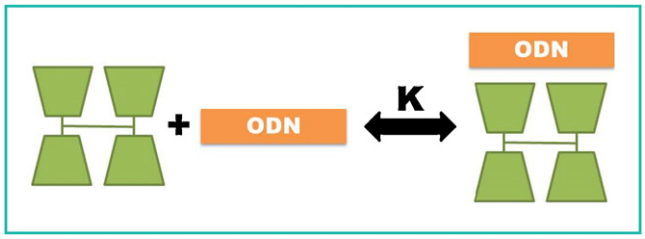

Figure 5. Illustration of the complexation process (Stoichiometry 1:1 and apparent formation constant K).
Illustration du processus de complexation (stoechiométrie 1:1 et constante apparente de formation K).

comparable to polyplex or polycationic existing systems. Toxicity experiments reveal that tetrapod is poorly and less cytotoxic than existing polyethyleneimine (PEI) cationic polymers.

Analytical strategies for oligonucleotide-cyclodextrin supramolecular edifices

Numerous analytical methods have been developed and applied in the study of the interactions between small ligands and biomacromolecules. Equilibrium dialysis or ultrafiltration has been widely used because of their simplicity. Spectroscopic methods such as fluorescence, NMR18, or microcalorimetric methods have been also applied [19]. In addition, some chromatographic methodologies, such as affinity chromatography or size exclusion could be employed to determine binding parameters. All these methods were based on the differences in the properties of bound and unbound ligands/analytes.

In this part of this account, we present our recent progress using capillary electrophoresis to describe a complexation process using ODN and an interesting bis-guanidinium-tetrakis-β-cyclodextrin tetrapod [18,20]. Capillary electrophoresis (CE) is an attractive method for such determination. Low sample consumption, rapid analysis speed, and high efficiency are advantages that make this technology a major tool for association constant measurement. Many reviews exist on the use of CE for the determination of binding parameters [21—23] and several CE modes are available for such measurement, such as affinity capillary electrophoresis (ACE), Hummel-Dreyer method (HD), vacancy affinity capillary electrophoresis (VACE), vacancy peak method (VP), frontal analysis (FA), or frontal analysis continuous capillary electrophoresis (FACCE). Measurement in the ACE mode is based on the change in electrophoretic mobility of the biomolecule due to complexation to the ligand added at various concentrations in the background electrolyte.

Surprisingly, very few examples deal with CyD/ODN using this simple and flexible instrumentation [20,24]. We recently show the interest of a CyD derivative (tetrapod CyD) for complexation with DNA or siRNA18, the formation of a 1:1 complex is illustrated in Fig. 5. ACE is the most simple and frequently used method. In this CE mode, the sample contains fixed amount of oligonucleotide, and the running buffer contains various amount of CyD. When

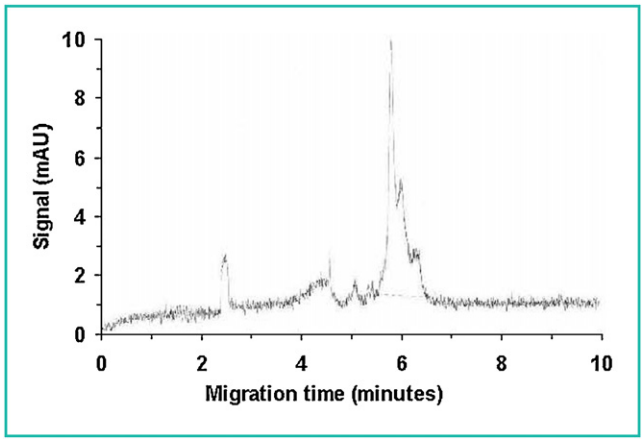

Figure 6. Electrophoregram of single strand siRNA (double strand open with temperature) with a background electrolyte (borate pH 9.3) containing 25 μM of CyD.
Électrophorégramme d'un siRNA monobrin (double brin dissocié par la température) avec un électrolyte de fond (borate pH 9,3) contenant 25 μM de CyD.

we assume that the stoichiometry of the formed complex is 1:1, the electrophoretic mobility of the injected analyte is shown dependent from the CyD concentration. The binding constant could be estimated using several linear least squares plotting methods (x-reciprocal, y-reciprocal or double-reciprocal plot).

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)\,K}\,\frac{1}{[CyD]} + \frac{1}{(\mu_c - \mu_f)} \qquad (1)$$

The double-reciprocal plot is known as the Benesi-Hildebrand plot in spectrophotometry. For 1:1 association, the change in solute mobility with changing ligand concentration is related to the following equation (Eq. (1)). Where μ_i is the experimentally measured electrophoretic mobility of the solute, μ_f is the mobility of the free (uncomplexed) solute, μ_c is the electrophoretic mobility of the solute-ligand complex, K is the binding constant, and [CyD] is the equilibrium ligand concentration.

The assumption is usually made that buffers do not interact with ODN but it is now well known that the effective charge of DNA is higher in borate buffers due to a possible complexation with borates.

Such mistakes could lead to the determination of a wrong binding constant value due to a complexation competitor in the background electrolyte. The determination of the association constant with phosphate and borate buffers (same pH) with a defined analyte, a single strand DNA primer for example, could avoid the borate error [20]. The tetrapod CyD was used with both DNA and siRNA (double strand and single strand) to show its ability to form stable complexes with oligonucleotides. An example of electrophoregram using a single strand RNA with 25 μM of CyD is illustrated in Fig. 6.

Without cyclodextrin in the background electrolyte (pH 9.3), the apparent mobility of the ODN anionic molecule (free) is depending from its own mobility and the electroosmotic flow. When the cationic CyD specie (due to the guanidino moiety) was added to the migration buffer, the formation of an association complex with ODN lead to a

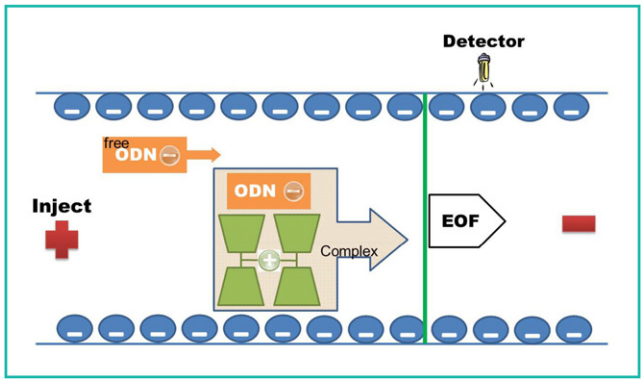

Figure 7. Schematic illustration of apparent motilities of a free ODN and a CyD/ODN complex inside the capillary with a borate pH 9.3 running buffer (EOF for electro-osmotic flow, injection at the anode on the left, cathode on the right).
Illustration schématique des mobilités apparentes d'un ODN libre et d'un complexe CyD/ODN dans un capillaire dans un tampon borate pH 9,3 (FEO pour flux électro-osmotique, injection à gauche à l'anode, la cathode à droite).

partial neutralization (less anionic) and finally to an increase of the corresponding apparent mobility. A schematic illustration of the capillary is shown on Fig. 7.

The increase of the complex electrophoretic mobility leads to a decrease of the migration time, as observed on Fig. 8, from 0 and 25 μM of CyD added. The apparent binding constant K was estimated by varying the ligand concentration at constant solute concentrations and fitting the data by linear regression to the Eq. (1).

When siRNA was used without temperature treatment (double strand not open), no modification of the migration time was observed indicating that the complexation process was not effective. When siRNA was opened with temperature (single strand), a complexation constant near 16,000 M^{-1} was obtained (Fig. 9) indicating the ability of our home made tetrapod to form strong association with such oligonucleotide. Identification of biomolecule interactions is crucial in understanding biological and biochemical

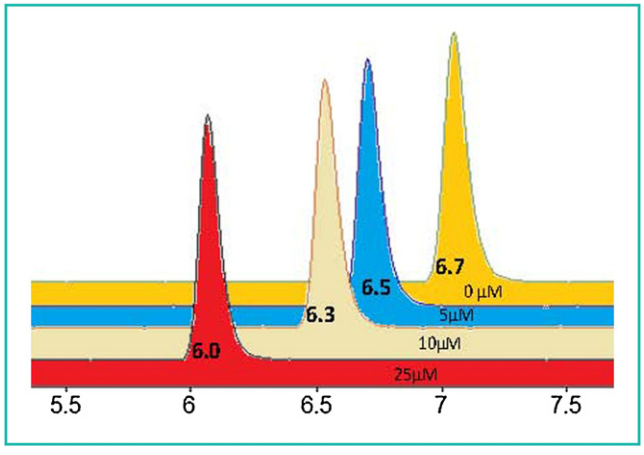

Figure 8. Schematic electrophoregrams of single strand siRNA (with a borate running buffer containing 0 to 25 μM of CyD).
Électrophorégrammes schématiques d'un siRNA monobrin (dans un tampon borate contenant de 0 à 25 μM de CyD).

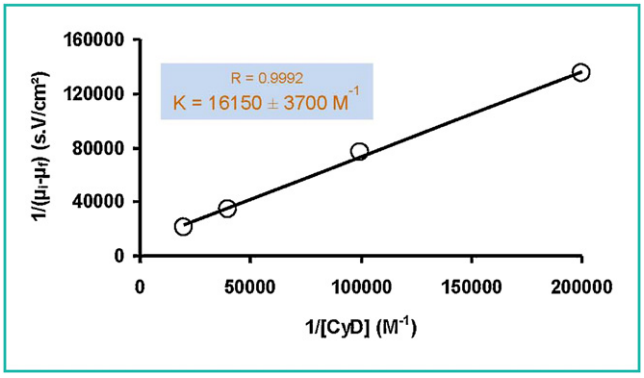

Figure 9. Application of Eq. (1) for single strand siRNA with [CyD] from 0 to 25 μM in borate buffer pH 9.3.
Application de l'équation (1) au siRNA monobrin avec une concentration en [CyD] de 0 à 25 μM dans un tampon borate pH 9,3.

processes. Capillary electrophoresis is demonstrated to be a simple and rapid analytical tool for binding constant evaluation. It is evidence that this technique is promising to a great future which must lead to miniaturization by the use of microchip CE [25]. Microfluidic technology (lab-on-a-chip) provides a means to improve performance. Adapting standard assays to the microscale assays might lead to enhanced speed, smaller required sample and reagent volumes.

Valuable biological approaches to study gene delivery in vitro

In this part, we present the different biological methods that can be used, in vitro, to study gene delivery, and more precisely ones we have performed to evaluate the capability of our original model, i.e. bis-guanidinium-tetrakis-β-cyclodextrin dendrimeric tetrapod, to deliver efficiently DNA or siRNA [18,20]. Before evaluating the delivery capabilities of your vector, the first question you may ask is: ''The vector which I use, can it be responsible for a toxic phenomenon?'' There are then two possibilities, either you use a commercial vector or already described in the literature (you can then take into account provider information's or previous published results), or you work on a completely new vector, and you have to make sure of its safety. To do so, two kinds of protocols exist: the first one allows you to study the cellular viability: the MTT assay, firstly described by *Mosmann* [26]; the other one allows you to study the cytotoxicity: here we can cited the Neutral Red assay, firstly described by Borenfreund and Puerner [27]. For both tests, one very large number of commercial kits is available, but you can also perform ''in-house'' protocols. Then, the second parameter which it is necessary to define is the cellular model. Indeed, at present, one very wide selection of eukaryotic cells is available, and it is crucial to choose a model easy to cultivate and of course adapted to the theme of research.

In our approach, we have performed the MTT assay and used MRC-5 cells [20]. The MTT assay is a widely used protocol, both in academic or pharmaceutical area, inexpensive, and less time consuming. The assay is based on reduction of

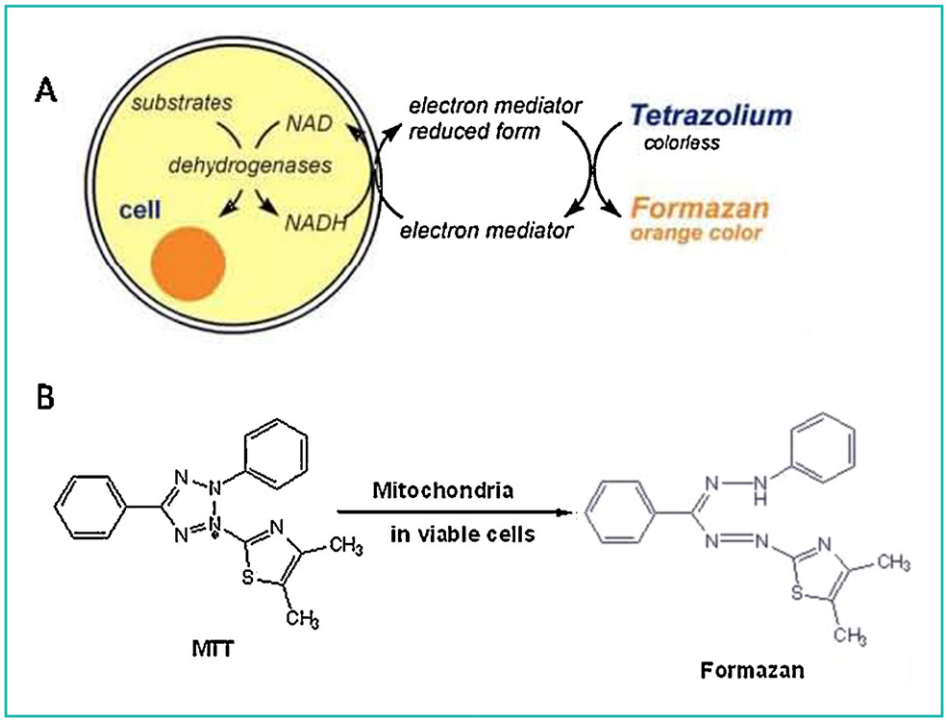

Figure 10. A. MTT assay principle. B. Chemical structures of MTT and Formazan.
A. Test MTT principe. B. Structures chimiques de MTT et du Formazan.
From: http://www.dojindo.com (A), http://www.mclab.com (B).

the tetrazolium salt MTT by active mitochondrial enzymes (i.e. succinate dehydrogenases) to produce an insoluble purple formazan salt (Fig. 10). As this conversion only occurs with viable cells, it directly correlates with cell count. Then, insoluble purple formazan was dissolved by adding an adapted volume of dissolvent (Sodium Dodecyl Sulfate or equivalent). The absorbance A540 was measured with a reference wavelength of A690, using an ELISA reader. The results (i.e. cell viability) were classically expressed as 50% inhibitory concentration (IC_{50}, mol L^{-1}).

We have measured cellular viability, in the presence of increasing amounts of our vector (i.e. bis-guanidinium-tetrakis-β-cyclodextrin tetrapod).

After 24 h and 48 h of treatment (Fig. 11), IC_{50} values were approximately of the same order of magnitude (6.9×10^{-4} mol L^{-1} and 6.7×10^{-4} mol L^{-1}, respectively); while at 168 h, we observed a slight decrease of IC_{50} value (3.9×10^{-4} mol L^{-1}). Hence we demonstrated that our vector poorly affects viability of MRC-5 cells, with a greater impact for prolonged exposure times (e.g. 168 h), and finally, we concluded that our vector weakly affects MRC-5 cell viability. In the end, once we have made viability/cytotoxicity studies, one can undertake the transfection experiments. To visualize, to follow the transfection experiments, the simplest protocol, is to use marked guest molecule. It exists different kind of labeling, but at the moment the most used system and the safest is the use fluorescent probes to label the guest molecules. This approach obliges you to use microscopy techniques: fluorescence microscopy will just allow you to verify if the transfection succeeded (i.e. were cells transfected?); the confocal microscopy will allow you to see exactly in which cellular compartment is located the labeling (i.e. cytoplasmic, nuclear...); and finally you can even use flow cytometry which will allow you to determine, more rapidly than a manual counting, your efficiency of transfection (i.e. how many cells were transfected?)... However, the use of fluorescent guest molecules obliges you to several checks: the first one, to verify that the guest molecule labeling does not modify the physico-chemical properties of this molecule; the second, to choose and to use fluorescent probes which are compatible with the microscopic system and between them. Indeed, to visualize the cell transfection, it is necessary, not only to label the molecule, but also to counterstain the cells (e.g. we use DNA intercalating dyes such as DAPI, the Hoechst dyes...).

Finally, it is also necessary to define and to test the experimental conditions to have the most effective transfection: the duration, the concentration in complex vs. the number of eukaryotic cells, the ''contact conditions'' with cells, the composition of the culture medium. For example, fetal calf serum, element indispensable to the culture of eukaryotic cells, was known to interfere on the efficiency of certain commercial vectors.

In our approach, after the validation of the operating conditions, we performed our transfection experiments on MRC-5 cells, with an oligonucleotide (i.e. siRNA) labeled by Cy3 fluorescent probe (red fluorescence), and we used Hoechst 33342 (blue fluorescence) to counterstain nuclei of MRC-5 cells (we had a microscope with fluorescence with the adequate filters).

We demonstrated (Fig. 12A—D) that a final concentration of 100 μM of siRNA is sufficient for an efficient cell transfection until 6 h of incubation with our tetrapod

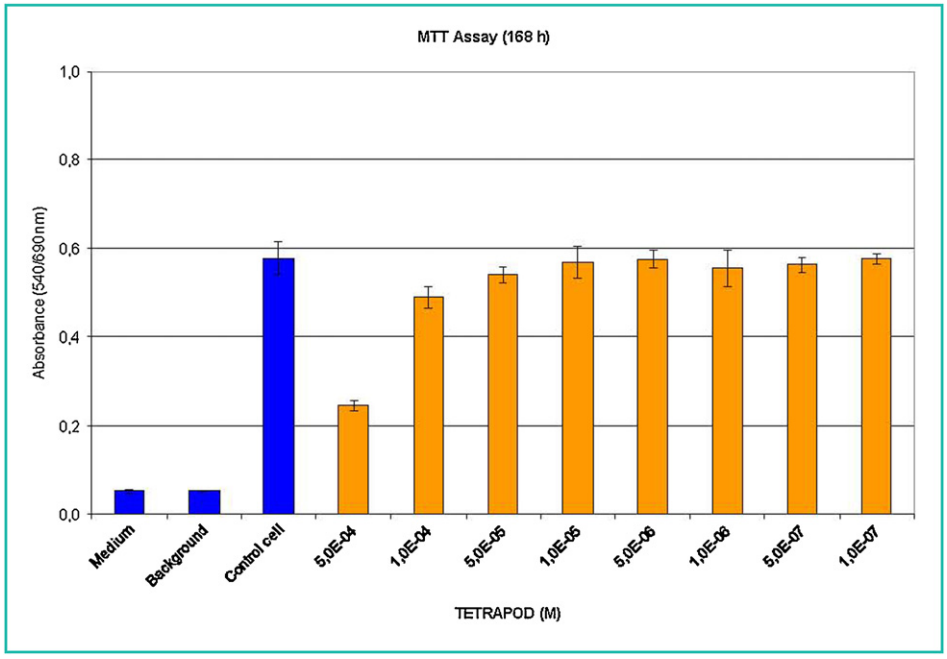

Figure 11. MTT assay performed with CyD tetrapod on MRC-5 cells at 168 h. Histograms were typical of three independent experiments. Medium = MEM alone; Background = MEM with drugs, Control cells = untreated MRC-5 cells.
Test MTT réalisé avec le tétrapode CyD sur des cellules MRC-5 à 168 heures. Les histogrammes sont caractéristiques de trois expériences indépendantes. Moyen = MEM seul ; Fond = MEM avec principes actifs ; Contrôle = cellules MRC-5 non traitées.

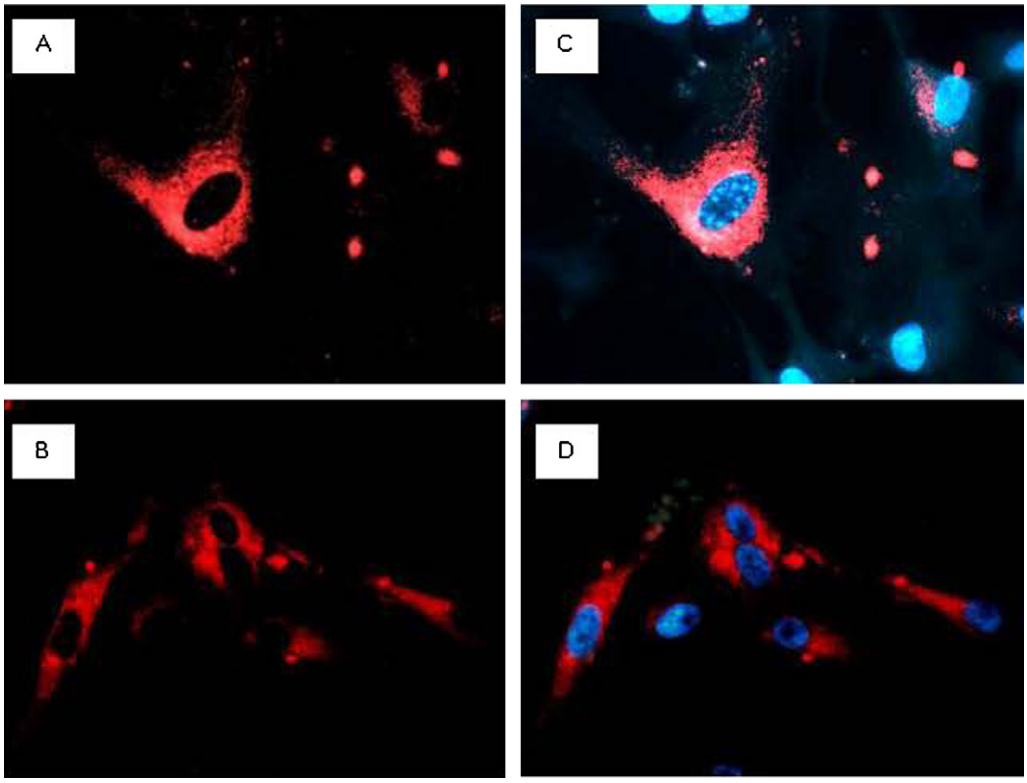

Figure 12. Fluorescence imaging depicting the time-dependent transfection and localization in cytoplasm of a siRNA in MRC-5 cells. A. Cy3 labelled-siRNA after 6 h of incubation. B. After 12 h of incubation. C. Double staining (i.e. Cy3 labelled-siRNA and Hoechst staining used to visualize nuclei), after 6 h of incubation. D. After 12 h of incubation.
Images en fluorescence montrant la transfection dépendante du temps d'un siRNA et sa localisation intracytoplasmique dans les cellules MRC-5. A. siRNA marqué au Cy3 après six heures d'incubation. B. Après 12 heures d'incubation. C. Double coloration (siRNA marqué Cy3 et contre-coloration de Hoechst du noyau cellulaire), après six heures d'incubation. D. Après 12 heures d'incubation.

(Fig. 12A). Nevertheless, the transfection reaction is clearly time-dependent, with a more intense fluorescence for 12 h transfected cells (Fig. 12C). We observed the same staining at 24 h post-transfection. Fig. 12B, D, showing parallel Hoechst 33342 (e.g. blue fluorescence) and Cy3 (e.g. red fluorescence) staining on the same field, at both 6 and 12 h, respectively.

Hence, we demonstrated an efficient and rapid (i.e. from 6 h) siRNA transfection in the cytoplasm (e.g. at the periphery of nucleus) of MRC-5 cells, using our original vector.

Conclusion

In this account, we have summarized our recent achievements on chemical, analytical and biological strategies for designing cyclodextrin tools as efficient gene delivery systems. The CyD commercial availability, easy and relatively inexpensive synthesis of appropriate derivatives in large scale, robustness, biocompatibility and lack of immunogenicity match important criteria for future development of nonviral vectors. Despite all the above success, there are, however, several challenges that remain to resolve:

- a persistent orders of magnitude poorer efficiency compared to viral vectors;
- growing understanding of the CyD-based gene delivery mechanisms;
- improve the theoretical understanding of the DNA packaging processes and characterization of the corresponding CyDplexes;
- ensure the best possible furtivity of the CyD-based vectors with respect to the immune system.

To complete this, in vivo data on our systems above-described, but also on each new CyD-based gene vector, should be collected and will be critical to achieve the ultimate goal: the construction of models of artificial viruses with a high level of transfection.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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