MARCELO AUGUSTO SZYMANSKI DE TOLEDO

TRÁFEGO INTRACELULAR DE VETORES NÃO-VIRAIS: DESENVOLVIMENTO DE PROTEÍNAS DE FUSÃO PARA TRANSPORTE DE DNA PLASMIDIAL ATRAVÉS DA INTERAÇÃO COM PROTEÍNAS MOTORAS

INTRACELULLAR TRAFFIC OF NON-VIRAL VECTORS : DEVELOPMENT OF RECOMBINANT FUSION PROTEINS TO MEDIATE PLASMIDIAL DNA TRANSPORT BY INTERACTION WITH MOTOR PROTEINS

CAMPINAS
2013

UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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Thesis presented to the Institute of Biology of The University of Campinas in partial fulfillment of the requirements for the degree of PhD in Genetics and Molecular Biology in the field of Genetics of Microorganisms.

Tese apresentada ao Instituto de Biologia da UNICAMP para obtenção do título de Doutor em Genética e Biologia Molecular, na área de Genética de Microorganismos.

Orientador: Prof. Dr. Adriano Rodrigues Azzoni

Co-orientadora: Profa. Dra. Anete Pereira de Souza

CAMPINAS
2013

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

T575t

Toledo, Marcelo Augusto Szymanski de, 1987-
Trafego intracelular de vetores não-virais : desenvolvimento de proteínas de fusão para transporte de DNA plasmidial através da interação com proteínas motoras / Marcelo Augusto Szymanski de Toledo. – Campinas, SP : [s.n.], 2013.

Orientador: Adriano Rodrigues Azzoni.
Coorientador: Anete Pereira de Souza.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Entrega gênica. 2. Dineínas. 3. Transfecção. 4. Terapia gênica. 5. Plasmídeos. I. Azzoni, Adriano Rodrigues. II. Souza, Anete Pereira de,1962-. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Intracelullar traffic of non-viral vectors : development of recombinant fusion proteins to mediate plasmidial DNA transport by interaction with motor proteins
Palavras-chave em inglês:
Gene delivery
Dyneins
Transfection
Gene therapy
Plasmids
Área de concentração: Genética de Microorganismos
Titulação: Doutor em Genética e Biologia Molecular
Banca examinadora:
Adriano Rodrigues Azzoni [Orientador]
Lucimara Gaziola De La Torre
Katlin Brauer Massirer
Luis Antônio Peroni
Sandra Aparecida Cororato dos Santos
Data de defesa: 17-12-2013
Programa de Pós-Graduação: Genética e Biologia Molecular

Campinas, **17** de **dezembro** de **2013**

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RESUMO

Apesar de seguros e simples de produzir, o uso de vetores não virais como o DNA plasmidial (DNAp) em estudos de terapia gênica e vacinação por DNA tem sido limitado pela baixa eficiência quando comparados aos vetores virais. Essa limitação provém principalmente da reduzida capacidade de superar as barreiras físicas, enzimáticas e difusionais encontradas durante o tráfego intracelular para o interior do núcleo das células alvo. Dentro deste contexto, o presente trabalho demonstra a utilização de cadeias leves modificadas de Dineína (Lc8 e Rp3) como vetores não-virais de entrega gênica. A escolha de cadeias leves de Dineína justifica-se pela possibilidade de utilizar o transporte retrógrado celular mediado por complexos motores de Dineína para facilitar o tráfego de material genético exógeno através do citoplasma em direção à periferia nuclear. Através da adição de pequenos domínios peptídicos, ricos em aminoácidos polares positivos (arginina e lisina), ao N-terminal de cadeias leves de Dineína foi possível conferir a estas proteínas a habilidade de interagir com material genético condensando-o em partículas. Ensaios de transfecção demonstraram que tais partículas apresentam elevada eficiência de entrega do material genético exógeno ao núcleo de células HeLa, eficiência esta superior àquela apresentada pelo peptídeo protamina, amplamente estudado como vetor não-viral de entrega gênica. A formação de complexos ternários utilizando-se DNA plasmidial, cadeias leves de Dineína modificadas e lipídios catiônicos apresentou eficiência de entrega superior àquelas apresentadas na ausência do lipídio. Adicionalmente, complexos de entrega formados apenas com DNA plasmidial e cadeias leves de Dineína modificadas apresentaram baixo efeito citotóxico em células HeLa, característica esta de grande relevância uma vez que a toxicidade dos vetores de entrega gênica atua como importante fator limitante em sua aplicação clínica.

O mecanismo envolvido no processo de entrega gênica mediado por cadeias leves de Dineína modificadas também foi estudado, podendo ser observado que (1) a entrada dos complexos de entrega na célula é altamente dependente do processo de endocitose, (2) a eficiência de entrega observada depende da rede de microtúbulos e (3) parte significativa dos complexos de entrega é degradada na via de endossoma/lisossomo celular.

Os vetores não-virais de entrega gênica descritos no presente estudo associam elevada eficiência de transfecção, baixa toxicidade celular e relativo baixo custo de produção, uma vez que as cadeias leves de Dineína recombinantes são produzidas em sistema heterólogo utilizando-se *Escherichia coli*. Ressalta-se ainda a possibilidade de adição de novos domínios peptídicos às cadeias leves de Dineína modificadas, agregando novas funções/capacidades que poderiam resultar em maior eficiência de entrega gênica através da otimização dos processos de internalização celular ou escape endossomal.

A abordagem de se utilizar a via de transporte retrógrado celular para o desenvolvimento de vetores não-virais para entrega gênica é pouco explorada pela comunidade científica e o presente estudo apresenta-se entre os poucos da área, esperando assim contribuir para o desenvolvimento de vetores não-virais mais eficientes e seguros.

ABSTRACT

The use of non viral vectors such as plasmidial DNA (pDNA) in gene therapy and DNA vaccination protocols has been limited due to its low transfection efficiency when compared to viral vectors. This limitation occurs mainly due to the physical, enzymatic and diffusion barriers faced during the transport of the genetic material to the nucleus of target eukaryotic cells.

Regarding this subject, the present work demonstrates the feasibility of using modified Dynein light chains (Lc8 and Rp3) as non viral vectors for gene delivery. The use of Dynein light chains relies on the possibility to exploit the Dynein based cellular retrograde transport in order to improve the exogenous genetic material transport across the citosol towards the nuclear periphery.

By adding small peptide domains, based in positively charged aminoacids (arginine and lysine) to the N-terminal of Dynein light chains, the resulting recombinant proteins were able to interact and condense genetic material into delivery particles. Transfection assays demonstrated that these particles are highly efficient to delivery plasmidial DNA to nucleus of HeLa cells when compared to the transfection efficiency presented by protamine, a well characterized non viral vector peptide.

Ternary complexes formed by modified Dynein light chains, pDNA and a cationic lipid showed even higher transfection efficiency. Additionally, the light chain based non viral delivery vectors presented low citotoxic effect to HeLa cells, a valuable feature as toxicity is regarded as one of the main concerns on delivery vectors development.

The mechanism by which the modified Dynein light chain based vectors mediates gene delivery was also investigated and we could observe that (1) the internalization process deeply

relies on endocytosis, (2) it depends on the microtubule network and (3) a significant fraction of the delivery complexes are trapped and degraded in the endocytic pathway.

The non viral vectors developed in the present study combine high transfection efficiency, low toxicity and relative low production cost, as all modified proteins were produced in *Escherichia coli* prokaryotic host. Its noteworthy that additional peptide domains can be further associated to the delivery vectors described providing it with new abilities such as higher internalization or endosomal escape capacity.

The approach to use the cellular retrograde transport in order to develop non viral vectors is poorly exploited by the scientific community and the present study stands among few in the field hopefully contributing to the development of more efficient and safer non viral vectors for gene delivery.

SUMÁRIO

"Do not indoctrinate your children. Teach them how to think for themselves, how to evaluate evidence, and how to disagree with you."

Richard Dawkins, The God Delusion

Agradecimentos

Gostaria de ressaltar o meu agradecimento e a importância de algumas pessoas que, de alguma forma contribuíram e contribuem para a realização do presente projeto e certamente de outros por vir.

Ao Prof. Adriano Rodrigures Azzoni, pela oportunidade de desenvolver o projeto em sua linha de pesquisa, pela orientação, pela amizade e por permitir que eu explorasse minhas idéias e por sempre estar ali, para puxar de volta quando eu me perdia muito. Minha eterna gratidão pela oportunidade e pelo aprendizado.

À Profa. Anete Pereira de Souza por ter me aceitado oito anos atrás em seu grupo. Por todos os ensinamentos, toda a ajuda, pelos conselhos, pelo modelo de pesquisador e pessoa serei eternamente grato. Seu entusiasmo pela ciência é inspirador e contagiante, sua maneira positiva de encarar os problemas e desafios é admirável. Obrigado pela oportunidade de conviver e aprender com a Sra. ao longo desses anos.

À minha mãe Carmen, pelo carinho e pelo exemplo de pessoa. Se conseguir espelhar uma fração do que vi e vejo, me considerarei satisfeito.

Ao meu irmão, Marco e minhas irmãs Maria Carolina e Maria Angélica pelo carinho, apoio e ajuda.

Ao Sr. Octávio J. Leite de Moraes, pela amizade e carinho paternais. Que a sua memória seja eterna.

Aos companheiros do LAGM, tanto aqueles que já passaram pelo laboratório, quanto aqueles que ainda estão presentes. Obrigado pela convivência.

À Profa. Maricilda P. de Melo, pelo auxílio na área de cultura de células humanas.

À Profa. Lucimara G. de la Torre, pelo auxílio com as medidas de potencial zeta e DLS.

Ao Laboratório Nacional de Luz Sincrotron (LNLS) pelas instalações que possibilitaram as análises de dicroísmo circular e SAXS.

À todos do CBMEG/Unicamp pela convivência e auxílio.

À Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP - pelo apoio financeiro e bolsa de estudos para o desenvolvimento deste trabalho.

Sem dúvidas serei grato a todos vocês pela convivência e aprendizado que levarei comigo para as próximas etapas. Obrigado!

Marcelo A. S. de Toledo, Dezembro de 2013

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CD	Dicroísmo circular (*Circular Dichroism*)
C-terminal	Extremidade carboxila terminal da proteína
D1	Domínio de ligação ao DNA 1
D2	Domínio de ligação ao DNA 1
D3	Domínio de ligação ao DNA 1
D4	Domínio de ligação ao DNA 1
DNAp	DNA plasmidial
DNAp-Cy3	DNA plasmidial marcado com o fluoróforo Cy3
FITC	Fluoróforo acoplado ao anticorpo secundário
Lc8	Cadeia leve de Dineína Lc8
LD1	Cadeia leve de Dineína Lc8 com domínio de ligação ao DNA 1
LD2	Cadeia leve de Dineína Lc8 com domínio de ligação ao DNA 2
LD3	Cadeia leve de Dineína Lc8 com domínio de ligação ao DNA 3
LD4	Cadeia leve de Dineína Lc8 com domínio de ligação ao DNA 4
N-terminal	Extremidade amino terminal da proteína
Rp3	Cadeia leve de Dineína Rp3
Rp3-Db	Cadeia leve de Dineína Rp3 com domínio de ligação ao DNA 4
SAXS	Espalhamento de raio-X à baixo ângulo (*Small angle X-ray Scattering*)

PREFÁCIO

A presente tese de doutoramento descreve as atividades desenvolvidas referentes ao projeto intitulado *"Tráfego intracelular de vetores não-virais: desenvolvimento de proteínas de fusão para transporte de DNA plasmidial através da interação com proteínas motoras"*. Inicialmente, é apresentado um capítulo introdutório sobre terapia gênica e vetores de entrega, bem como uma contextualização do presente trabalho. Os principais resultados obtidos ao longo do desenvolvimento do presente trabalho são apresentados na forma de artigos já publicados na literatura científica internacional. O "Capítulo 1" apresenta os resultados obtidos no desenvolvimento de vetores de entrega gênica não-viral baseados na cadeia leve de Dineína Lc8 ressaltando-se a eficácia da abordagem adotada para a melhora da eficiência de entrega gênica. O "Capitulo 2" descreve, também na forma de artigo científico, os resultados obtidos explorando-se a capacidade da cadeia leve de Dineína Rp3 em mediar a entrega gênica. Resultados complementares, obtidos ao longo do desenvolvimento do presente trabalho também são apresentados e demonstram abordagens adicionais para a otimização dos vetores não-virais desenvolvidos.

A viabilidade e eficiência da abordagem utilizada no presente trabalho para o desenvolvimento de vetores não-virais mais eficientes e seguros, explorando-se a maquinaria de transporte intracelular, bem como estudos futuros que se mostram necessários são abordados no tópico "Conclusões e Perspectivas".

1. Introdução

1.1 Terapia gênica

Terapia gênica pode ser definida, em uma forma ampla, como a administração de material genético exógeno (DNA ou RNA) às células de um paciente de forma a corrigir um quadro patológico de base genética ou amenizar o quadro clínico (Patil *et al.*, 2012). Tal efeito pode ser alcançado através de três principais abordagens: (1) adição de uma cópia "saudável" do gene defeituoso que pode ser mediada por recombinação homóloga ou através de um vetor episssomal, sendo tal abordagem a mais empregada em testes clínicos; (2) correção/alteração do gene defeituoso por mutação de ponto específica. Embora esta última abordagem tenha sido pouco explorada, o recente desenvolvimento de novas técnicas de inserção de mutações ponto-específico impulsionaram novos estudos envolvendo este tipo de terapia (Kay, 2011; Urnov *et al*., 2010); (3) regulação no nível de expressão gênica envolvendo a aplicação de moléculas de RNA de interferência e micro RNAs, possibilitando o silenciamento da expressão de genes alvos bem como a regulação dos níveis de expressão e alteração do processo de *splice* destes durante o processo de maturação do RNA mensageiro (Lu *et al.*, 2011; Janowski & Corey, 2010; Zhou & Rossi, 2010). Ressalta-se ainda que combinações destas três abordagens podem ser utilizadas em protocolos terapêuticos (Kay, 2011). Adicionalmente, dependendo do vetor utilizado para a entrega do material genético exógeno, a alteração ou correção do quadro genético da célula alvo pode ser permanente, caso a terapia leve a uma modificação no genoma celular, ou transiente, caso o vetor terapêutico exista fora do genoma como um episssoma (Kay, 2011).

O primeiro caso bem documentado da aplicação de terapia gênica em humanos data do início da década de 90, realizado pelo grupo do Dr. W. French Anderson no National Institutes of Health's Clinical Center, Bathesda, Maryland. Anderson e seus colaboradores coletaram

linfócitos de um paciente diagnosticado com deficiência da enzima adenosina deaminase (*Adenosine Deaminase Deficiency* ou *ADA deficiency*), que leva a um quadro patológico de insuficiência generalizada do sistema imune (*Severe Combined Imuno Deficiency - SCID)* e, utilizando um vetor de entrega retroviral (LASN), inseriram a cópia correta do gene nestas células. Os linfócitos "corrigidos" foram então transferidos para o paciente que apresentou melhora significativa no seu quadro clínico (Blaese *et al.*, 1995; Anderson *et al.*, 1990).

Após mais de duas décadas, diversos trabalhos tem ressaltado a importância da terapia gênica como ferramenta para o tratamento de diversas doenças e como a metodologia que promete bons resultados e avanços no tratamento de diversas patologias (Kay *et al.*, 2010; Mastrobattista *et al.*, 2006; Lee & Davidson, 2011). Em consonância, um aumento do número de testes clínicos utilizando terapia gênica tem sido observado nas últimas duas décadas (Figura 1.1). Contudo, embora conceitualmente simples, a terapia gênica não apresentou o desenvolvimento e aplicabilidade esperados quando do seu anuncio pela comunidade científica. Tal fato decorre, principalmente, da dificuldade de desenvolvimento de técnicas eficientes e seguras para a entrega de material genético exógeno ao núcleo de células em cultura (*ex vivo*) ou em um organismo (*in vivo*) (Li & Huang, 2000; Scanlon, 2004; Martin & Boulikas, 1998; Parker *et al*., 2003; Patil *et al*., 2012; Pouton & Seymour, 2001).

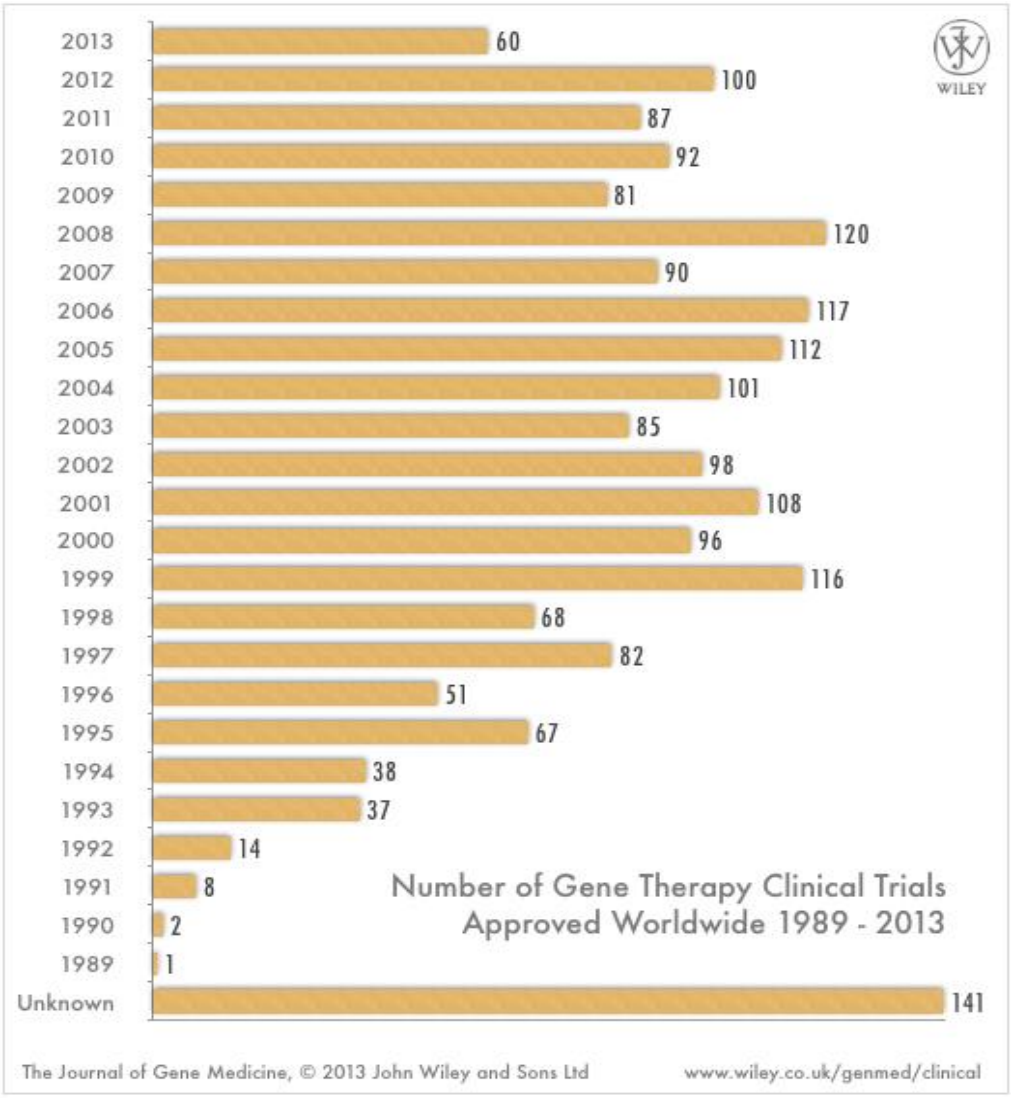

Figura 1.1 - Testes clínicos aprovados envolvendo o uso de terapia gênica no período de 1989 à 2013. Fonte: www.wiley.co.uk/genmed/clinical.

1.2 Vetores virais e não virais

Dentre as técnicas desenvolvidas até o presente momento para mediar o processo de internalização celular de material genético exógeno observa-se uma dicotomia das abordagens adotadas: (1) técnicas de transfecção que exploram a habilidade de diversos vírus em infectar e levar o material genético ao núcleo das células, gerando vetores de transfecção virais e, (2) diversas classes de moléculas capazes de interagir e condensar o material genético e, através de

diversos mecanismos, facilitar a entrada deste no citoplasma e núcleo de células eucarióticas, denominados como vetores não-virais ou vetores sintéticos (Nayerossadat *et al*., 2012; Cevher *et al*., 2012).

Vetores virais exploram a capacidade desenvolvida por diversos vírus de infectar células eucarióticas e transferir seu material genético para o núcleo destas de maneira eficiente. Utilizando-se ferramentas de biologia molecular e engenharia genética é possível substituir seqüências do genoma viral, responsáveis por deflagrar o quadro patogênico, por seqüências terapêuticas. O vírus então modificado atua como "transportador" do material genético exógeno (Cevher *et al.*, 2012).

Os principais tipos virais utilizados no desenvolvimento de vetores para terapia gênica são retrovírus, lentivírus, adenovírus, vírus associado ao adenovírus, vírus da herpes, poxovírus e HFV (*human foamy virus*) (Nayerossadat *et al*., 2012). Por apresentarem elevada capacidade de entrega de material genético exógeno, os vetores virais tem sido mais estudados e explorados em testes clínicos (Figura 1.2). Contudo, diversos fatores tem contribuído para o desenvolvimento de técnicas de entrega gênica que não utilizem vetores virais: (1) embora apresentem elevada capacidade de entrega do material genético, vetores virais, como a maioria dos *retrovirus,* são capazes de infectar apenas células em divisão; (2) diversos vetores virais requerem a inserção do material genético no genoma do hospedeiro, podendo gerar diversos efeitos colaterais incluindo a ativação de oncogenes; (3) podem deflagrar fortes reações imunológicas no hospedeiro e esta pode variar entre indivíduos, dificultando assim o desenvolvimento de vetores-virais seguros; (4) podem readquirir sua virulência uma vez dentro da célula do hospedeiro; (5) enfrentam problemas durante o processo de escalonamento de produção de partículas virais, apresentando baixo rendimento e alto custo; (6) a reprogramação

de vetores-virais quanto à sua especificidade celular tem se mostrado difícil (Kay, 2011; Nayerossadat *et al*., 2012, Thomas *et al*., 2003).

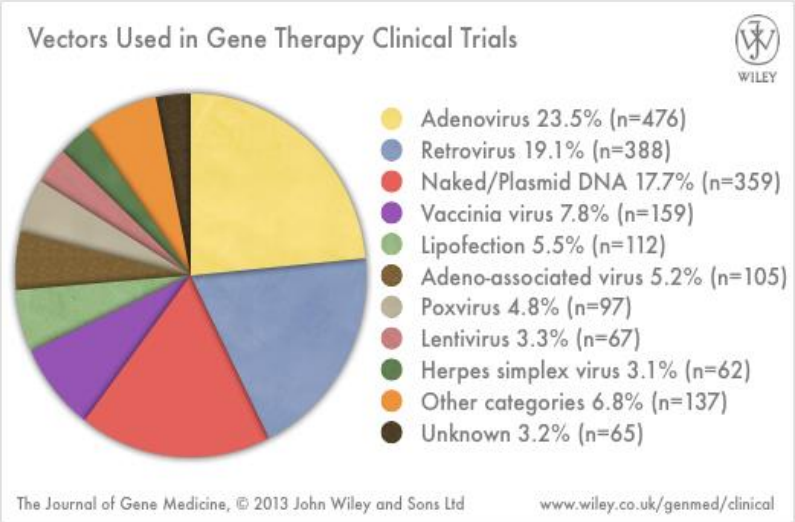

Figura 1.2 - Representatividade numérica e percentual dos principais vetores de entrega gênica utilizados em testes clínicos. Fonte: www.wiley.co.uk/genmed/clinical.

Dentro deste contexto, destacam-se os estudos focados no desenvolvimento de vetores de entrega gênica que não envolvam a utilização de partículas virais modificadas. Os vetores não-virais, também denominados como vetores sintéticos, englobam diversas classes moleculares dentre as quais diversos tipos de lipídios catiônicos (Zhi *et al*., 2013; Medvedeva *et al*., 2009; Martin *et al*., 2005; Montier *et al*., 2008), polímeros catiônicos como polietilenoimina - PEI (Lemkine & Demeneix, 2001) e poli-L-lisina - PLL (Park *et al.*, 2006; Smedt *et al.*, 2000; Mintzer & Simanek, 2009), diversos peptídeos catiônicos como a protamina (Fominaya *et al*., 2000; Tsuchiya *et al*., 2006), nanopartículas (Panyam & Labhasetwar, 2012; Ghosh et al., 2008; Bowman & Leong, 2006), bem como combinações destas moléculas (Maitani & Hattori, 2009; Tam *et al*., 2013; Martin-Herranz *et al*., 2004). A carga negativa da moléculas de DNA, em decorrência dos grupamentos fosfatos presentes nos desoxiribonucleotídeos, impede sua interação eletrostática com a membrana celular composta por lipídios aniônicos (Parker *et al*., 2003). De tal modo, as diversas moléculas a serem utilizadas como vetores de entrega devem

ser capazes de interagir com a molécula de DNA compactando-o em partículas com carga superficial positiva e tamanho próximo de 100 nm, permitindo o contato com a superfície celular e subseqüente internalização do complexo de entrega (Parker *et al*., 2003; Amini *et al*., 2012). Adicionalmente, os vetores não-virais devem ser capazes de desempenhar todo o processo de internalização celular, tráfego através do citoplasma e internalização nuclear. A dificuldade em mimetizar os vetores virais em todos estes processos torna-se evidente pela baixa eficiência de internalização do material genético terapêutico apresentada por vetores não-virais. Contudo, por serem mais seguros uma vez que são pouco imunogênicos/tóxicos, compostos por moléculas de fácil manipulação e de baixo custo e, perante aos riscos encontrados na utilização de vírus modificados, maior atenção tem sido dada ao desenvolvimento de vetores não-virais mais eficientes (Pack *et al*., 2005; Parker *et al*., 2003; Kay, 2011)

Complexos de entrega gênica formados por lipídios catiônicos e moléculas de DNA, denominados lipoplexos foram descritos inicialmente em 1987 (Felgner *et al*., 1987) e diversas formulações lipídicas tem sido descritas desde então bem como variações quanto às relações de carga de lipoplexos, decorrente da molaridade do lipídio catiônico utilizado (positivo) e da molaridade do vetor plasmidial (negativo). Ambas características, composição lipídica e relação de carga, mostraram afetar a eficiência de transfecção (Parker *et al*., 2003). O processo de internalização de lipoplexos pode ocorrer *via* endocitose, muito embora o processo de fusão com a membrana celular ou ruptura de sua camada bilipídica tenha sido observado durante o processo de internalização (Felgner *et al*., 1994; Friend *et al*., 1996). Uma vez internalizados, parte dos lipoplexos dentro de endossomos é degradada pela maturação destes com a fusão por lisossomos. Durante tal processo, a interação dos lipídios de entrega com a membrana de vesículas endossomais leva a uma instabilidade desta, levando a sua ruptura. Isso permite que o

material genético terapêutico seja liberado no citoplasma e siga para o núcleo celular (Figura 1.3) (Parker *et al*., 2003). Embora tenha sido demonstrado que lipoplexos são eficientes na transfecção de células em cultura, sua aplicação *in vivo* é limitada pela interação de sua superfície hidrofóbica e positiva com proteínas do plasma sangüíneo (Liu *et al*., 2003), pela sua inespecificidade de reconhecimento celular (Wiethoff & Middaugh, 2003) e pela sua baixa eficiência em transfectar células que não estão em divisão celular, fato este atribuído pela incapacidade de se transpor a membrana nuclear (Parker *et al*., 2003).

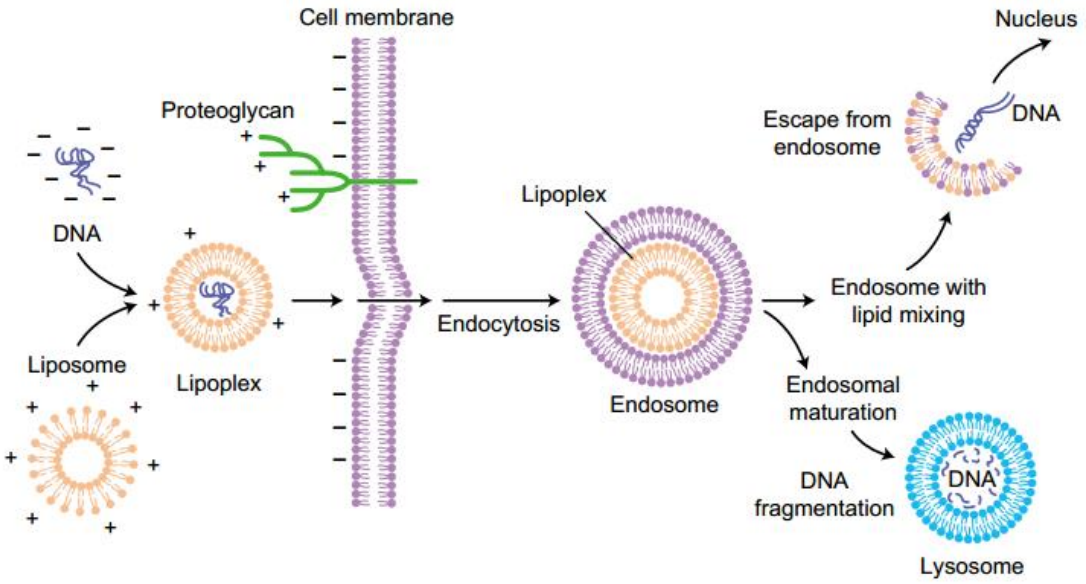

Figura 1.3 - Representação gráfica da utilização de lipídios catiônicos como vetor não-viral de entrega gênca. As principais etapas do processo de entrega gênica são demonstrados: complexação com DNA, internalização celular, escape endossomal e internalização nuclear. Fonte: Parker *et al.* (2003).

Polímeros catiônicos complexados com moléculas de DNA, também denominados de poliplexos, também tem sido amplamente estudados como vetores de entrega gênica não-viral. Destes, polietilenoimina ou PEI tem se apresentado como o mais promissor (Parker *et al*., 2003; Pack *et al*., 2005). Sua capacidade de transfecção é superior à capacidade de vetores lipídicos, que é creditada à sua habilidade em mediar o escape endossomal do material genético exógeno. Tal capacidade decorre de uma possível propriedade tamponante ("*proton sponge*")

desempenhada pelas moléculas de PEI que impedem a acidificação lisossomal, levando à ruptura da vesícula e liberação do material genético no citoplasma (Creusat *et al*., 2010; Boussif *et al*., 1995; Pack *et al*., 2005). Outra possível explicação seria que a protonação de moléculas de PEI dentro das vesículas lisossomais levem à uma expansão de sua estrutura polimérica causando a ruptura física da membrana vesicular (Behr, 1997). Adicionalmente, foi proposto que a carga positiva presente na estrutura de moléculas de PEI mimetizam sinais de localização nuclear (*nuclear localization signals - NLS*) o que poderia também contribuir para a maior eficiência de transfecção e pelo fato de que complexos de PEI-DNA serem capazes de transfectar células que não estão em divisão (Brunner *et al*., 2002). De maneira similar ao observado para lipoplexos, a carga positiva superficial de poliplexos favorece sua interação com proteínas do plasma sanguíneo e garante inespecificidade quanto ao reconhecimento celular (Parker *et al*., 2003). Contudo, poliplexos podem ser modificados adicionando-se ligantes peptídicos que são reconhecidos por receptores presentes na membrana de células alvo, tornando-os assim mais específicos (Figura 1.4) (Won *et al.*, 2013; Wang *et al.*, 2013).

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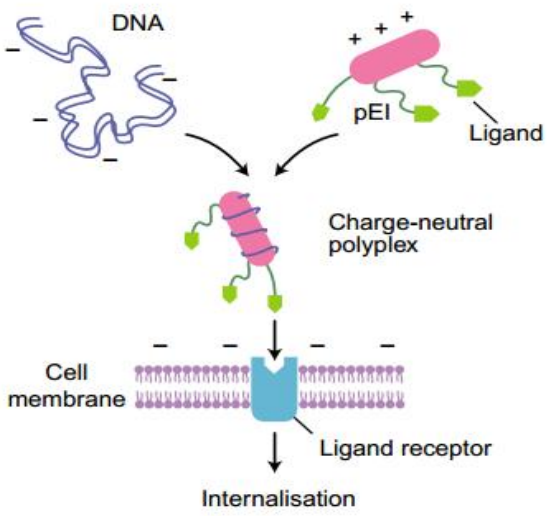

Figura 1.4 - Representação gráfica da utilização de polímeros catiônicos como vetor não-viral de entrega gênica. A complexação de PEI funcionalizado com peptídeos para reconhecimento celular com o material genético exógeno é representada bem como o processo de reconhecimento celular específico. Fonte: Parker *et al.* (2003).

Proteínas ou peptídeos ricos em aminoácidos polares positivos como arginina (R) e lisina (K) também são utilizados como vetores não-virais de entrega gênica. A protamina é um peptídeo de 5,1 kDa, responsável pela compactação do DNA gênomico durante o processo de espermatogênese. Por sua carga altamente positiva devido a presença de diversos clusters de resíduos de arginina, tem sido estudada como vetor não-viral de entrega gênica. Sua capacidade de interação e compactação de moléculas de DNA plasmidial foi verificada, muito embora tenha apresentado baixa eficiência de transfecção (Tsuchiya *et al*., 2006). Entretanto, diversos estudos relatam o desenvolvimento de vetores não-virais utilizando-se protamina associada com lipídios, polímeros catiônicos e outros peptídeos, demonstrando o potencial deste peptídeo no desenvolvimento de vetores não-virais (Maruyama *et al.*, 2004; Fujita *et al.*, 2008; Bruckheimer *et al.*, 2004).

Diversos outros peptídeos têm sido utilizados associados à lipídeos ou polímeros catiônicos e nanopartículas, visando a criação de vetores não-virais mais eficientes, específicos e seguros. Dentre estes destacam-se: (1) peptídios catiônicos compostos por arginina (oligoarginina) e lisina (poli-lisina), utilizados na compactação de moléculas de DNA (Opanasopit *et al*., 2011; Yamanouchi *et al*., 2008; Zhang *et al*., 2010); (2) peptídeos membrano-ativos como TAT (peptídeo do fator de transativação de transcrição do vírus HIV) e penetratina (Torchilin, 2008); (3) seqüências peptídicas correspondentes à diversos sinais de localização nuclear (NLS), que favorecem o processo de internalização nuclear do material genético exógeno (Cartier & Reszka, 2002) e; (4) peptídeos específicos do processo de reconhecimento celular cuja utilização garante mais especificidade aos vetores de entrega (Kuriyama *et al*., 2009; Schaffer & Lauffenburger, 2000).

1.3 Barreiras à entrega gênica mediada por vetores não-virais

A capacidade das partículas virais de transferir seu material genético para o núcleo de células hospedeiras foi desenvolvida ao longo do seu processo evolutivo como patógeno, justificando a elevada eficiência dos vetores-virais em transferir material genético terapêutico ao núcleo de células eucarióticas (Maetzig et al., 2011). Em contrapartida, os vetores não-virais não compartilham da mesma eficiência, uma vez que tem que enfrentar diversas barreiras, facilmente contornada pelos vetores-virais, no processo de entrega gênica (Figura 1.5). Diversos trabalhos tem abordado as barreiras enfrentadas por vetores não-virais no processo de entrega gênica (Parker *et al*., 2003; Khalil *et al*., 2006; Wiethoff & Middaugh, 2003; Morille *et al*., 2008) destacando-se as seguintes:

(1) o complexo formado pelo material genético com o vetor ou molécula adjuvante do processo de entrega deve ser estável o suficiente para garantir a integridade do material genético no meio extracelular. A presença de endonucleases no meio extracelular, leva à rápida degradação do material genético terapêutico quando estes são administrados por via intravascular ou intramuscular;

(2) o comportamento coloidal do complexo de entrega afeta diretamente sua dispersão pelo sistema vascular ou tecido, podendo limitar sua capacidade em atingir células alvo. A diferença de força iônica presente no plasma sanguíneo ou no espaço intercelular pode afetar a interação do vetor não-viral com a molécula de DNA, expondo esta à degradação por endonucleases ou favorecer agregação dos complexos de entrega, em ambos os casos levando à uma redução dos complexos terapêuticos disponíveis para internalização;

(3) complexos de entrega baseados em lipídios, polímeros e peptídeos catiônicos possuem carga de superfície positiva. Uma vez dentro do espaço vascular ou extracelular, tais partículas de entrega se associam à proteínas aniônicas presentes no plasma sanguíneo, tais como

glicosaminoglicanos e albumina. Estas, por sua vez, podem levar à agregação dos complexos de entrega ou competem com o material genético pela interação com o vetor catiônico;

(4) a capacidade de associação dos complexos de entrega com a superfície celular esta diretamente relacionada com a capacidade de internalização e eficiência de entrega destes. A carga superficial positiva do complexo de entrega mostrou ser essencial para o processo de associação com a superfície celular aniônica, bem como o seu comportamento coloidal, uma vez que complexos de entrega que apresentam agregação mostraram ser mais eficientes no processo de adesão celular. Acredita-se que o processo de adesão à superfície celular realizada por complexos de entrega lipídicos ou poliméricos seja mediada pela associação com proteoglicanos de superfície celular contendo cadeias de sulfato de heparana;

(5) diversas vias de internalização celular dos complexos de entrega não-virais tem sido consideradas, entre elas o processo de endocitose mediado ou não pela associação de clatrinas, endocitose mediada por caveolas e macropinocitose. O entendimento de qual ou quais destes processos contribuem de forma significativa para a internalização de complexos de entrega pode permitir o desenvolvimento de vetores especializados para recrutar tal processo de internalização;

(6) no citoplasma, os complexos de entrega não-virais encontram-se aprisionados em vesículas da via endocítica presentes na periferia celular. O fusionamento destas com vesículas lisossomais leva a acidificação do compartimento e degradação do material genético exógeno. A incapacidade de vetores não-virais em escapar das vesículas endossomais é considerada como sua principal limitação e causa direta de sua baixa eficiência de transfecção.

(7) considerando-se que uma pequena parte do material genético terapêutico internalizado consiga "escapar" para o citoplasma, este ainda enfrenta dois principais obstáculos, a degradação por endonucleases citoplasmáticas e a reduzida mobilidade no plasma celular.

Inicialmente, acreditava-se que o movimento de moléculas de DNA no interior de células transfectadas ocorria por difusão livre ou interações não específicas com componentes do citoplasma (Lechardeur *et al*., 2005; Vaughan and Dean, 2005). Porém, trabalhos demonstraram que vetores de DNA plasmidial injetados no citoplasma (microinjeção) permanecem perto do local de injeção, e que moléculas de DNA maiores que 2000 pares de base (pb) não são capazes de se mover livremente por difusão (Lukacs *et al*., 2000), ficando expostas à degradação por nucleases citoplasmáticas;

(8) o processo de internalização nuclear do material genético terapêutico pode ocorrer por duas vias: através dos poros nucleares ou por associação como a cromatina da célula durante o processo de divisão celular. A utilização dos poros nucleares tem sido explorada através da associação de fatores de localização nuclear ou regiões promotoras que se associam a fatores de transcrição citoplasmáticos como a região *enhancer* do SV40;

(9) dentro do núcleo da célula alvo, o material genético exógeno deve ser reconhecido pela maquinaria de transcrição celular de maneira eficiente para produzir o RNA mensageiro e por final a molécula terapêutica (proteína ou RNA).

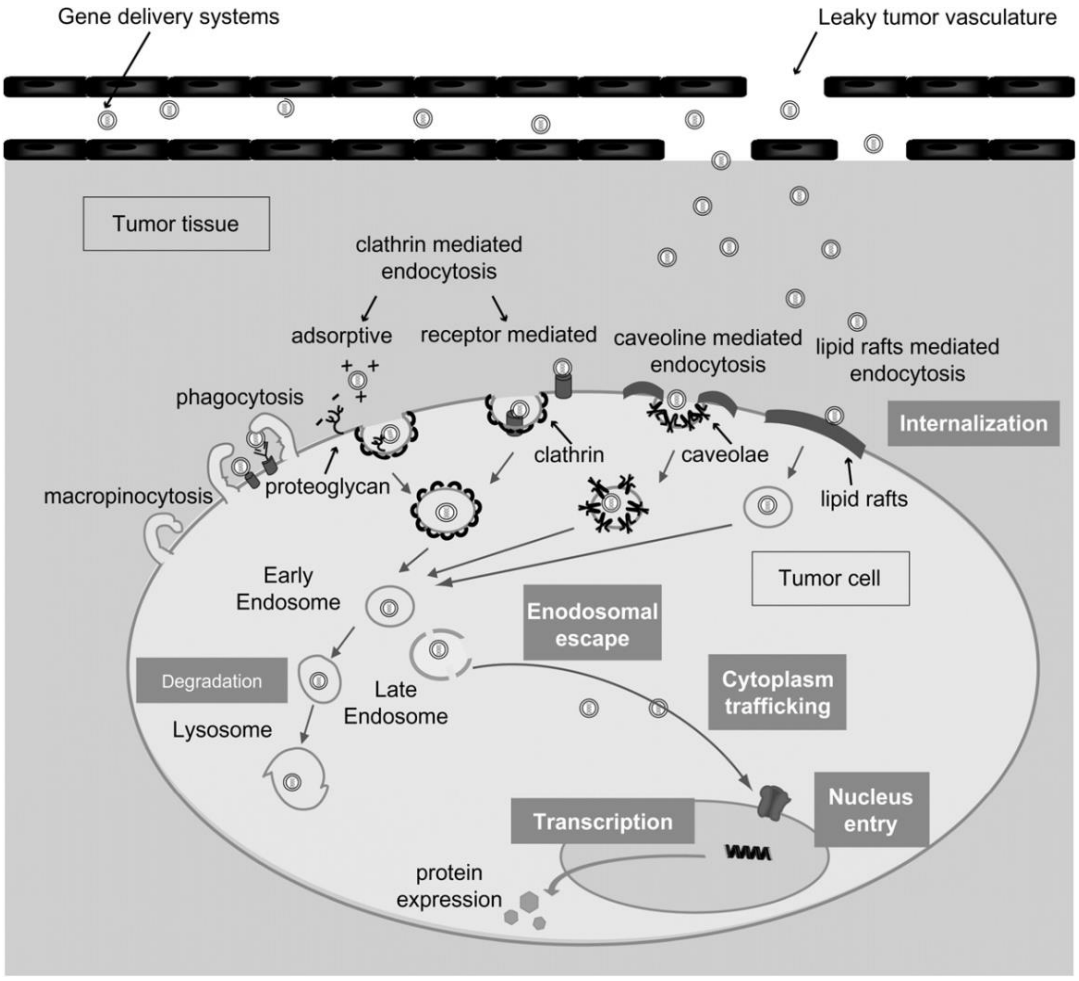

Figura 1.5 - Representação gráfica das principais barreiras enfrentadas durante o transporte de DNA por vetores não-virais: estabilidade do complexo de entrega, tamanho de partícula, biodisponibilidade, internalização, escape endossomal, tráfego citoplasmático até a periferia nuclear, internalização nuclear, transcrição e tradução do material terapêutico. Fonte: Morille *et al.* (2008).

1.4 O complexo motor Dineína e sua importância no desenvolvimento de vetores não-virais

O complexo motor Dineína (Figura 1.6) possui aproximadamente 1,2 MDa, sendo composto por diversas subunidades: duas cadeias pesadas de 530 kDa responsáveis pela interação com a rede de microtúbulos e hidrólise de ATP, duas cadeias intermediárias de 74 kDa, quatro cadeias intermediarias leves de 52-61 kDa e diversas cadeias leves de 10-25 kDa (Carter *et al*., 2011).

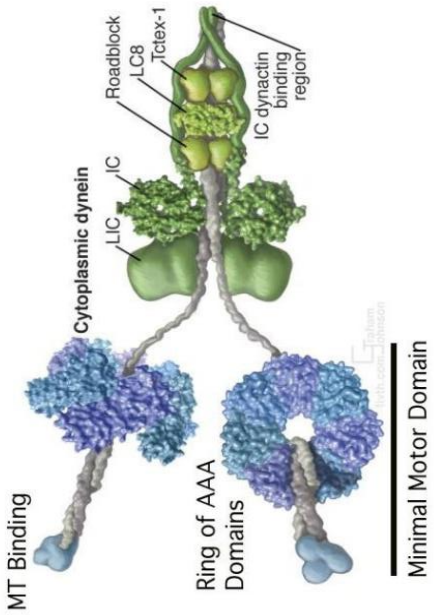

Figura 1.6 - Estrutura do complexo motor Dineína baseado em estruturas de ressonância magnética nuclear (RMN). Fonte: Yildiz LAb, Molecular Motors Research Group, Berkeley, Copyright (c) 2012, University of California.

Através da hidrólise de ATP nas cadeias pesadas este complexo motor é responsável pelo transporte de vesículas, proteínas e partículas virais infectantes da periferia celular ao centrossoma, localizado próximo ao núcleo. As cadeias leves de Dineína são responsáveis por realizar a interação da carga a ser transportada com o complexo motor (Holzbaur & Vallee, 1994).

Diversos estudos relatam (sumarizados na Tabela 1.1) que partículas virais recrutam o complexo motor Dineína para atravessar o citoplasma em direção ao núcleo durante o processo de infecção de células eucarióticas hospedeiras. Tal associação ocorre através da interação de proteínas da superfície viral com as cadeias leves do complexo motor Dineína.

A possibilidade de recrutar o transporte retrógrado celular (mimetizando partículas virais) para facilitar o tráfego de material terapêutico pelo citoplasma, através do desenvolvimento de vetores não virais que se associariam com o complexo motor Dineína, foi considerada em alguns poucos trabalhos (Bergen & Pun, 2007; Bergen et al., 2005; Bergen et

al., 2008). De tal forma, novas abordagens e estudos que explorem o transporte retrógrado celular no desenvolvimento de vetores não-virais mostram-se necessários.

Tabela 1.1 - Exemplos de partículas virais que se utilizam do transporte retrógrado celular mediado pelo complexo motor Dineína através da interação com cadeias leves.

Vírus	Interação (cadeia leve de Dineína)	Referência
Rabies virus	Lc8	Raux *et al.*, 2000
Poliovirus	TcTex	Mueller *et al.*, 2002
Mason-Pfizer monkey virus(M-PMV)	TcTex	Vlach *et al.*, 2008
Herpex simplex	TcTex	Douglas *et al.*, 2004

1.5 Cadeias leves de Dineína

As cadeias leves de Dineína mais estudadas na literatura são a Lc8, a TcTex e a Rp3. A Lc8 é amplamente caracterizada na literatura científica, com modelos estruturais resolvidos por cristalografia e diversas análises funcionais indicam que tal proteína possui funções não apenas no complexo Dineína mas também atua no complexo Miosina V, óxido nítrico sintase neural, estando ainda associada com fatores apoptóticos Bim e Bmf e com o fator de transcrição NF-κB em um mecanismo responsivo ao estado oxi-redox da célula (King, 2008). Adicionalmente, diversas moléculas citoplasmáticas foram identificadas como possíveis ligantes de Lc8 (Rodríguez-Crespo et al., 2001, Benison et al., 2007) e proteínas virais como do vírus da raiva foram identificadas interagindo com Lc8 (Raux *et al*., 2000).

As cadeias leves Rp3 e TcTex apresentam elevada identidade (52%) e similaridade (75%) em seqüência de aminoácidos. Contudo, ambas apresentam afinidade por diferentes moléculas carreadas pelo complexo Dineína, indicando que tais cadeias estão envolvidas em diferentes funções do complexo (Tai *et al.*, 1999). É digno de nota que, em células epiteliais polarizadas

de rim canino Madin-Darby, foi demonstrado que as cadeias leves Rp3 e TcTex competem pela mesma cadeia intermediária no complexo Dineína e a superexpressão de Rp3 foi capaz de deslocar cadeias TcTex do mesmo (Tai *et al*., 2001).

Estudos adicionais foram realizados demonstrando, similarmente ao descrito para Lc8, que a Rp3 possui funções dentro da célula que não estão associadas ao motor molecular Dineína. Tal cadeia leve foi encontrada tanto no citosol como no núcleo associada ao fator de transcrição STATB1 e à elementos da matriz nuclear. O complexo protéico rp3-SATB1 foi encontrado associado a uma região do DNA do gene Bcl2 relacionada à adesão na matriz nuclear. Por fim, foi demonstrado que a cadeia leve Rp3 está envolvida na repressão de Bcl2 (Yeh *et al*., 2005).

A estrutura cristalográfica da cadeia leve de Dineína TcTex de *Drosophila melanogaster* foi elucidada (Williams *et al*., 2005) e diversos trabalhos relatam a interação desta cadeia leve com diversas proteínas citoplasmáticas, tais como a kinase Fyn (Campbell *et al*., 1998; Kai *et al*., 1997), a proteína associada a lipídio DOC2 (Nagano *et al*., 1998), a GTPase FIP1 (Lukashok *et al*., 2000), o receptor do poliovírus (Mueller *et al*., 2002), poliproteínas do Mason–Pfizer monkey virus (M-PMV) (Vlach *et al*., 2008), a cauda citoplasmática da rodopsina (Tai *et al*., 1999) entre outros.

1.6 Abordagens experimentais para a avaliação da eficiência de entrega gênica por vetores não-virais

A eficiência de entrega gênica de vetores não-virais é frequentemente avaliada pela capacidade de transfecção *in vitro* de células de mamífero, utilizando-se para isso plasmídeos modelos contendo genes repórteres (por exemplo, *Luciferase* ou *GFP*) cuja expressão é facilmente quantificada. Adicionalmente, de maneira a se obter um critério de comparação, são

utilizados como controles positivos agentes de entrega gênica amplamente caracterizados e utilizados na literatura, como a Lipofectamina ou a protamina. A Lipofectamina 2000^{TM}, formulação lipídica catiônica comercializada pela Life Technologies (Invitrogen), apresenta elevada eficiência de transfecção de diversos tipos celulares tais como Cos-7, HEK293, HeLa e CHO-K1. Já a protamina, mencionada anteriormente no presente estudo, é um peptídeo (5,1 kDa) catiônico cuja capacidade de mediar entrega gênica tem sido estudada, podendo ser utilizado como único vetor de entrega ou em formulações na presença de lipídeos ou outros polímeros (ver tópico 1.2 Vetores virais e não-virais) (Figura 1.7A).

Além da quantificação da transfecção através da expressão de genes repórteres, é frequente a avaliação dos mecanismos de entrada na célula e tráfego intracelular utilizados pelos vetores não-virais de entrega gênica utilizando-se drogas que inibem mecanismos específicos. Por exemplo, para averiguar se de fato a eficiência de entrega gênica de vetores não-virais desenvolvidos é dependente do processo de transporte retrógrado celular, drogas como o nocodazol e a citocalasina D são utilizadas. O **nocodazol** é um composto que apresenta elevada afinidade pelas subunidades de tubulina, bloqueando a polimerização da rede de microtúbulos (Figura 1.7B). Sua utilização permite avaliar a contribuição da rede de microtúbulos, utilizada pelo complexo motor Dineína no transporte retrógrado celular, na eficiência de entrega gênica dos vetores não-virais desenvolvidos. A **citocalasina D**, agente despolimerizante dos filamentos de actina através da sua ligação com a subunidade actina F. Os filamentos de actina estão diretamente relacionados com o processo de endocitose, de tal modo sua utilização permitiu averiguar se os vetores de entrega desenvolvidos no presente estudo dependem do processo de endocitose para sua entrada na célula (Figura 1.7B). Além destes, a **cloroquina** também é uma droga bastante utilizada. Trata-se de uma base fraca que se acumula em vesículas endossomais impedindo sua acidificação quando estas se fusionam com

lisossomas (Figura 1.7B). A protonação da cloroquina diminui sua capacidade de atravessar membranas lipídicas favorecendo seu acúmulo em lisossomas, levando-os à ruptura em decorrência da pressão osmótica gerada. Seu uso permite observar a capacidade de escape de vesículas endocíticas dos complexos de entrega estudados. Outro composto utilizado é a **ciliobrevina D**, trata-se de um inibidor específico da atividade de ATPase das cadeias pesadas de Dineínas citoplasmáticas (Figura 1.7). Tal composto, descrito por Firestone et al. (2012), mostrou ser um eficiente inibidor do tráfego retrógrado mediado por Dineínas. Dessa forma, sua utilização permite averiguar se a atividade motora de Dineínas esta relacionada com a eficiência de entrega dos vetores não-virais desenvolvidos.

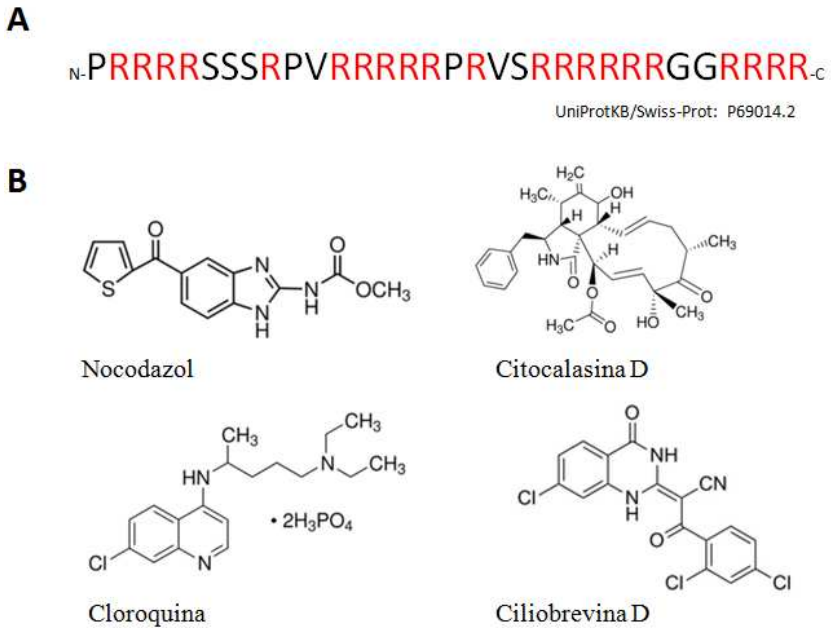

Figura 1.7 - A: Sequência em aminoácidos da protamina utilizada no presente trabalho. **B:** Estruturas moleculares dos compostos químicos utilizados no presente estudo. Fonte: www.sigmaaldrich.com

2. Objetivos

Objetivos gerais

- Desenvolvimento de proteínas de fusão capazes de atuar como transportadoras e facilitadoras do transporte intracelular de vetores plasmidiais, em estudos de terapia gênica e vacinação por DNA.
- Contribuir para o conhecimento dos mecanismos de transporte intracelulares de transgenes através do citoplasma e membrana nuclear, especialmente no que diz respeito ao uso de proteínas recombinantes especificamente desenhadas para esse fim.

Objetivos específicos

- Clonagem e expressão em *E. coli,* e purificação de proteínas de fusão contendo um dominío C-terminal de ligação à proteínas motoras Dineínas (cadeias leve de Dineína) e um domínio ou seqüência N-terminal capaz de interagir com pDNA e favorecer seu translado para o interior do núcleo de células de mamífero.
- Estudo da eficiência das proteínas de fusão no transporte de pDNA em células de mamífero transfectadas.
- Estudo da contribuição dos componentes do citoesqueleto para a eficiência de transfecção dos vetores não-virais desenvolvidos.
- Estudos de microscopia confocal para avaliar a interação dos complexos de entrega não-viral desenvolvidos com a maquinaria de transporte retrógrado celular (Dineína).

3. Estratégia experimental

3.1. Estratégia experimental para o desenvolvimento de vetores não-virais baseados em cadeias leves de Dineína modificadas

Nosso grupo de pesquisa tem se concentrado no desenvolvimento de vetores não-virais de natureza protéica. Assim, o objetivo principal do presente estudo é o desenvolvimento de proteínas recombinantes de fusão capazes de atuar eficientemente como vetores não-virais de entrega gênica. Essas proteínas de fusão devem associar a capacidade de domínios peptídicos ricos em resíduos de arginina (R) e lisina (K) de interagir e compactar moléculas de DNA plasmidial com o transporte retrógrado celular mediado pelo complexo motor Dineína. Tal associação é obtida através do fusionamento dos domínios peptídicos catiônicos à região N-terminal de cadeias leves de Dineína (Figura 3.1). Ao recrutar o motor celular Dineína para mediar o transporte do material genético exógeno, espera-se que este ocorra de maneira mais eficiente permitindo que um maior número de moléculas de DNA chegue ao núcleo celular intactas resultando em maior eficiência de transfecção.

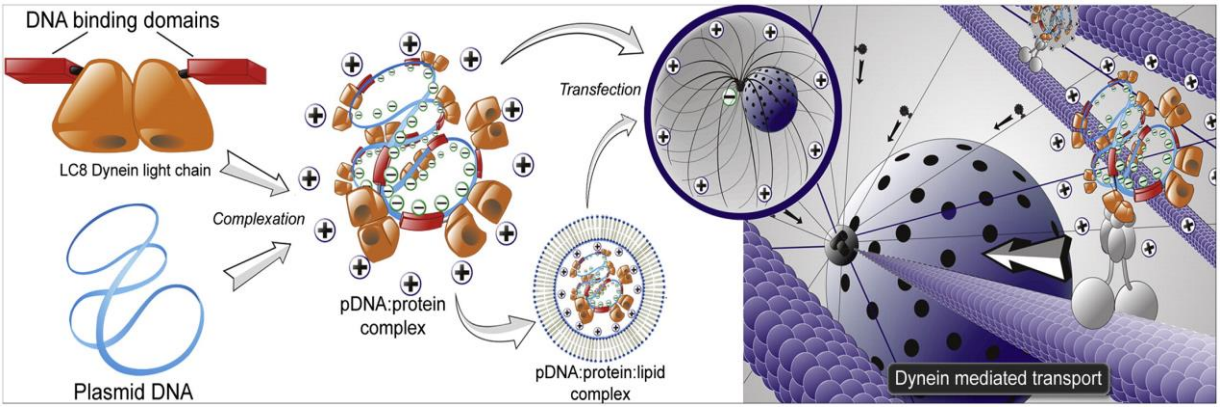

Figura 3.1 - Esquematização da abordagem adotada no presente trabalho para o desenvolvimento de vetores não-virais baseados em cadeias leves de Dineína. Através do fusionamento de domínios de ligação ao DNA ao N-terminal de cadeias leves de Dineína estas seriam capaz de simultaneamente formar complexos com DNA plasmidial e mediar seu tráfego através do citoplasma interagindo com o motor molecular Dineína. A utilização de lipídicos catiônicos também pode ser adotada na formação de complexos ternários.

"Development of a recombinant fusion protein based on the dynein light chain LC8 for non-viral gene delivery"

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Artigo publicado na Revista Journal of Controlled Release, Volume 159 (2) (2012), Páginas: 222–231.

GENE DELIVERY

Journal of Controlled Release 159 (2012) 222–231

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Development of a recombinant fusion protein based on the dynein light chain LC8 for non-viral gene delivery

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ARTICLE INFO

Article history:
Received 21 September 2011
Accepted 12 January 2012
Available online 20 January 2012

Keywords:
Non-viral gene delivery
LC8 dynein light chain
Microtubules
DNA vaccines
Gene therapy

ABSTRACT

The low efficiency of gene transfer is a recurrent problem in DNA vaccine development and gene therapy studies using non-viral vectors such as plasmid DNA (pDNA). This is mainly due to the fact that during their traffic to the target cell's nuclei, plasmid vectors must overcome a series of physical, enzymatic and diffusional barriers. The main objective of this work is the development of recombinant proteins specifically designed for pDNA delivery, which take advantage of molecular motors like dynein, for the transport of cargos from the periphery to the centrosome of mammalian cells. A DNA binding sequence was fused to the N-terminus of the recombinant human dynein light chain LC8. Expression studies indicated that the fusion protein was correctly expressed in soluble form using *E. coli* BL21(DE3) strain. As expected, gel permeation assays found the purified protein mainly present as dimers, the functional oligomeric state of LC8. Gel retardation assays and atomic force microscopy proved the ability of the fusion protein to interact and condense pDNA. Zeta potential measurements indicated that LC8 with DNA binding domain (LD4) has an enhanced capacity to interact and condense pDNA, generating positively charged complexes. Transfection of cultured HeLa cells confirmed the ability of the LD4 to facilitate pDNA uptake and indicate the involvement of the retrograde transport in the intracellular trafficking of pDNA:LD4 complexes. Finally, cytotoxicity studies demonstrated a very low toxicity of the fusion protein vector, indicating the potential for *in vivo* applications. The study presented here is part of an effort to develop new modular shuttle proteins able to take advantage of strategies used by viruses to infect mammalian cells, aiming to provide new tools for gene therapy and DNA vaccination studies.

1. Introduction

Gene therapy and DNA vaccination protocols demand efficient and safe mechanisms to deliver therapeutic genetic material to the patient cell nucleus. So far, viral-based vectors have been preferred as delivery vehicles since they are naturally efficient in receptor-mediated recognition and cell internalization, endosomal escape, nuclear transport and DNA integration [1,2]. Nevertheless, the use of viruses as gene delivery systems continues to rise safety concerns and the future development of viral gene therapy continue to generate intense scientific debates [2,3]. The alternative approach, non-viral vectors, is considered safer and has been also attracting significant attention of the scientific community. Efforts have been made to increase the delivery efficiency of non-viral vectors, including the creation of sophisticated vehicles able to mimic some of the viral properties regarding both size and biological properties [2]. These vectors are called "artificial viruses" and include polymeric constructs [4–6], protein-only shells and virus-like particles [4]. Among the properties of these vectors are the ability to condense and protect DNA from nuclease degradation, low systemic and cellular toxicity, membrane crossing abilities, and steady expression of the therapeutic gene [7]. However, the inability of these vectors to efficiently traverse the target cell cytoplasm and reach the nucleus has largely been overlooked [8]. It has been also reported that non viral vectors face several extra- and intracellular barriers before DNA can be delivered to the cell's nucleus [9] and that cytosolic proteins may bind to the delivery complex acting as another barrier [10].

The majority of the non-viral vectors studied so far rely on passive diffusion or non specific transport for trafficking within the cytoplasm, and this limited mobility represents a significant barrier to gene delivery [11]. Since it has been shown that diffusion of DNA

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doi:10.1016/j.jconrel.2012.01.011

fragments larger than 2000 base pairs through the crowded cytoplasmic environment is greatly restricted [12], an ideal vector should, like most viruses, include the ability to exploit the host cell machinery to rapidly traverse the cytoplasm. Many authors have suggested that an ideal form to actively transport drugs, including transgenes, from the site of cytoplasmic entry to the nuclear periphery would include recruitment of the minus end-directed motor dynein [8,13–15]. Cytoplasmic dynein is a multisubunit protein complex (~1.2 MDa) composed of two heavy chains (~530 kDa) responsible for microtubule attachment and ATP hydrolysis [16], two 74-kDa intermediate chains (IC74), four light intermediate chains (52–61 kDa), and several light chains (10–25 kDa) which are responsible for cargo attachment to the dynein motor complex and hence, its transport through the cytosol toward the nucleus [17,18]. However, so far no successful strategies to exploit motor proteins' retrograde transport for efficient gene delivery have been demonstrated. In an attempt to design synthetic gene carriers that display dynein-binding peptides for enhanced intracellular transport, Bergen and Pun (2007) studied the use of a peptide that binds to the dynein light chain LC8 subunit, as the first potential dynein-binding peptide [8]. It was demonstrated that, while the peptide readily bound free LC8, it could not bind to dynein-associated LC8, emphasizing the need to identify or design peptides that could mediate binding to the intact dynein motor complex. More recently, Moseley and collaborators (2010) reported that protein transduction can be enhanced by attachment to a dynein light chain association sequence [15]. These sequences were able to enhance nuclear accumulation of GFP-fusion proteins, with dependence on the LC8/microtubule (MT) network. This work provided the first successful evidence that dynein/MT-association can be exploited for DNA or drug delivery approaches.

Here, we propose the use of the dynein light chain LC8 itself as a cargo adaptor for plasmid delivery into mammalian cells, taking advantage of the dynein retrograde transport *via* the MT network. LC8, also called DYNLL1, is a small (10 kDa) and highly conserved globular protein reported as an essential component of the dynein and myosin V molecular motors [19,20]. The LC8 binds as a dimer directly to specific sites on the dynein intermediate chain IC74 or myosin V heavy chain, while some studies indicate additional roles for LC8 in multiple protein complexes unrelated to the motor proteins such as p53-binding protein 1 [21], neuronal nitric oxide synthase [22], the proapoptotic member of the Bcl-2 family proteins Bim and Bmf [23,24], the product of the *Drosophila* swallow gene [25], and a number of proteins with unknown functions [26]. We envision that LC8 could be modified with short DNA-binding sequences, rich in positively charged amino acids, which would interact and condense pDNA and facilitate its transport toward the nucleus periphery *via* interaction with the dynein complex. Finally, the work presented here intends to generate new information on plasmid delivery and presents a new strategy for the development of modular non-viral vectors potentially useful for gene therapy and DNA vaccination.

2. Materials and methods

2.1. Plasmid DNA vector

In the present study, a plasmid DNA model named pVAX1-Luc was constructed based on the previously reported pVAX1-GFP plasmid [27]. Using XbaI and EcoRI restriction endonucleases, the sequence coding for GFP was replaced by the luciferase gene obtained from the pGL3-Luc control vector (Promega) using the same restriction enzymes. The success of the reporter gene replacement was confirmed by the expression of the reporter enzyme after transfection of HeLa cells, as described in this work. Purification of the pVAX1-Luc plasmid used in all transfection studies was performed as described by Freitas and co-workers [28].

2.2. DNA binding domains design

The fusion protein DNA binding domains were designed based on the scientific literature available [2,29] for peptides and protein domains with high DNA binding and condensing capacity. We designed four domains to be cloned upstream of human dynein light chain LC8: DNAb1, WRRRGHGKKK; DNAb2, WRRRGFGKKK; DNAb3, WRRRGHGRRR; and DNAb4, WRRRGFGRRR. The corresponding single strand oligonucleotides were synthesized with optimized codons for *E. coli* expression and annealed before cloning. The DNA binding domains were cloned in the *NdeI* and *BamHI* sites in the pET28a expression vector (Novagen, Darmstadt, Germany). The clones containing the DNA binding domains were further used for LC8 cloning in the *BamHI* and *XhoI* restriction sites. In this work we define as LD4 the recombinant human LC8 fused to the DNA binding domain 4 (DNAb4), described above.

2.3. Recombinant proteins expression and purification

The human dynein light chain LC8 was amplified from HeLa cDNA with specific primers (forward:5′-ATAGGATCCATGTGCGACCGAAAG-3′, reverse: 5′ATACTCGAG TTAACCAGATTTGAACAGAAGA-3′) and cloned into pET28a with and without DNA binding domains previously cloned at the N-terminal. Recombinant LC8 with or without DNA binding domains were expressed in *E. coli* BL21(DE3). Briefly, cells were grown in 1 L LB medium at 37 °C, 300 rpm and up to an optical density of 0.8 AU (600 nm). Protein expression was induced with 5.6 mM lactose or 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for further 12 h at 25 °C, 200 rpm. After centrifugation, the cell pellet was suspended in 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 15 mM β-mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride). Cell lysis was performed by sonication and the recombinant LC8 was purified by a single Ni-NTA affinity chromatography step. This method was successfully used to purify recombinant LC8 with and without the four different DNA binding domains.

For *in vitro* interaction of recombinant dynein light chain LC8 and LD4 with human dynein intermediate chain DYNIC2 its N-terminal domain (the first 300 amino acids) and the dynein light chain TcTex were cloned using the same methodology as described for LC8. The DYNIC2 domain was amplified with specific primers from HeLa cDNA and cloned into pET28a using *NdeI* and *BamHI* restriction sites (forward, 5′-ATTCATATGATGTCAGACAAAAGTGAATT-3′, reverse, 5′-ATTGGATCCTTAGTTATAGGAAGCCAC-3′). The TcTex light chain was inserted in *BamHI* and *XhoI* restriction sites (forward, 5′-ATAGGATCCATGGAGGAGTACCATCG-3′, reverse, 5′-ATACTCGAGTTAAAGAACAATAGCAATGG-3′). Both recombinant proteins were expressed in *E. coli* BL21 (DE3) Rosetta strain. The expression of the N-terminal DYNIC2 was induced with 0.2 mM IPTG at 20 °C and 200 rpm for 12 h. For all recombinant proteins, purity was evaluated by SDS-PAGE and concentration was measured by absorbance at 280 nm.

2.4. Circular dichroism studies

Circular dichroism (CD) spectra of the purified recombinant proteins were obtained using a Jasco J-810 Spectropolarimeter dichrograph (Japan Spectroscopic, Tokyo, Japan). The far-UV CD spectra were generated at 20 °C using 10–20 μM of each protein in 10 mM sodium phosphate buffer pH 8.0. The assays were carried out using a quartz cuvette with a 1 mm path length. Ten accumulations within the 185–260 nm range at a rate of 50 nm/min were recorded. Data was processed using OriginLab 8.0 software.

2.5. Size exclusion chromatography

To assess the oligomeric state of purified LC8 with and without DNA binding domains, size exclusion chromatography was performed using a Superdex 200 GL10/300 or Superdex 75 GL10/300 prepacked column

(GE Healthcare, Uppsala, Sweden). After equilibration of the column with extraction buffer or PBS (8.0 g NaCl, 0.2 g KCl, 1.4 g Na_2HPO_4, 0.24 g $KH2PO_4$, pH 7.4 per liter), oxidized and reduced (10 mM DTT) samples (250 μl) were loaded at a flow rate of 0.75 ml/min. The calibration curve was prepared using High and Low molecular calibration kits (GE Healthcare). Gel permeation chromatography was also used to evaluate LC8-TcTex-Intermediate chain complex formation.

2.6. In vitro interaction of recombinant dynein light chains LC8 and TcTex with dynein intermediate chain 2, isoform C

In order to evaluate the ability of recombinant LC8 and LD4 to interact *in vitro* with the human dynein intermediate chain DYNIC2, isoform C, the N-terminal of this protein (first 300 amino acids) was cloned, expressed and purified as described. We also used in this assay the TcTex light chain since its presence in the complex may enhance the interaction between LC8 and the intermediate chain, as discussed in the Results and discussion section. LC8 (19.2 μM) or LD4 (8.9 μM) and TcTex (6.3 μM) light chains were incubated with purified intermediate chain domain, IC_{DOM} (7.23 μM), at 8 °C for 20 h under reducing conditions (10 mM DTT). The purified proteins were used immediately after purification procedure and complex was formed in 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 0.1 mM EDTA and approximately 200 mM imidazole. Complex formation was evaluated by gel permeation chromatography using a Superdex 75 GL10/300 prepacked column (GE Healthcare, Uppsala, Sweden) and collected samples corresponding to the complex elution peaks were concentrated (10-fold concentration) using a Amicon Ultra Centrifugal Filter (3 kDa) (Millipore, Ireland) and visualized by SDS-PAGE.

2.7. Evaluation of DNA-protein interaction by gel retardation assay

To evaluate the ability of LC8, LC8 with different DNA binding domains and protamine to interact and condense pDNA, we performed a gel retardation assay. Proteins were dialyzed in PBS (protamine sulfate powder was ressuspended in PBS) and incubated with 250 ng of pVAX-Luc vector (previously in PBS) at different pDNA:protein molar ratios (1:400, 1:800, 1:1000, 1:2000, 1:4000 and 1:8000) in a final volume of 50 μL. The samples were incubated at room temperature for 1 h following by the addition of 50 μL of non supplemented F-12 media and additional incubation for 20 min. Samples were run in a 0.8% agarose gel and visualized by ethidium bromide staining.

2.8. Zeta potential and particle size assays

Zeta potential measurements were performed to comparatively evaluate the surface charge of complexes formed by pDNA:LC8 and pDNA:LD4. Complexes were formed as described for gel retardation assay but without F-12 media addition and using the same pDNA:protein molar ratios. Each sample was measured in triplicate using Zetasizer 3000 (Malvern, England). Particle size measurements were performed to evaluate the behavior of different complexes regarding its size along time. pDNA:protamine (pDNA:protein molar ratio of 1:8000) and pDNA:LD4 (pDNA:protein molar ratio of 1:100 and 1:8000) complexes with and without Lipofectamine™ and pDNA:Lipofectamine™ complexes were formed by 1.0 μg of pDNA and the corresponding amount of protein in a final volume of 800 μL. When indicated, 1.5 μL of Lipofectamine™ was added to the complex. Each sample was submitted to multiple readings during a 60 min period. Particle diameter was plotted against time for each sample.

2.9. Atomic force microscopy (AFM)

Plasmid DNA:protein complexes at different molar ratios (1:400, 1:1000 e 1:2000 for LC8 and 1:100, 1:200 e 1:500 for LD4) were prepared in 10 mM Tris–HCl buffer pH 7.4 in a final DNA concentration of 600 pg/μl by incubation at room temperature for 1 h. After complex formation, a final concentration of 5 mM $MgCl_2$ was added to each sample and 60 μl of the solution was physisorbed for 1 min on freshly cleaved muscovite mica (Ted Pela, California, USA). After adsorption, the surface was washed for 10 s in ultrapure water and dried in a weak nitrogen stream. The AFM imaging was performed in air a room temperature in acoustic mode at a scanning speed of 300 nm/s with an Agilent 5500 (Agilent, Santa Barbara, USA) using commercial silicon cantilevers (MicroMash, NSC-14/ALBS) with a tip radius of approximately 10–20 nm. The topography images were treated using the Open-Source software Gwyddion (www.gwyddion.net).

2.10. Culture and transfection of HeLa cells

HeLa cells were grown in F-12 (Ham) nutrient mixture (Gibco, UK) containing 10% (v/v) fetal bovine serum (growth medium, Gibco, UK). All cell cultures were performed in 75 cm^2 culture flasks and incubated in 5% CO_2 humidified environment at 37 °C. Following growth up to confluence, cells were trypsinized and seeded in 24 well culture plates (5×10^4 cells per well). The cells were incubated for 48 h (to a 70% confluence) and then transfected with pDNA:protein complexes formed as described for gel retardation assays and using the same molar ratios. When indicated, transfection was carried out using the Lipofectamine 2000™ reagent (Invitrogen, USA) according to the manufacturer instructions (1.0 μg pDNA plus 1.5 μL reagent in 100 μL of medium per well), protamine sulfate (Sigma Aldrich, Germany) or PEI (polyethylenimine, branched, MW ~25,000) (Sigma Aldrich, Germany). The medium containing the transfection solution remained on the cell containing wells for 6 h and was then replaced by regular growth medium. Cells were collected after 24 h post-transfection for determination of luciferase activity using the luciferase Assay System (Promega, USA), following the manufacturer's instructions. Luminescence intensity was normalized against protein concentration in each transfection sample, as determined by the Micro BCA Protein Assay Kit (Thermo Scientific, USA).

To evaluate the contribution of the microtubule network and actin filaments in the intracellular trafficking of the complexes, cells were pre-incubated for 2 h with nocodazole (25 μM) or cytochalasin D (25 μM) for microtubule and actin filaments disruption, respectively. Both drugs were dissolved in DMSO and an equal volume of drug-free DMSO was used as control (0.5% for cytochalasin D and 0.4% for nocodazole). Transfections in the presence of chloroquine were performed to evaluate the contribution of lysosomal degradation of protein-pDNA complexes. Cells were pre-incubated for 4 h with chloroquine (100 μM). For all assays with the mentioned drugs, pre-treated cells were incubated afterwards in the presence of the different complexes for 4 h, when the medium was replaced by freshly growing medium. After 24 h, cells were collected and the luciferase activity was assayed as described above.

Cytotoxicity assays of delivery vectors studied in the present work were performed using Cell Proliferation Reagent WST-1 (Roche Applied Science, USA) following manufacturer's instructions. Briefly, HeLa cells were grown on 96 wells plates to a confluence of 70%. Transfection was performed as described before with pDNA:protein complexes (molar ratio of 1:8000) with and without Lipofectamine™, with pDNA:Lipofectamine™, and also with naked pDNA as control. Finally, 10 μL of the WST-1 reagent was added to each well, and cells were further incubated for 2 h. Absorbance was read after 60 s agitation at 440 nm using a Spectramax 384 Plus UV/VIS Microplate Reader (Molecular Devices, USA).

3. Results and discussion

3.1. Recombinant human dynein light chain LC8 was successfully produced with N-terminal DNA binding domains

Using the methodology described above we were able to produce recombinant LC8 and recombinant LC8 fused to DNA binding domains

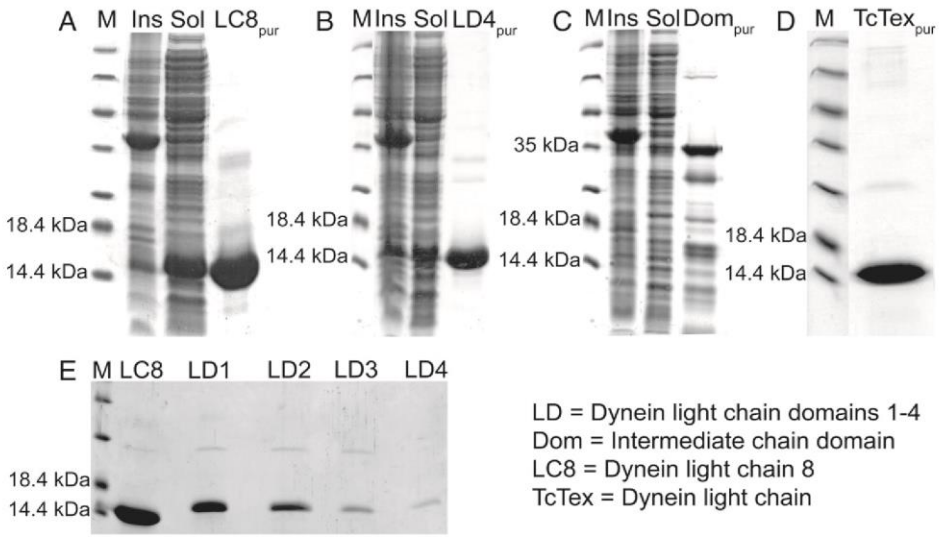

Fig. 1. SDS-PAGE analysis of purified recombinant proteins used in the present study. A: Purified LC8. B: Purified LD4. C: Purified intermediate chain N-terminal domain. D: Purified TcTex. E: LC8 and LC8 with the four different DNA binding domains at the N-terminal after dialysis against PBS. M: Broad Range molecular weight marker (Fermentas). Ins: Insoluble fraction after cell lysis. Sol: Soluble fraction after cell lysis. $LC8_{pur}$: purified LC8. $LD4_{pur}$: purified LD4. Dom_{pur}: purified N-terminal domain of DYNIC2 intermediate chain. $TcTex_{pur}$: purified TcTex. LC8, LD1,LD2,LD3,LD4: LC8 without and with each DNA binding domain.

(DNAb1, DNAb2, DNAb3 and DNAb4), all proteins efficiently expressed in *E. coli* BL21(DE3) strain. All constructs were obtained in the soluble fraction after cell disruption and purified by a single Ni-NTA affinity chromatography step (Fig. 1). Circular dichroism analysis of the secondary structures of the proteins showed that addition of the DNA binding domains had little effect on the secondary fold of LC8 probably due to the addition of the domain to its N-terminal (Fig. 2 and Supplementary Data). The oligomeric state of LC8 and LC8 fused with DNAb4 (LD4) was evaluated in phosphate saline buffer (PBS) by gel permeation chromatography under reduced and non reduced conditions. We could observe that the human LC8 is dimeric under reducing conditions, as reported for the *Drosophila* homologue [30], and appears as a tetramer under non reducing conditions (results not shown). The LD4 presented a higher propensity to aggregate under low ionic strength (such as in PBS), as judged by the fact that a significant portion of the purified protein precipitated during dialysis against PBS, even under reducing conditions. Gel permeation chromatography shows that LD4 in PBS under reducing and non reducing conditions is polidispersed in diverse oligomeric subpopulations. However, analysis of small angle X-ray scattering (SAXS) data for Lc8 and LD4, collected in 50 mM sodium phosphate, 500 mM NaCl, 0.1 mM EDTA and 500 mM Imidazole, allowed us to assume that the recombinant proteins were folded in solution under reducing conditions. A bell shaped Kratky plot was observed, which is characteristic of folded proteins (Supplementary Data). Therefore, the observed aggregation for LD4 may be due to the relative low ionic strength of PBS. In summary, our results indicate that the recombinant LC8 and its variations with different DNA binding domains were successfully and correctly expressed and purified (Fig. 1) allowing us to proceed with pDNA interaction and transfection assays. It is noteworthy that LD4 (as well with other DNA binding domains), despite appearing as a population of aggregates in gel permeation assays, was successfully used in pDNA condensation assays, indicating that upon DNA addition, the protein aggregates were disrupted allowing protein interaction with the pDNA.

3.2. Recombinant LC8 and LD4 are able to interact in vitro with human dynein intermediate chain

The human LC8 dynein light chain interacts with the dynein intermediate chain and some other proteins not related to the dynein motor complex as mentioned before (see Introduction). Additionally, the interaction between LC8, TcTex and the intermediate chain IC74 is well characterized for the *Drosophila* homologues. In a recent work, Hall and collaborators (2009) showed that when one of the light chains (LC8 or TcTex) is previously bound to the intermediate chain IC74, the binding of the second light chain is enhanced by 50-fold [31]. In the context of the gene delivery mechanism envisioned in

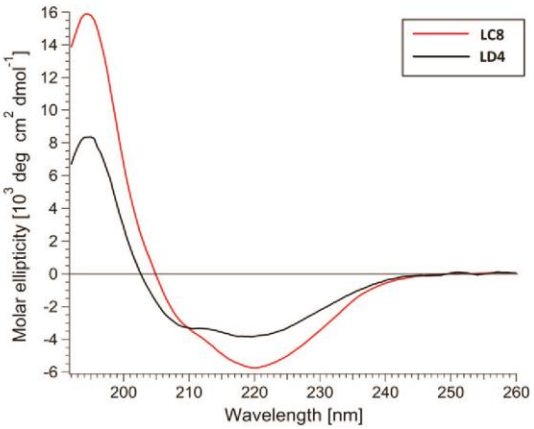

Fig. 2. Circular dichroism curves for dynein light chain LC8 and for LC8 fused with DNA binding domain 4. LC8: LC8 dynein light chain. LD4: LC8 dynein light chain fused with DNAb4 binding domain. Little variation between the spectra can be observed probably due to the addition of the DNA binding domain.

the present work, the recombinant dynein light chain LC8, whether fused or not to DNA binding domains, should be able to interact with the dynein machinery. Thus, in order to analyze the functionality of recombinant LC8 and LD4, we assayed their interaction with the N-terminal domain (300 first amino acids) of the human dynein intermediate chain 2, isoform C (DYNIC2), in combination with TcTex dynein light chain. We could observe that both, LC8 and LD4 were able to interact *in vitro* with dynein intermediate chain by gel permeation chromatography and subsequent SDS-PAGE analysis of the eluted peak (Fig. 3). This result shows that the recombinant LC8 light chain, with or without the N-terminal DNA binding domain retains its functionality once it is able to interact with human intermediate chain *in vitro*.

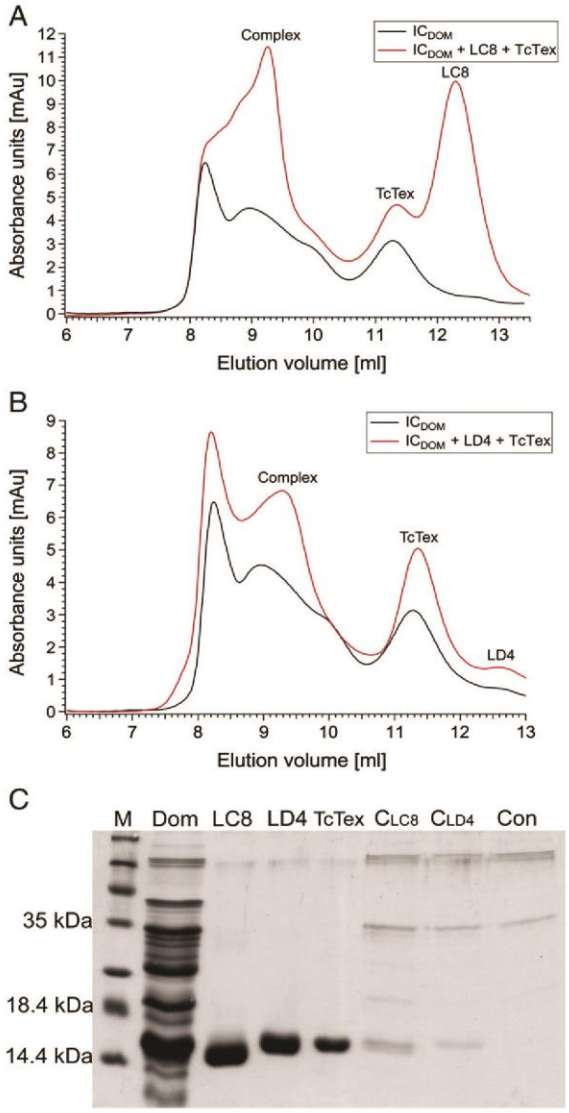

Fig. 3. Interaction of recombinant LC8 and LC8 with DNA binding 4 with the N-terminal domain of DYNIC2 and with the dynein Light Chain Tctex. A: Gel permeation chromatography elution curves for LC8-DYNIC2-TcTex. B: Gel permeation chromatography elution curves for LD4-DYNIC2-TcTex. C: SDS-PAGE 15% for elution samples from gel permeation chromatography showing that DYNIC2 and Light Chains are eluted together indicating interaction. Dom: purified N-terminal domain of Intermediate Chain (premature translation end can be observed for this protein). LC8: purified LC8. LD4: purified LD4. TcTex: purified TcTex. C_{LC8}: elution peak collected at 9 ml and concentrated 10 fold for the LC8-DYNIC2-TcTex complex. C_{LD4}: elution peak collected at 9 ml and concentrated 10-fold for the LD4-DYNIC2-TcTex complex. Con: Control experiment, elution peak collected at 9 ml and concentrated 10-fold for DYNIC2 alone.

3.3. Addition of DNA binding domains at the N-terminal of LC8 light chain leads to enhanced interaction and condensation of plasmid DNA

A gel retardation assay was performed to evaluate the effect of DNA binding domains on the LC8 ability to interact and condense pDNA. We tested the same gradient of DNA:protein molar ratio for all DNA binding constructs, for LC8 alone and used protamine as control. Despite being non quantitative, this assay clearly shows an enhanced capacity of LC8 with DNA binding domains to interact and condense pDNA when compared to LC8 alone or to protamine (Fig. 4). At low pDNA:protein molar ratios (1:400), shifted bands can be observed for all constructs with DNA binding domains whereas only at higher molar ratios (1:2000) some shift can be observed for LC8 alone and for protamine. In addition, at higher molar ratios (1:4000), uncomplexed pDNA can still be observed for LC8 whereas for LC8 with DNA binding domains, all pDNA is complexed and has its migration retarded.

3.4. LC8 with DNA binding domain 4 interacts with pDNA generating positively charged complexes

An efficient vector for gene delivery must mediate the condensation and uptake of the genetic material by the cell. Specifically, the delivery vector must help to overcome the charge incompatibility between the negatively charged cell surfaces and pDNA molecules, by generating complexes with a positive net charge. We thus evaluated the surface charge of complexes formed between pDNA and LC8 or LD4. Zeta potential of pDNA-protein complexes was measured at different pDNA:protein molar ratios (Fig. 5A). The plot of zeta potential against each molar ratio shows that the addition of the DNA binding

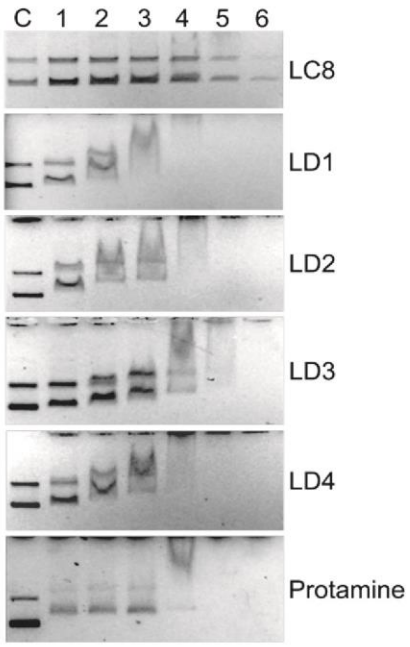

Fig. 4. Protein–DNA interaction analysis by gel retardation assay. The ability of protamine, LC8 and LC8 fused to DNAb1 (LD1), DNAb2 (LD2), DNAb3 (LD3) and DNAb4 (LD4) binding domains to interact and condense plasmid DNA was analyzed by a gel retardation assay. Six pDNA:protein molar ratios were used (lanes 1 to 6): 1:400, 1:800, 1:1000, 1:2000, 1:4000 and 1:8000. C: control pDNA with no protein.

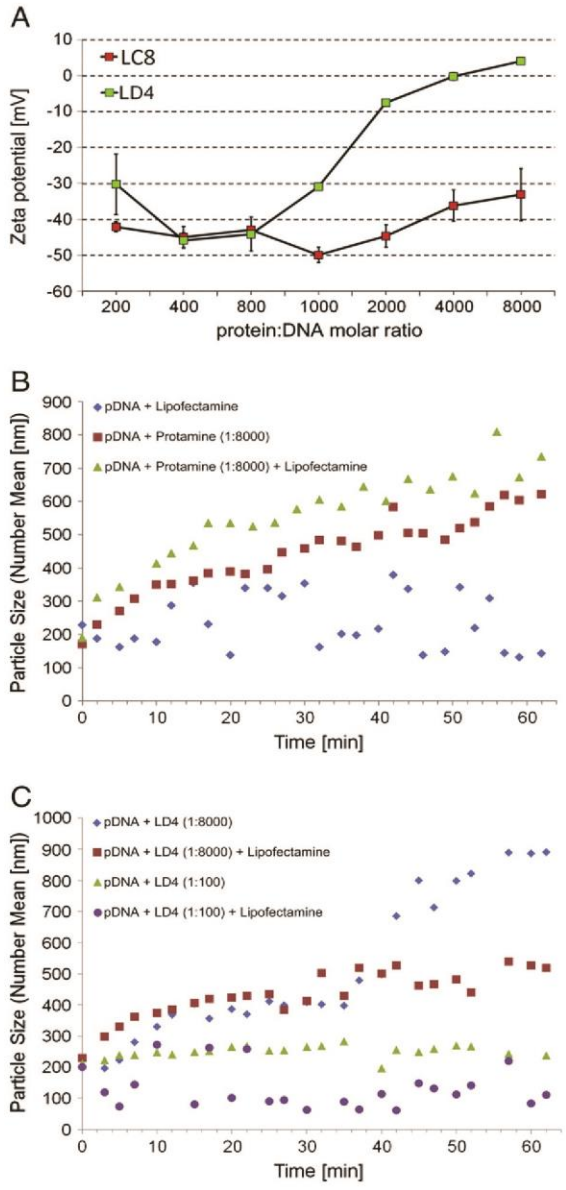

Fig. 5. A: Net surface charge of pDNA complexes formed by LC8 and LC8 fused to DNA binding domain 4 (LD4). The zeta potential of pDNA:protein complexes was measured at seven different molar ratios (1:200, 1:400, 1:800, 1:1000, 1:2000, 1:4000 and 1:8000). LD4 clearly shows higher ability to form positively charged complexes at molar ratios above 1:4000, while LC8 alone generated negatively charged complexes in all conditions tested. B and C: Time course of particle size for the different pDNA:delivery vector complexes studied.

sequence 4 to LC8 enabled the formation of complexes with positive net surface charge when a molar ratio above 1:4000 was used. For complexes formed with LC8 without DNA binding sequence, the net surface charge remained negative in all molar ratios tested.

We also monitored the particle size of pDNA:protamine (1:8000 molar ratio) and pDNA:LD4 (1:8000 and 1:100 molar ratio) with and without Lipofectamine™ and pDNA:Lipofectamine™ complexes during 60 min of incubation at room temperature (Fig. 5B and C). This analysis showed that addition of Lipofectamine™ to pDNA:protein complexes (LD4 or protamine) slightly increased the particle size, especially in the first 40 min of incubation. After that, pDNA:LD4 complexes, in especial, presented a sharp increase in size, probably due to aggregation caused by the lack of electrostatic repulsion, as indicated by the low values of zeta potential. Interestingly, the smallest complex which also showed no significant variation during the analysis was pDNA:LD4 (1:100) with Lipofectamine™. Particle size and particle surface charge are two main factors that dictates internalization of the delivery complex by the cell and is directly correlated to transfection efficiency [32,33]. Thus, by monitoring both variables we are able to correlate and perhaps explain the results found during transfection.

In order to evaluate the pDNA condensation, we performed atomic force microscopy (AFM) assays. The methodology allowed us to visualize the effect of the DNA binding domain 4 on the LC8 light chain affinity for the pDNA molecule. As it can be observed (Fig. 6), LD4 presented enhanced capacity to interact and condense plasmid DNA in contrast to LC8. In excess of both pDNA binding proteins, the pDNA:protein complexes form nearly spherical particles. In comparison, LD4 condenses pDNA to compact particles with sizes in the range of 75 ± 8 nm at lower molar ratios (1:500) than LC8 (1:2000) with size in the range of 101 ± 9 nm. However, it is important to note that the complexes observed by this method were formed at a lower ionic force and different buffer salt (10 mM Tris–HCl buffer) than those shown in gel retardation and Zetasizer experiments (PBS). The excess of salt in PBS buffer strongly affects the AFM imaging procedure. Despite the difference in ionic strength that probably lead to DNA condensation at lower pDNA:protein ratios, the AFM images clearly indicate the effect of the DNA binding domain.

3.5. Transfection experiments demonstrated that LD4 is able to deliver pDNA to HeLa cells

After the interaction tests *in vitro*, we assayed the ability of the fusion protein LD4 to deliver pDNA to HeLa cells in culture. Firstly, we tested complexes formed by pDNA and recombinant LC8 with and without the four different DNA binding domains. These preliminary transfections assays showed that LD4 was the most efficient shuttle protein among all the constructs (results not shown). Therefore, we focused our efforts on LD4, and used LC8 and the arginine rich protamine as controls. The results show that LD4 is more efficient in cell transfection when compared to protamine (Fig. 7A), a well characterized nuclear protein that is known for its ability to condense DNA molecules, facilitate DNA uptake by the cells, and transposition of the nuclear barrier due to presence of a nuclear localization signal [34,35]. LD4 mediated transfection presented a 30-fold higher luciferase expression compared to protamine at the same pDNA:protein molar ratio (1:8000), while LC8 presented even lower efficiency (4-fold lower than protamine). It is noteworthy that pDNA:LD4 (1:8000) complexes formed particles with bigger size than pDNA:protamine (1:8000) complexes as shown by size measurements and a mildly positive surface charge, as shown by Zeta potential measurements. Therefore, the observed enhanced transfection efficiency presented by pDNA:LD4 complexes compared to protamine complexes might be related to an additional property of the recombinant LD4 that facilitates pDNA delivery. Interestingly, when pDNA:LD4 complexes were formed at a molar ratio of 1:16,000, the transfection efficiency dropped 3-fold. It is possible that the decrease in transfection efficiency was caused by protein saturation and formation of bigger complexes by non-specific protein–protein interaction leading to a lower complex uptake by the cell [36].

We also tested multicomponent complexes formed by pDNA and protein (LC8, LD4 and protamine), Lipofectamine™ or PEI (Fig. 7B). Cationic lipids like Lipofectamine™ are known as efficient pDNA delivery vectors in culture cells, enabling the internalization of several thousand pDNA copies per cell during *in vitro* transfection [34]. As expected, binary complexes formed by pDNA and Lipofectamine™

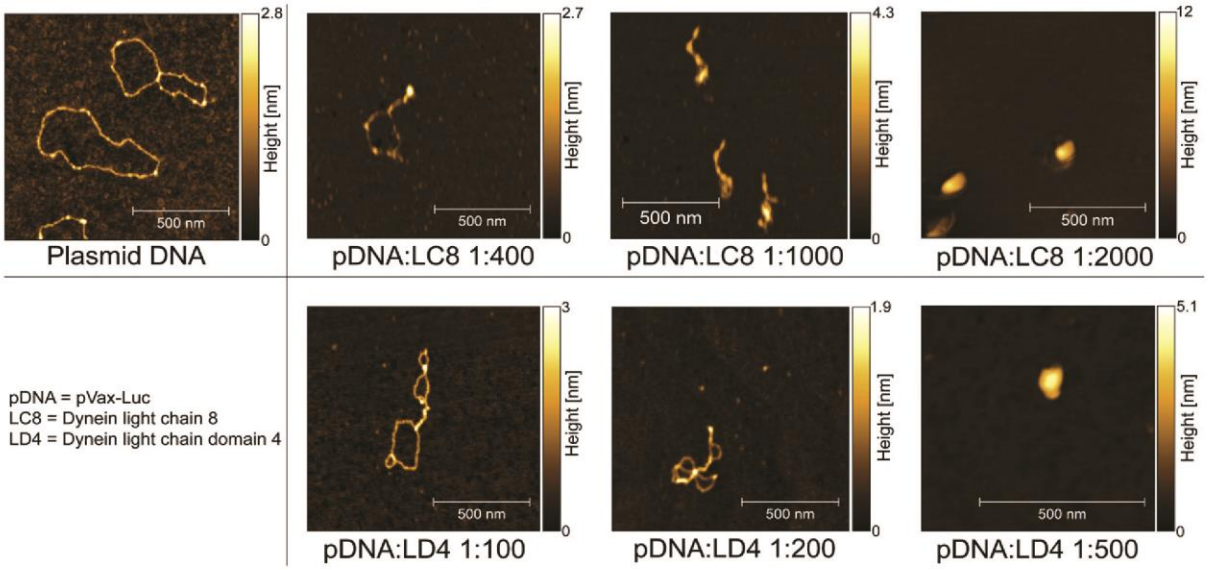

Fig. 6. Visualization of pDNA:LC8 and pDNA:LD4 complexes by atomic force microscopy (AFM). pDNA:protein complexes formed at different molar ratios (1:400,1:1000 and 1:2000 for LC8 and 1:100, 1:200 and 1:500 for LD4) allowed us to visualize the condensation process mediated by the protein. This methodology clearly shows that addition of a DNA binding domain 4 to the LC8 Dynein Light Chain enhanced its ability to interact and condense plasmidial DNA.

promoted higher transfection efficiencies than pDNA:protein complexes (using either LD4 or protamine). Interestingly, ternary complexes formed by pDNA, Lipofectamine and LD4 presented even more efficient pDNA delivery. In this case, we observed that in the presence of Lipofectamine™, complexes formed at 1:100 pDNA:protein molar ratio were more efficient than those formed at 1:8000. One possible explanation is that addition of Lipofectamine™ to the already compact complex formed by pDNA:LD4 (1:100) lead to the formation of more stable and even smaller complexes of approximately 100 nm, as can be seen in Fig. 5.

It is interesting to note that the addition of protamine to the ternary complex (1:8000:8000 pDNA:LD4:protamine), resulting in the formation of quaternary complexes, restored the transfection efficiency, indicating that synergic effects between the lipid and proteins are responsible for the increase in transfection efficiency. The increase in transfection efficiency promoted by protamine in multicomponent complexes have already been reported and was credited to its nuclear localization signal [35,37]. Despite this may also be the case of LD4, the observed increase in pDNA delivery of complexes containing LD4 at a low molar ratio (1:100) can also be explained by changes in complex stability, size, and the ability to overcome different barriers to the intracellular trafficking, including the recruitment of dynein motors. Synergic effects resulted from the combination of proteins and the cationic polymer PEI were also observed (Fig. 7B). Therefore, these multicomponent complexes may combine the high capacity of pDNA internalization of the cationic lipids or polymers with the enhanced capacity of transport through the cytoplasm and nuclear entrance of the protein vectors.

3.6. Cytoskeleton and endossome/lysosome involvement in the transfection efficiency of pDNA:LD4 complexes

Our expectation on the study of LD4 protein for pDNA delivery is set on the possibility that this protein could mediate pDNA trafficking throughout the cytoplasm *via* dynein retrograde transport following escape from endosomes. The insertion of a DNA binding domain at the protein N-terminus was decided based on the structural data of the LC8 protein, since interaction with the dynein motors occurs *via* C-terminus and dimer interface [38]. Since the dynein motor complex relies on the microtubule network to mediate the cellular transport, the use of microtubule depolymerizing agents, such as nocodazole, would abort the active transport resulting in the decrease of transfection efficiency.

Therefore, to evaluate the role of microtubules on pDNA:LD4 complexes (1:8000 pDNA:protein molar ratio) mediated gene transfer into HeLa cells, nocodazole was used. By using this drug, we observed a 49% decrease in the transfection efficiency (Fig. 8). Similarly, cytochalasin D was used to evaluate the effect of actin filaments disruption on transfection efficiency (Fig. 8). The disruption of the actin filaments lead to a decrease in transfection efficiency of 99%. The results indicate that pDNA:LD4 complexes strongly rely on the cells cytoskeleton for intracellular trafficking and pDNA delivery. However, the decrease in transfection efficiency caused by the disruption of the cytoskeleton has already been described in the literature using pDNA:Lipofectamine™ and pDNA:PEI (polyethyleneimine) complexes [39,40]. Despite microtubules tend to facilitate intracellular trafficking of vectors *via* active retrograde transport of the vesicles formed after endocytosis, they can also contribute to their degradation since most of the vectors remain entrapped inside late endosomes and lysosomes and are finally destroyed in these vesicles [41,42]. On the other hand, the observed decrease in transfection efficiency when cells were pre-treated with cytochalasin D can be credited to the role attributed to actin filaments in the early steps of complex entry in the cell [40] as they are important to receptor-mediated endocytosis and might be involved in other pathways of internalization [43]. Disruption of actin filaments tends to decrease the internalization of complexes and hence lower transfection efficiency.

In order to further investigate the endosomal entrapment, which stands as a limiting step for gene delivery efficiency [44] of the pDNA:LD4 complexes, we performed transfections of HeLa cells treated with the lysosomotropic agent chloroquine (Fig. 8). Cloroquine is a weak base that accumulates in acidic organelles such as late endosomes and lysosomes, raising the luminal pH of the organelles and avoiding enzymatic degradation of non-viral vectors [45]. This drug is frequently used to investigate the effect of the endosomal/lysosomal entrapment as a barrier to gene delivery, since its action causes a

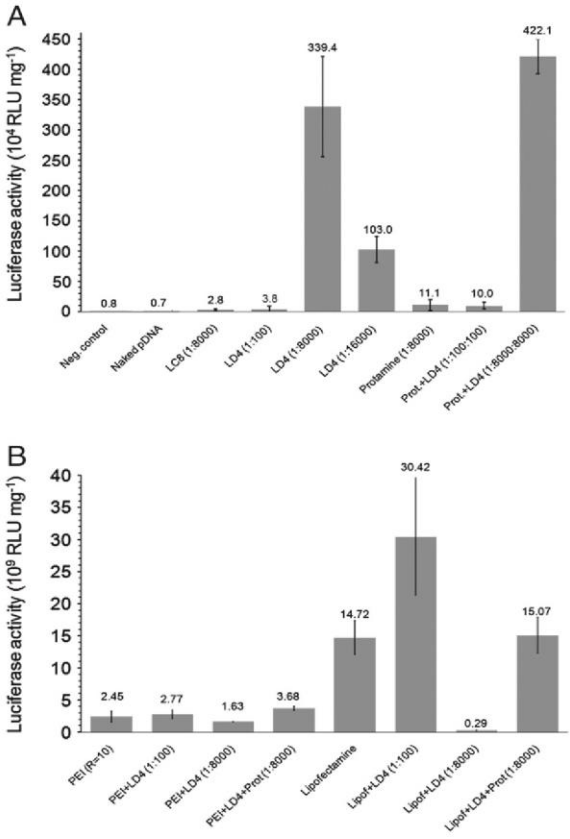

Fig. 7. A: Gene transfer to HeLa cells mediated by pDNA:LC8 and pDNA:LD4 complexes. Transfection efficiency was assessed by measuring the activity of the luciferase reporter gene. B: Comparison of the transfection efficiency of the LD4 fusion protein with the traditional delivery vectors PEI and Lipofectamine™. The combination of proteins (LD4 and protamine) with Lipofectamine™, resulting in ternary vectors, was also evaluated. Error bars indicate standard deviation between triplicates.

rise in intraendosomal osmolarity and its eventual lysis [46,47]. In our case, pre-treatment of HeLa cells with chloroquine (100 μM) enhanced the LD4 mediated transfection efficiency by 383%. Considering

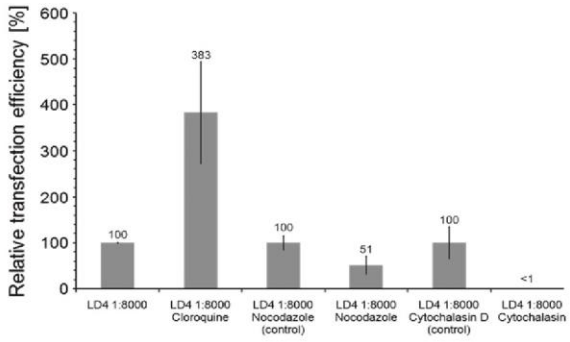

Fig. 8. Involvement of microtubules and endosomes/lysosomes on transfection efficiency of HeLa cells using LD4:pDNA complexes. The involvement of microtubules and actin were studied using the drugs nocodazole and cytochalasin D, respectively. The lysosomotropic agent chloroquine was used to investigate the effect of the endosomal/lysosomal entrapment as a barrier to gene delivery. Experiments were performed in triplicate as described in the Materials and methods section. Error bars indicate standard deviation between triplicates.

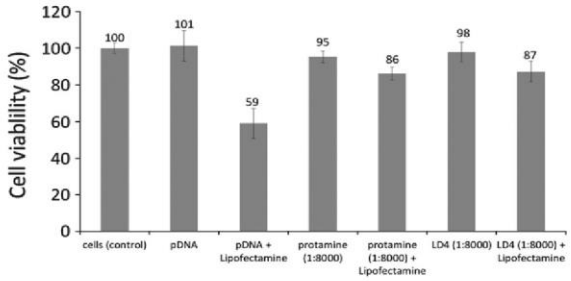

Fig. 9. Evaluation of the cytotoxicity of the different delivery vectors for HeLa cells performed using WST-1 reagent (Roche Applied Science). We assayed the cell viability after transfection with complexes formed by pDNA with lipofectamine, protamine and LD4 according to the procedure described in the Materials and methods section. Error bars indicate standard deviation between six replicates.

this data, escape from endosomes and lysosomes seems critical for the efficiency of LD4 mediated gene delivery and also for the understanding of the mechanisms of the intracellular trafficking of these pDNA:protein complexes. Beside this, we should also consider that an early release of pDNA:LD4 complexes in the cytosol promoted by chloroquine has another positive effect, since the complex may still rely on the retrograde transport to get closer to the cell nucleus *via* direct interaction between LD4 and dynein. In fact, we performed transfections using pDNA:protamine complexes in the presence of chloroquine and we found that the drug promoted an increase of 11-fold in Luciferase expression (Supplementary Data). Taking this result into account, LD4 seems to be more efficient in endosomal/lysosomal escape than protamine.

Finally, since both, pDNA:LD4 and pDNA:protamine, complexes used in this study have similar physical-chemical characteristics (size, charge ratio, nature, etc.), we believe that the differences found in transfection efficiency between LD4 and protamine mediated transfection (30-fold) may also be related to the natural ability of the LD4 to interact with the dynein motor and facilitate the intracellular trafficking. A complete study of the differences in intracellular trafficking of the different complexes studied here is still in progress by our research group. However, due to the complex nature of the vectors and the diversity of cellular entry pathways and intracellular trafficking, this is a very challenging task.

3.7. Plasmid DNA:LD4 complexes presented reduced cytotoxicity when compared to pDNA:Lipofectamine™ complexes

Cytotoxicity of the delivery vectors used in the present work was evaluated using the Cell Proliferation Reagent WST-1 (Roche Applied Science, USA) and cultivated HeLa cells. As expected, protein vectors proved to be far less toxic (98% and 95% cell viability for LD4 and protamine, respectively) than Lipofectamine™ (59% cell viability) (Fig. 9). Interestingly, the combination of LD4 and Lipofectamine™ lead to a reduced cell mortality comparing to the complex formed by cationic lipid and pDNA. So far, it is not clear the mechanisms behind the reduction of toxicity caused by the presence of the proteins in the ternary complexes. A possible explanation could be the reduction of the cationic lipid content in these complexes caused by competition with the proteins for the negatively charged plasmid backbone. Anyway, an important attribute of a gene delivery vehicle is the low toxicity, opening the possibility for a secure *in vivo* utilization, and the results indicate that pDNA:LD4 is a promising vector.

4. Conclusion

We presented here an innovative approach for the non-viral delivery of plasmid DNA. By combining the recombinant dynein light chain LC8 with a synthetic N-terminal DNA binding domain we were able to

construct a modular fusion protein specifically designed for gene delivery. The modified LC8, named LD4, was able to: i) interact and condense pDNA *in vitro* forming positively charged complexes and ii) to interact *in vitro* with dynein intermediate chain, confirming its functionality. Despite still far from being an optimized vector like the commercial lipids Lipofectamine™, LD4 proved to be 30-fold more efficient in transfection than protamine and 485-fold more efficient than naked DNA – this one the delivery method used in most of the non-viral clinical trials conducted so far. Transfection and cytotoxity assays also indicated that LD4 can be associated to cationic lipids to generate even more efficient delivery vectors for *in vitro* applications. Pre-treatment of the transfected HeLa cells with different drugs showed a major involvement of the cell's cytoskeleton in the intracellular trafficking, indicating the involvement of the dynein molecular motor in the transport of the pDNA:LD4 complexes. Despite the transfection results found using the LD4 vector are promising, the results also indicate the possibility of different forms of vector optimization, particularly particle charge and the ability to escape from endosomes/lysosomes. Finally, we believe that the work presented here add new information on the development of recombinant modular proteins specifically designed for gene delivery. By taking advantage of strategies used by virus to infect mammalian cells these vectors may, in the near future, increase the efficiency of non viral vectors and provide new tools for DNA vaccination and gene therapy studies.

Supplementary materials related to this article can be found online at doi:10.1016/j.jconrel.2012.01.011.

Acknowledgements

The authors gratefully acknowledge the financial support of the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (São Paulo, Brazil). We also thank the Laboratório de Espectroscopia e Calorimetria (LEC), Laboratório Nacional de Biociências – LNBio (Campinas, Brazil), for the support on the circular dichroism studies and Professor Maricilda Palandi de Mello, CBMEG, UNICAMP (Campinas, Brazil), for the support on the HeLa cells culture. Finally, the authors thank Professor Ricardo Aparício, IQ, UNICAMP, for the help with the preliminary studies of SAXS and Professor Lucimara Gaziola de la Torre, FEQ, UNICAMP, for the help with potential zeta and particle size measurements.

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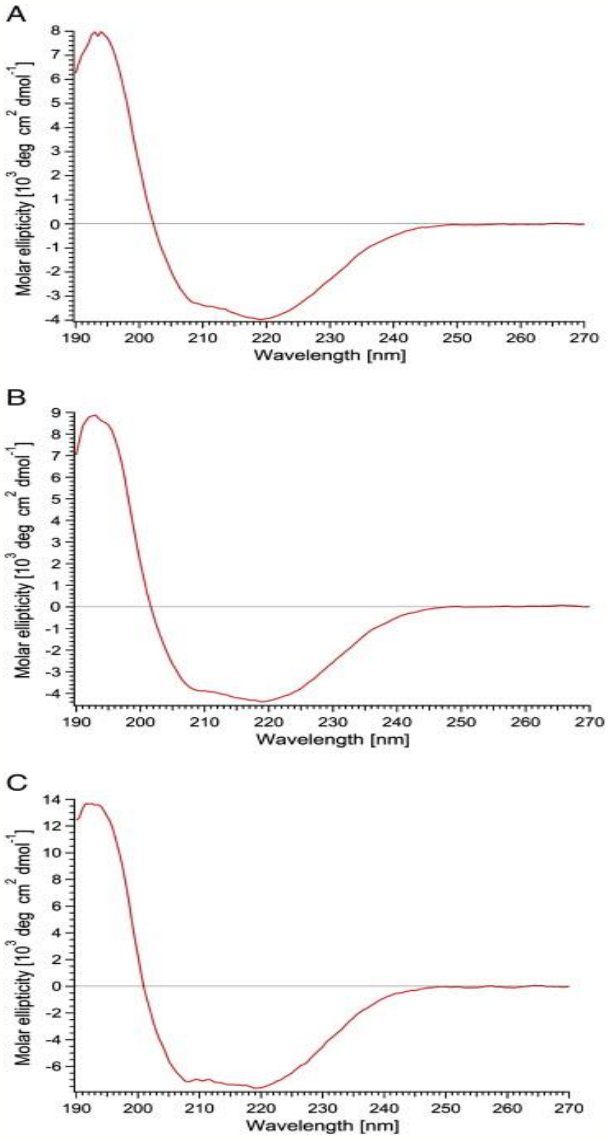
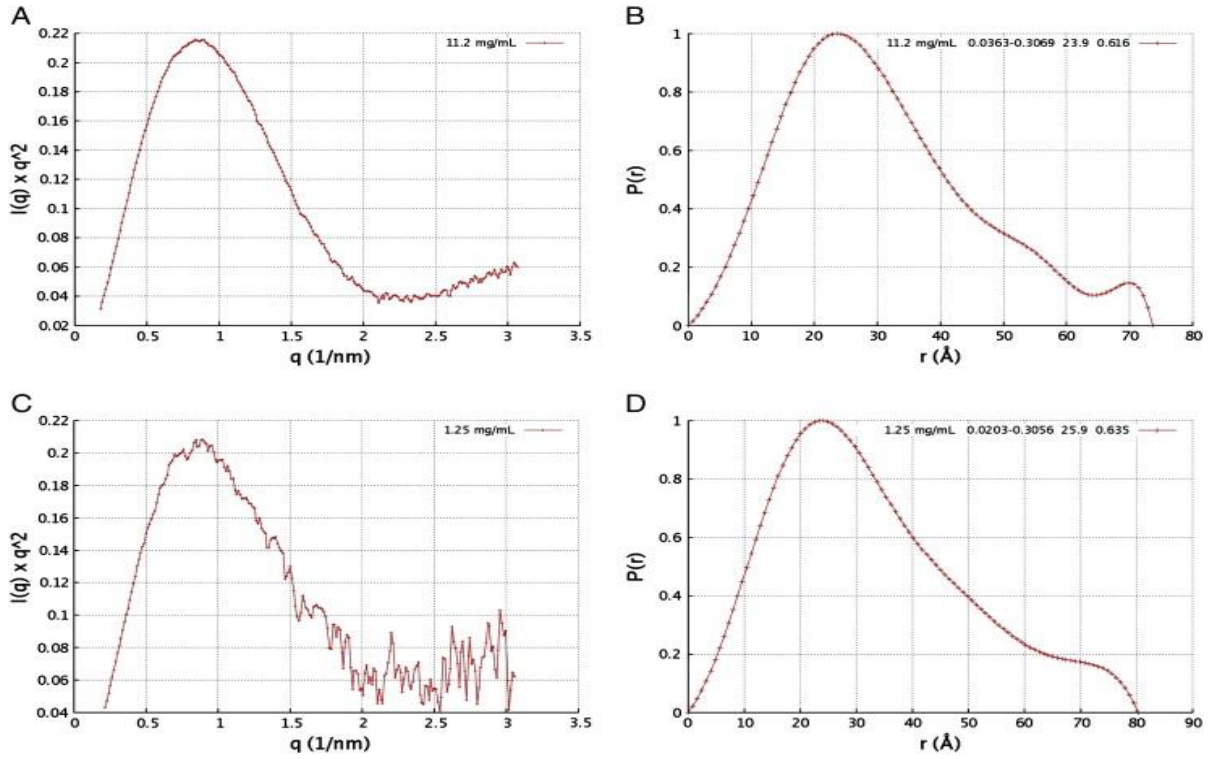
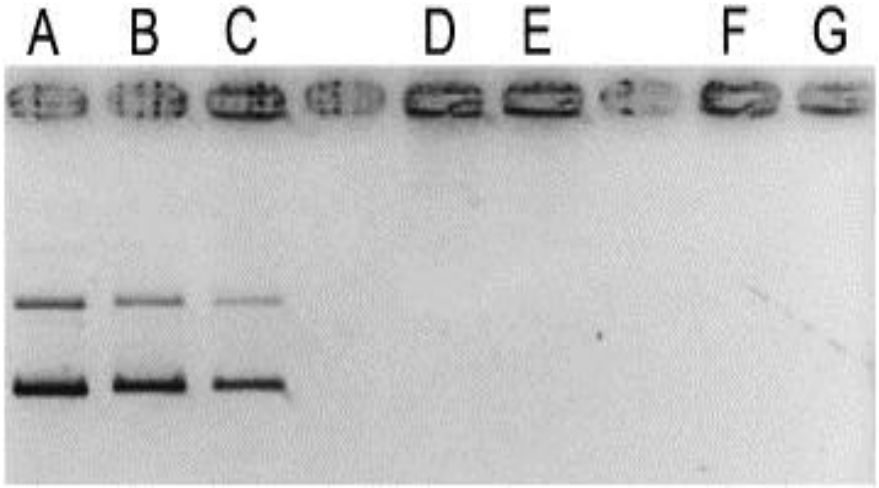


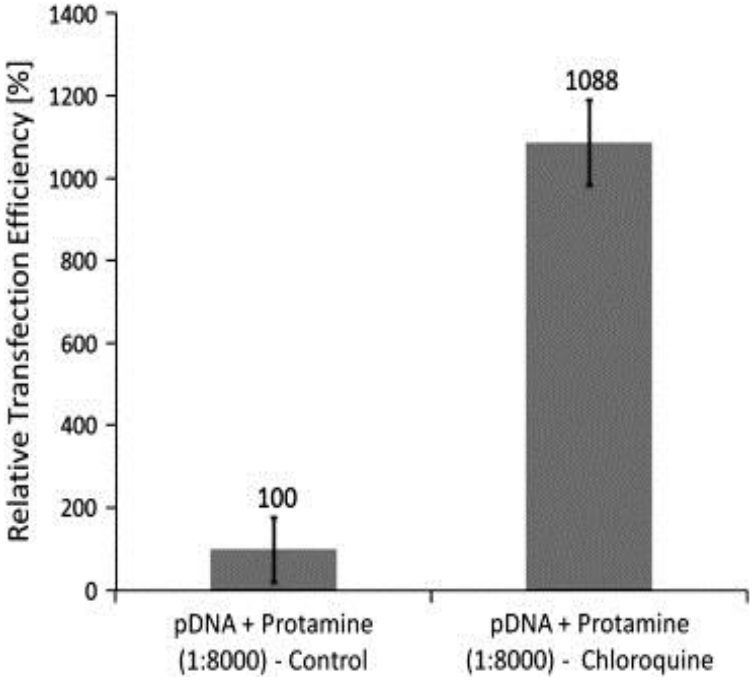
Supplementary Figure 1. Circular Dichroism spectra for LC8 dynein light chain with DNA binding 1 (A), DNA binding 2 (B) and DNA binding 3 (C).



Supplementary Figure 2. Small angle X-ray scattering (SAXS) data obtained for LC8 and LC8 with DNA binding 4. A: Krakty plot for LC8 alone. B: P(r) plot for LC8 alone. C: Krakty plot for LC8 with DNA binding 4. D: P(r) plot for LC8 with DNA binding 4. The bell shaped Krakty plot indicates that both proteins are folded and the P(r) plots indicate a spherical shape in solution.



Supplementary Figure 3. Gel retardation assay for different pDNA:LD4 complexes with and without Lipofectamine. A: pDNA (control), B: pDNA:LD4 (1:100), C: pDNA:LD4 (1:100) + Lipofectamine, D: pDNA:LD4 (1:4000), E: pDNA:LD4 (1:4000) + Lipofectamine, F: pDNA:LD4 (1:8000), G: pDNA:LD4 (1:8000) + Lipofectamine.



Supplementary Figure 4. Transfection assay for pDNA:protamine complexes in a pDNA:protein molar ratio of 1:8000 with and without previous chloroquine treatment of HeLa cells. This assay clearly shows that a great portion of pDNA:protamine complexes are trapped inside endosomes/lysosomes after cellular uptake.

"Characterization of the human dynein light chain Rp3 and its use as a non-viral gene delivery vector"

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Artigo publicado na revista Applied Microbiology and Biotechnology. *E-pub ahead of print*, 2013

Appl Microbiol Biotechnol
DOI 10.1007/s00253-013-5239-5

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of the human dynein light chain Rp3 and its use as a non-viral gene delivery vector

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Received: 27 July 2013 / Revised: 30 August 2013 / Accepted: 4 September 2013

Abstract Dynein light chains mediate the interaction between the cargo and the dynein motor complex during retrograde microtubule-mediated transport in eukaryotic cells. In this study, we expressed and characterized the recombinant human dynein light chain Rp3 and developed a modified variant harboring an N-terminal DNA-binding domain (Rp3-Db). Our approach aimed to explore the retrograde cell machinery based on dynein to enhance plasmid DNA (pDNA) traffic along the cytosol toward the nucleus. In the context of non-viral gene delivery, Rp3-Db is expected to simultaneously interact with DNA and dynein, thereby enabling a more rapid and efficient transport of the genetic material across the cytoplasm. We successfully purified recombinant Rp3 and obtained a low-resolution structural model using small-angle X-ray scattering. Additionally, we observed that Rp3 is a homodimer under reducing conditions and remains stable over a broad pH range. The ability of Rp3 to interact with the dynein intermediate chain in vitro was also observed, indicating that the recombinant Rp3 is correctly folded and functional. Finally, Rp3-Db was successfully expressed and purified and exhibited the ability to interact with pDNA and mediate the transfection of cultured HeLa cells. Rp3-Db was also capable of interacting in vitro with dynein intermediate chains, indicating that the addition of the N-terminal DNA-binding domain does not compromise its function. The transfection level observed for Rp3-Db is far superior than that reported for protamine and is comparable to that of the cationic lipid Lipofectamine™. This report presents an initial characterization of a non-viral delivery vector based on the dynein light chain Rp3 and demonstrates the potential use of modified human light chains as gene delivery vectors.

Keywords Rp3 · Dynein · Gene delivery · SAXS · Transfection

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Introduction

Non-viral vectors for gene delivery are expected to efficiently interact, condense, and deliver exogenous DNA to the nucleus of target cells. To accomplish this task, non-viral vectors must overcome a series of physico-chemical barriers that drastically reduce the efficiency of transgene delivery (Lundstrom and Boulikas 2003). There are several requirements for an efficient delivery vector: (1) protection from exogenous endonucleases, (2) maintenance of the stability of the delivery complex in the blood or extracellular environment, (3) enhanced cellular uptake, (4) the evasion of endosomal entrapment, (5) protection from cytoplasmic nucleases, (6) enhanced exogenous DNA transport towards the nucleus, and (7) the facilitation of nuclear membrane transposition (Niidome and Huang 2002; Guo and Huang 2012). Viral vectors are naturally capable of efficiently performing all of these tasks but have the drawback of safety concerns, such as the generation of immune responses and adverse side effects (Barbar et al. 2001). Therefore, there has been an ongoing effort to study and develop non-viral vectors with an efficiency comparable to that of viral-based vectors. Delivery complexes based on

Published online: 29 September 2013

cationic lipids, cationic polyplexes, peptides, and metal nanoparticles have been reported (Amand et al. 2012; Zhang et al. 2008; Ryou et al. 2011; Tang and Hughes 1998; Morishita et al. 2005; Mintzer and Simanek 2009; Lemkine and Demeneix 2001; Parker et al. 2003).

Our research is focused on exploiting the cellular transport machinery based on the dynein motor complex to enhance plasmid DNA (pDNA) trafficking via the cytosol, thereby increasing the transfection efficiency. The dynein molecular motor is a large multisubunit complex (~1.5 MDa) formed by heavy chains, intermediate chains, light intermediate chains, and light chains (King 2000). The latter group of subunits is responsible for the interaction between the cargo and the motor complex. With the exception of the heavy chains, each of the subunits has multiple isoforms, which lead to the generation of different motor complexes with specific cargo affinity and regulation capabilities, enabling the dynein motor complex to be involved in the retrograde transport of many types of molecules and vesicles (Tai et al. 1998; Lo et al. 2007).

To take advantage of the cellular transport machinery to enhance exogenous DNA transport to the cell nucleus, we have recently focused our efforts on characterizing and modifying dynein light chains. We have added a DNA-binding sequence at its N-terminal region (involved in cargo recognition and binding), which is expected to interact and condense plasmid DNA molecules. Once bound to the pDNA, the light chain is expected to mediate cellular uptake and interact with the dynein molecular motor to enhance the transport of the exogenous DNA toward the nucleus. We recently demonstrated the feasibility of this approach using a modified human dynein light chain Lc8 protein (Toledo et al. 2012).

In the present study, we report the characterization of the recombinant human dynein light chain Rp3 and evaluate the ability of the modified Rp3 (harboring an N-terminal binding domain) to act as a gene delivery vector. The human dynein light chain Rp3 shares 55 % identity with the human TcTex light chain. Rp3 is reported to be involved in dynein -mediated transport and in functions outside the cellular transport pathway. This light chain mediates the retrograde transport of the herpes simplex virus type 1 through the interaction with the capsid protein VP26 (Douglas et al. 2004). Rp3 also competes with TcTex for binding in the intermediate chain in polarized epithelial Madin-Darby canine kidney cells (Tai et al. 2001). In this system, Rp3 overexpression was able to replace TcTex in endogenous dynein complexes. Rp3 was also found to be associated in the cell nucleus with the transcription factor SATB1 at nuclear matrix elements. This association is dynein motor complex independent, and Rp3 localization in the nucleus is SATB1-dependent. The Rp3–SATB1 interaction is reported to be involved in the *Bcl2* gene repression and indicates a role of Rp3 outside the dynein motor complex (Yeh et al. 2005).

Here, we present the first structural characterization of the recombinant human light chain Rp3. We successfully expressed and purified recombinant Rp3 in *Escherichia coli* and demonstrated that its dimeric structure occurs only under reducing conditions and is maintained over a broad pH interval. Small-angle X-ray scattering analysis revealed that the Rp3 dimer has a fold structure that is very similar to that of human TcTex and Lc8 and that its N terminus is exposed in the quaternary structure surface, which is an ideal site for the addition of a DNA-binding domain. Additionally, the modified Rp3 with an N-terminal DNA-binding domain (referred to as Rp3-Db) was evaluated as a non-viral delivery vector and exhibited a transfection efficiency far superior to that of our previously reported Lc8-based vector.

Methods

Plasmids

The plasmid pVAX1-Luc was used as a model pDNA vector as previously described by Toledo et al. (2012). This vector was constructed by replacing the *GFP* reporter gene from the pVAX1-GFP plasmid with the *Luciferase* gene obtained from the pGL3-Luc control vector (Promega, USA). All assays, plasmid extractions, and purification steps were performed as described by Freitas et al. (2007).

Cloning, expression, and purification of Rp3 and Rp3 with a DNA-binding domain

The human dynein light chain Rp3 was amplified from HeLa cDNA (forward primer with a *BamH*I restriction site: 5′-ATAGGATCCATGGAGGAGTACCATCG-3′; reverse primer with a *Xho*I restriction site: 5′-ATACTCGAGTTAAAG AACAATAGCAATGG-3′) and cloned into pET28a vector previously digested with the indicated restriction endonucleases. The same sequence was cloned (using the same restriction sites) into a pET28a vector harboring a previously cloned unspecific DNA-binding domain (WRRRGFGRRR) between NdeI and BamHI restriction sites, as described previously (Toledo et al. 2012). Recombinant protein was expressed in the *E. coli* BL21(DE3) Rosetta strain. Briefly, cells were grown in 2.0-L Erlenmeyer flasks containing 1.0 L of LB medium. The cells were cultured at 37 °C with 300 rpm shaking to an optical density of 0.8 AU (600 nm). Protein expression was induced with 5.6 mM lactose or 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for an additional 12 h at 25 °C and 200 rpm. The cells were then recovered by centrifugation at 6,000×*g* for 10 min. Recombinant protein extraction was performed by resuspending the cell pellet in 50 mM Tris–HCl buffer (pH 8.0 for Rp3 and pH 6.3 for Rp3

with DNA-binding domain), 500 mM NaCl, 0.1 mM EDTA, 1.0 mM PMSF, and 15 mM β-mercaptoethanol. Protein purification was performed in a single-affinity chromatography step in Ni-NTA Superflow resin (Qiagen). The protein size and purity were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Size-exclusion chromatography

Size-exclusion chromatography was performed using a Superdex 75 10/300 GL or Superdex 200 10/30 GL column (GE Healthcare, USA) coupled to an ÄKTA FPLC system (GE Healthcare, USA). Prior to each run, the column was equilibrated with the same buffer used for dialysis depending on the desired pH. We evaluated the oligomeric behavior of Rp3 at the following pH values: pH 7.5 (20 mM Tris–Cl); pH 6.0, 5.0 and 4.0 (20 mM sodium acetate); and pH 3.0 (20 mM glycine–HCl). All buffers also contained 200 mM NaCl and 15 mM β-mercaptoethanol. Samples (250 μL) were injected at a flow rate of 0.6 mL/min. High-molecular-weight and low-molecular-weight gel filtration calibration kits (GE Healthcare) were used as calibration standards, and the results were analyzed according to the manufacturer's instructions. Ribonuclease A (13.7 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) and blue dextran 2000 (2,000 kDa) were used as standards.

Small-angle X-ray scattering analysis of recombinant Rp3

SAXS data were collected at the SAXS-1 beamline of the Brazilian National Synchrotron Light Laboratory (LNLS/Brazil) (Kellerman et al. 1997) using a Dectris-Pilatus (300 K; 84 mm×107 mm) 2D detector. The sample-to-detector distance was 1,174.89 mm, and the X-ray wavelength was 1.3808 Å, covering a momentum transfer interval of $0.0126<q<0.415$ Å^{-1}, where $q=4\pi\sin\theta\,\lambda$ and 2θ is the scattering angle. For each protein concentration, six successive frames were recorded with a 500 s exposure time and a sample holder temperature of 25 °C. After each protein sample analysis, the buffer solution was measured for the same duration. Correction based on the sample attenuation, normalization of the collected images to the intensity of the transmitted beam, buffer scattering subtraction, and radial integration were performed using FIT2D software (Hammersley et al. 1996). The radius of gyration, R_g, and the scattering intensity at zero angle, $I(0)$, were estimated using the Guinier approximation (Glatter and Kratky 1982) $I(q)=I(0)\exp(-q^2R_g^2\,3)$, which is valid for small angles ($qR_g\lesssim1.3$). A convenient q range was chosen to minimize potential aggregation effects observed at very low scattering angles. Additional estimates for R_g and $I(0)$ were obtained using the indirect Fourier transform method as implemented in GNOM (Svergun 1992), which also calculates the distance distribution function, $P(r)$, which allows for an assessment of the maximum intramolecular distance and particle anisometry. The conformational state of the protein in solution was evaluated based on the Kratky plot (Rambo and Tainer 2011; Doniach 2001; Semisotnov et al. 1996) ($Iq^2\times q$), in which the native state of a compact globular protein has a characteristic profile. An estimate for the molecular mass of the protein in solution was obtained using the method described by Fischer et al. (2009), which is independent of the sample concentration. The oligomeric state of the protein in solution was subsequently obtained from the known molecular mass of the protein monomer.

Ab initio dummy bead models were calculated from the experimental curves using the program DAMMIF (Franke and Svergun 2009) while imposing point-group P2 symmetry. The most probable model, which represents the final protein envelope, was obtained by averaging 40 independent DAMMIF runs using DAMAVER (Volkov and Svergun 2003) in the automatic mode. The relatively low normalized spatial discrepancy and low standard deviation (NSD=0.596±0.072) indicated that the individual models were very similar (only two were omitted when calculating the final average). To obtain a better visualization of the protein shape, the average model was represented by a surface model calculated using NCSMASK (Winn et al. 2011; Winn et al. 1997; CCP4 1994).

A theoretical scattering curve for the NMR solution dimer structure of the Tctex1 homologue from *Chlamydomonas* axonemal inner dynein arm I1 (chains A and B of the PDB entry 1XDX) (Wu et al. 2005) was calculated and fitted to the experimental data using CRYSOL (Svergun et al. 1995). A good agreement was obtained with a χ value of 1.59. The 1XDX dimer also presented a molecular mass (25.3 kDa) and maximum intraparticle distance (56.8 Å) comparable with the estimates obtained for Rp3 in solution, indicating that Rp3 was likely a dimer in solution. Therefore, to interpret the envelope recovered from the experimental data using *ab initio* methods, an Rp3 dimer structure was constructed as follows. In the absence of a high-resolution model determined by experimental techniques, five monomeric Rp3 three-dimensional homology models were obtained using the I-TASSER server (Roy et al. 2010; Zhang 2008). Each model was individually aligned to chains A and B of the 1XDX PDB structure using CEAlign (Shindyalov and Bourne 1998) implemented as a PyMOL plugin (The PyMOL Molecular Graphics System, version 1.4.1, Schrödinger, LLC), resulting in two chains that were subsequently combined to yield a dimer for a total of five putative RP3 dimers. The maximum intraparticle distance for each dimer was computed with the program NCONT (Winn et al. 2011, 1997; CCP4 1994). The closest distance (65.0 Å) to that predicted by the $P(r)$ function (74.0 Å) corresponded to the dimer built from the second-best solution output from I-TASSER (C-score=−1.26) The

discrepancy between the experimental data and the theoretical scattering curve calculated for this dimer was low, as indicated by a CRYSOL χ value of 1.50. Superpositions were performed using SUPCOMB (Kozin and Svergun 2001). Additional analyses were performed with the package ATSAS (Konarev et al. 2006) and GNUPLOT (http://www.gnuplot.info/), supported by C shell scripts running under Slackware Linux for processing automation. RASMOL (Sayle and Milner-White 1995; Bernstein 2000) and PyMOL (The PyMOL Molecular Graphics System, version 1.4.1, Schrödinger, LCC) were used for graphical analysis and visual inspection of the models.

Interaction assays with dynein intermediate chain

Purified recombinant Rp3 and Rp3-Db were assayed for their ability to interact with the dynein intermediate-chain DYNIC2 N-terminal domain in vitro. Dynein light chain Lc8 was also used in the interaction assay, as it has been reported that the interaction of *Drosophila melanogaster* Rp3 with the corresponding intermediate chain is enhanced in the presence of the Lc8 light chain. The DYNIC2 N-terminal domain and Lc8 light chain were produced as described previously (Toledo et al. 2012). Purified recombinant proteins (Rp3 or Rp3-Db) were incubated with Lc8 and DYNIC2 N-terminal domain. Incubation was performed overnight at room temperature under reducing conditions (20 mM DTT in elution buffer, as described in "Cloning, expression and purification of Rp3 and Rp3 with a DNA-binding domain"). Complexes were resolved by size-exclusion chromatography using a Superdex 200 10/30 GL column (GE Healthcare, USA) equilibrated with PBS. The chromatography was performed using an ÄKTA FPLC system (GE Healthcare, USA). We also evaluated each protein separately at the same concentrations and operating conditions. Chromatographic samples corresponding to the absorbance peaks were collected separately, and the proteins were precipitated using TCA followed by SDS-PAGE analysis.

Electrophoresis shift assay

An electrophoresis shift assay was performed to compare the ability of Rp3 and Rp3-Db to interact with and condense plasmid DNA. Purified Rp3 was first dialyzed against 20 mM sodium phosphate buffer, pH 8.0, whereas Rp3-Db was dialyzed against 20 mM MES buffer, pH 6.0; both buffers contained 100 mM NaCl and 15 mM β-mercaptoethanol. For each interaction assay, 200 ng of pDNA was incubated with varied amounts of each protein in a final volume of 50 μL. Protein/pDNA molar ratios of 100, 500, 1,000, 2,000, 4,000, 8,000, 10,000 and 12,000 were tested. Protein/pDNA complexes were incubated at room temperature for 1 h followed by analysis by 0.8 % agarose gel electrophoresis with ethidium bromide staining.

Transfection assays

HeLa (human epithelioid carcinoma) cells were used in transfection assays to compare the ability of Rp3 and Rp3-Db to act as delivery vectors. Briefly, cells were cultivated in 25 cm^2 flasks with F-12 Ham nutrient mixture (Gibco, England) in a humidified atmosphere with 5 % CO_2 at 37 °C. After the cells reached 70–90 % confluence, they were trypsinized and seeded in 24-well plates (5×10^4 cells per well). The cells were further incubated in the same growth medium for approximately 48 h (70–80 % cell confluence) and then transfected with pDNA/protein complexes at different molar ratios. Protein/pDNA molar ratios of 500, 2,000, 8,000, and 12,000 were used. Similar to the previously described gel shift protocol, 200 ng of pDNA was used in each well. The pDNA/protein complexes (50 μL) were incubated for 1 h at room temperature, followed by the addition of 50 μL of non-supplemented medium to the complex and further incubation for 20 min. A total volume of 100 μL of pDNA/protein complex at different molar ratios was then added to each well. After incubation for 6 h, the growth medium was replaced, and the cells were incubated for an additional 18 h. The cells were then trypsinized and collected for the determination of luciferase activity using the Luciferase Assay System (Promega, USA) as described previously (Toledo et al. 2012).

Results

Recombinant human Rp3 and Rp3 harboring a DNA-binding domain were successfully expressed in *E. coli* and purified in a single step

Using the above-described methods, we were able to successfully express and purify both recombinant proteins studied in this work. The dynein light chain Rp3 containing an N-terminal His-tag (16.6 kDa, theoretical pI of 6.3) and Rp3-Db containing the N-terminal His-tag as well as a DNA-binding domain (16.9 kDa, theoretical pI of 7.8) were purified in a single Ni-NTA affinity chromatography step (Fig. 1). It is noteworthy that the addition of the small DNA-binding domain at the N terminus of Rp3 altered its predicted isoelectric point from 6.3 to 7.8, which is explained by the presence of six arginine residues in this domain. This shift in the isoelectric point led us to use different buffers with varied pH values for each protein in all assays to avoid protein aggregation due to the proximity of the isoelectric point. Recombinant Rp3 was successfully purified and characterized at pH 8.0, whereas the optimal pH observed for Rp3-Db was 6.3.

Size-exclusion chromatography showed that recombinant Rp3 has an oligomeric state that is highly dependent on the redox state of the environment, as we obtained a single

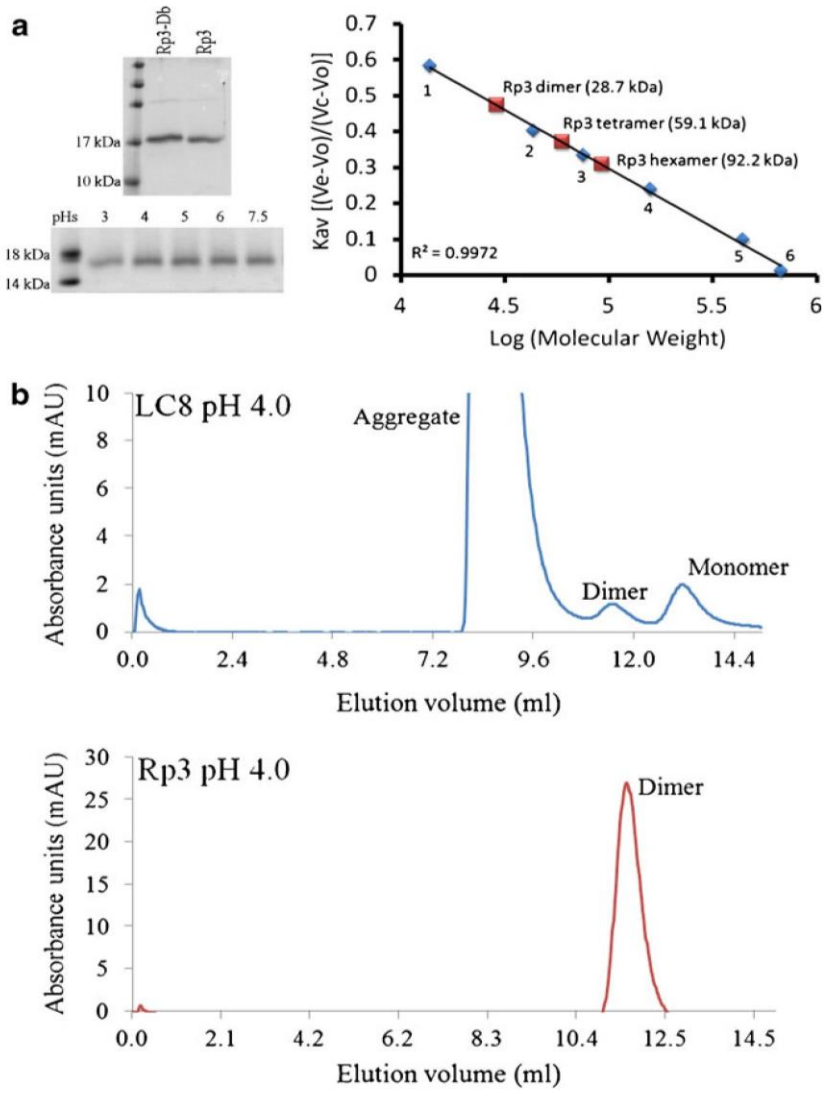


Fig. 1 **a** *Left*. SDS-PAGE 15 % showing purified Rp3 (16.6 kDa) and Rp3-Db (16.9 kDa) and Rp3 after dialysis against buffers at different pHs. *Right*. Size exclusion chromatography curve for purified Rp3 showing dimeric (28.7 kDa), tetrameric (59.1 kDa), and hexameric (92.2 kDa) subpopulations of Rp3 under non-reducing conditions. Reduced Rp3 is obtained as a single dimeric peak. *1*: ribonuclease A (13.7 kDa), *2*: conalbumin (75 kDa), *3*: aldolase (158 kDa), *4*: ferritin (440 kDa), *5*: thyroglobulin (669 kDa). **b** Size exclusion chromatography for dynein light chains Lc8 and Rp3 at pH 4.0. It can be observed that Lc8 has a monomeric intermediate at lower pH prior to denaturation unlike Rp3 that remains as a dimer

dimeric population only when the protein samples were reduced using β-mercaptoethanol, dithiothreitol (DTT) or Tris(2-carboxyethyl)phosphine. When the samples were not reduced, three peaks were observed, corresponding to dimers (30.1 kDa), tetramers (61.5 kDa), and hexamers (95.5 kDa) (Fig. 1).

Rp3 remains as a dimer at pH 4.0 and does not exhibit a monomeric intermediate before denaturation

We also evaluated the oligomeric behavior of recombinant Rp3 over a range of pH values. Previous studies reported that the dynein light chain TcTex from *D. melanogaster* (70 % sequence identity to human dynein light chain TcTex and 48 % sequence identity to human dynein light chain Rp3) does not exhibit a folded monomeric intermediate during protein unfolding by denaturing agents (or low pH values), as was reported for *D. melanogaster* dynein light chain Lc8 (Barbar et al. 2001; Talbott et al. 2006). We observed that human Rp3 remained as a dimer in solution at pH values ranging from 7.5 to 4.0, with the exception of pH 6.0, which is close to the predicted Rp3 isoelectric point (6.3), leading to protein aggregation after dialysis. At pH 3.0, Rp3 did not appear as a dimer or monomer in solution, as we observed a single peak corresponding to protein aggregates that may have formed by protein unfolding at low pH. As a control, we evaluated the oligomeric behavior of human dynein light chain Lc8, which was previously studied by our group (Toledo et al. 2012). At pH 4.0, we observed three peaks corresponding to monomers, dimers, and aggregates, which is in agreement with the reported data for *D. melanogaster* Lc8 (Barbar et al. 2001).

SAXS analysis indicates that RP3 conserves an interface analogous to that observed in the homologue Tctex1

SAXS scattering curves were collected for Rp3 protein samples at concentrations of 1.1, 4.3, and 6.4 mg/mL in the same protein extraction buffer. Samples were centrifuged for 10 min at 16,000×*g* prior to exposure to the X-ray beam. Radiation damage was clearly visible within the successive frames for the higher-concentration samples, which limited the analyses to six curves collected from the lower-concentration samples. Despite the relatively weak scattering profile at higher angles, consistent and reproducible results were obtained. Although no radiation damage was detected by visual inspection, individual processing of the frames indicated an increased intraparticle maximum distance for the last two curves. Due to the relatively noisy scattering at higher angles, the averaging of the first four curves did not result in a significant improvement. Therefore, more stringent criteria were adopted, and the final experimental curve (Fig. 2a) was obtained by averaging the curves from the first and second frames only and limiting the data to the range $0.0200<q<0.2503$ Å^{-1}.

The Guinier region exhibited a linear behavior characteristic of monodisperse samples, corresponding to an R_g of 25.2 Å obtained based on a linear regression in the region $0.0200<q<0.0517$ Å^{-1} (Fig. 2a). The Kratkyplot shown in Fig. 2b displays a well-defined maximum, indicating a compact globular protein in its native state.

The distance distribution function obtained from the experimental curve (Fig. 2c) corresponds to an elongated particle with 74.0 Å as maximum dimension and an R_g equal to 23.1 Å, which is consistent with the value obtained from the Guinier analysis. The molecular mass obtained from the integral of the Kratky function (Fischer et al. 2009) $I(q)q^2$ (q_{max}=0.25) calculated for the regularized curve output by GNOM (Svergun 1992) was 34.0 kDa. This result indicated, within the margin of error of the technique, the presence of an Rp3 dimer in solution, as anticipated based on the size-exclusion chromatography analysis.

The final envelope superposed onto the predicted Rp3 dimer is shown in Fig. 3. An excellent fitting of the dimer theoretical scattering curve to the experimental data was also obtained, as shown in Fig. 2a. A high structural similarity was obtained between the shape determined from the SAXS curve and the three-dimensional homology model, strongly suggesting that Rp3 conserves an interface analogous to that observed in the homologue Tctex1 dimer from *Chlamydomonas* axoneme (Wu et al. 2005). In addition, the protein probably possesses some degree of flexibility at its N terminus, as indicated by the structural analysis (not shown) of the individual monomers predicted by I-TASSER (Roy et al. 2010).

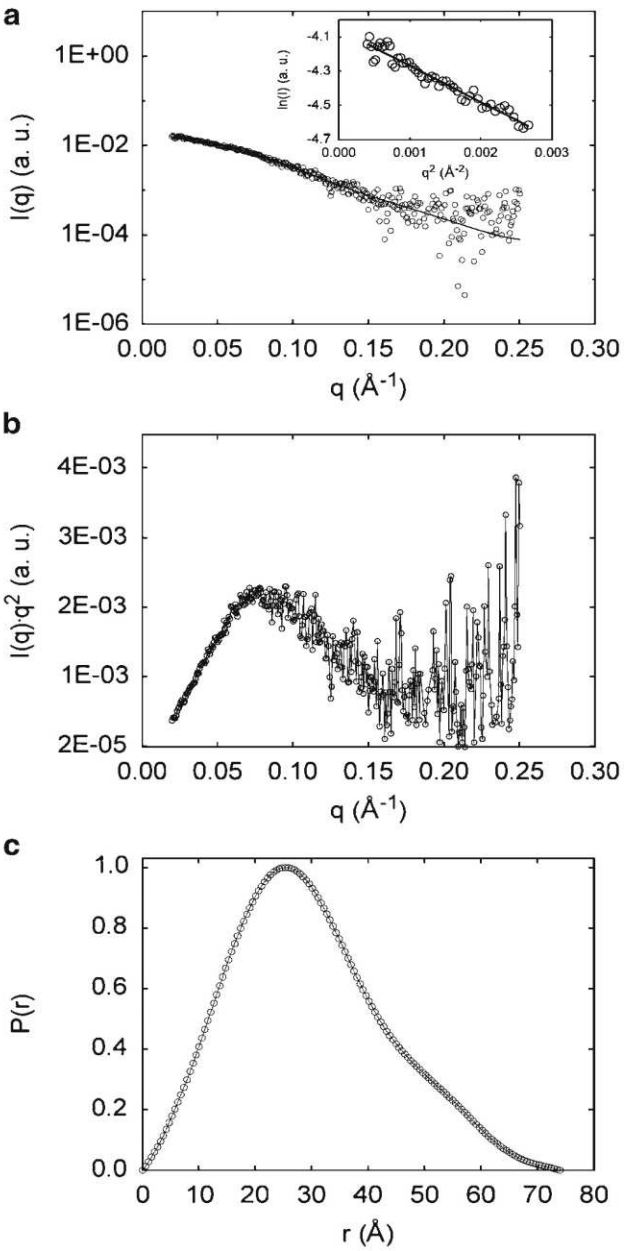


Fig. 2 SAXS results. **a** The theoretical curve computed for the predicted RP3 dimer (*solid line*) fitted into the experimental scattering curve (*open circles*). Due to the low concentration of the protein sample, weak scattering at higher angles led to a few data points with negative intensity for $q>\sim0.20$ Å^{-1} (far from the Guinier region), after buffer scattering subtraction, and were ignored during the composition of this and the next plot. The *inset* shows the linear fitting obtained from Guinier analysis. **b** Kratky plot calculated from the experimental data, normalized for $I(0)=1$. **c** Distance distribution function derived from the experimental curve in a procedure in which the few negative points mentioned were treated by GNOM and are absent in the final regularized curve used for shape recovery. For further details, see the text

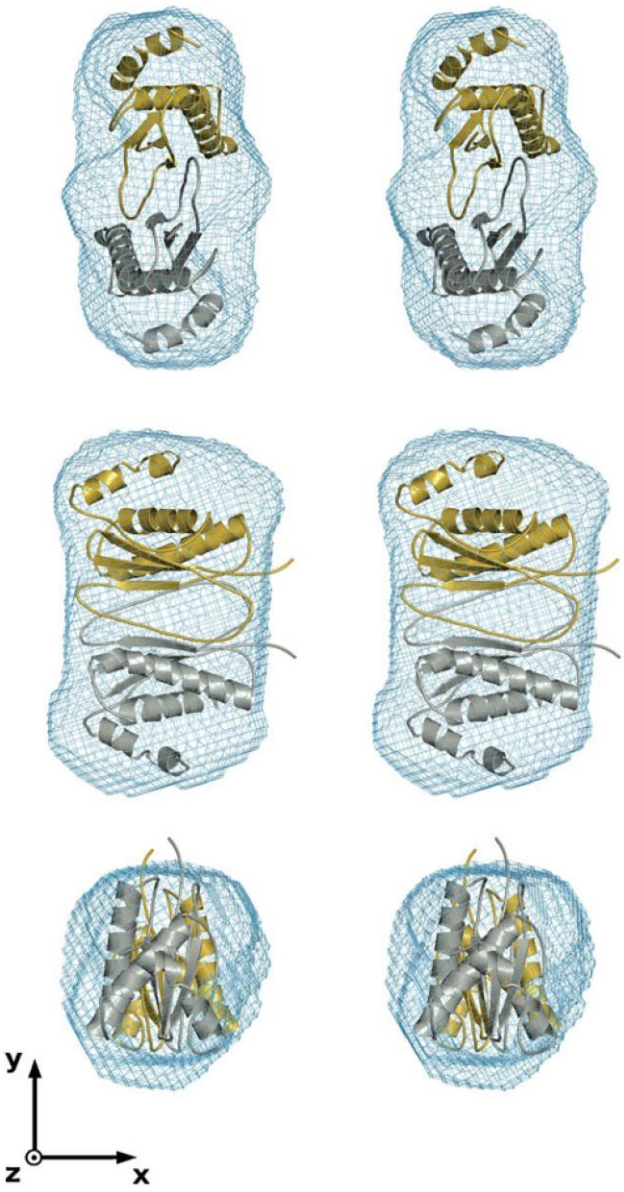


Fig. 3 Perspectives in stereo of a cartoon representation of the predicted RP3 dimer (protomers in gold and silver), superposed onto the molecular envelope (*blue mask*) recovered from the experimental curves by imposing symmetry P2. The *middle* and *bottom rows* are rotated clockwise by 90° around the *y*-axes and counterclockwise by 90° around *x*-axes, respectively. Drawings were prepared with PyMOL and edited using GIMP (http://www.gimp.org) under Slackware Linux

Both Rp3 and Rp3 with DNA-binding domain are able to interact with the dynein intermediate chain in vitro

Recombinant human Rp3 and Rp3-Db were evaluated for their ability to interact with the N-terminal domain of human dynein intermediate chain DYNIC2. This assay was important for determining whether Rp3 and Rp3-Db retain their correct folding and therefore the ability to interact with the dynein intermediate chain after recombinant expression in a prokaryotic host. As shown in Fig. 4, both recombinant proteins Rp3 and Rp3-Db formed complexes with dynein intermediate chain and Lc8 light chain. Size-exclusion chromatography (Fig. 4a) showed that at 13 mL of elution, an absorbance peak corresponding to the complex, was present for both Rp3 and Rp3-Db. The formation of a complex in both cases was further confirmed by TCA precipitation of the eluted protein sample followed by SDS-PAGE analysis. As shown in Fig. 4b, we observed the presence of the intermediate chain and the Lc8 light chain with the Rp3 light chain or Rp3-Db light chain eluted at the same elution fraction (13 mL). Additionally, no absorbance peak at the same elution value was observed when each purified protein was injected alone at the same concentrations (Fig. 4a).

The modified Rp3, harboring a DNA-binding domain, is able to interact and condense plasmid DNA

We comparatively evaluated the ability of recombinant Rp3 and Rp3-Db to interact with plasmid DNA using an agarose gel electrophoretic mobility shift assay (Fig. 5). The Rp3/pDNA or Rp3-Db/pDNA complexes at different protein/pDNA molar ratios were evaluated. The addition of a DNA-binding domain at the Rp3 N terminus (Rp3-Db) allowed the protein to efficiently interact and condense pDNA, as evidenced by shifted bands in the agarose matrix. We tested protein/pDNA molar ratios from 100 to 12,000, and it was possible to visualize shifted bands when using Rp3-Db at a molar ratio of 2,000 and above. For the molar ratios of 10,000 and 12,000, no bands were observed. This lack of banding may have occurred due to the formation of protein/pDNA particles with positively charged surfaces and/or large particles that could not enter the gel matrix, as previously reported (Toledo et al. 2012). It is noteworthy that no shift was observed for Rp3/pDNA complexes at any of the molar ratios tested, even when we used purified Rp3 dialyzed against the same buffer optimized for Rp3-Db, at pH 6 (data not shown). Therefore, the electrophoresis shift assays indicate that the presence of a small DNA-binding sequence at the N terminus of Rp3 confers the ability to interact with and condense DNA molecules.

Additionally, the 260/280 absorbance ratio for Rp3-Db, after the dialysis step, was verified to investigate DNA contamination in purified Rp3-Db samples, which might compromise interaction between protein and pDNA. Purified Rp3-Db, after dialysis, presented a 260/280 ratio of 0.96±0.21 which is consistent with a protein sample with low degree of DNA contamination.

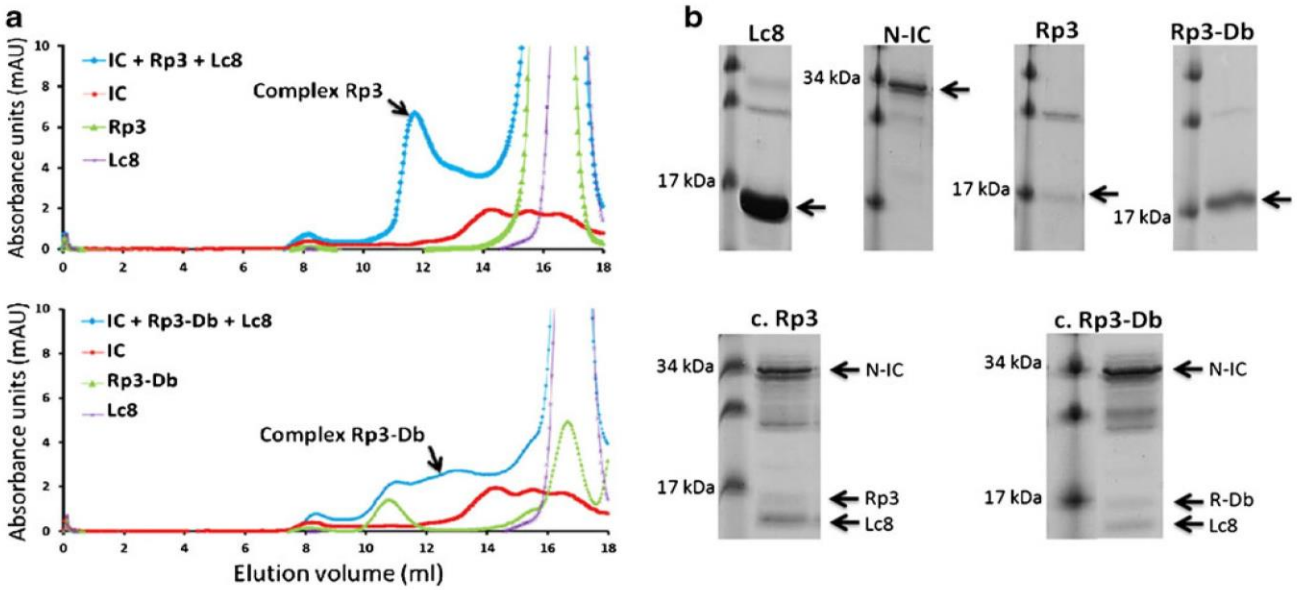


Fig. 4 Interaction assay of Rp3 and Rp3-Db with dynein intermediate chain and Lc8. **a** Size exclusion chromatography curves showing the absorbance peaks corresponding to Rp3/Lc8/intermediate chain N-IC complex (*above*) and Rp3-Db/Lc8/intermediate chain N-IC complex (*below*) after 13 ml of elution. **b** Confirmation of the complexes by TCA precipitation of eluted samples followed by SDS-PAGE analysis. Rp3, Rp3-Db, and Lc8 light chains eluted as dimmers when run alone whereas dynein intermediate chain N-terminal domain (N-IC) eluted as a monomer. Protein samples eluted at 13 ml contained either Rp3, Lc8, and N-IC or Rp3-Db, Lc8, and N-IC proving the interaction between these proteins. Please note that no absorbance peak was observed at 13 ml when each protein was run in the SEC column separately indicating that the observed complexes do not occur as protein oligomerization or method artifact

Transfection assays demonstrated that Rp3-Db is an efficient gene delivery vector

After we confirmed that Rp3-Db is able to interact with and condense plasmid and is able to interact with the dynein intermediate chain in vitro, we performed transfection assays using cultured HeLa cells to evaluate its ability to mediate gene delivery to eukaryotic cells. Recombinant Rp3 without the DNA-binding domain was used as a negative control because our results demonstrated that this protein is not able to interact with pDNA. Four protein/pDNA molar ratios were tested (500, 2,000, 8,000, and 12,000), and the transfection efficiency was evaluated at 24 and 48 h post-transfection. The results indicated that Rp3-Db was far more efficient in the delivery of pDNA to HeLa cells, especially at protein/pDNA ratios of 8,000 and 12,000. At the latter molar ratio, Rp3-Db presented a Luciferase expression approximately 13,000-fold higher than that of Rp3 (Fig. 6a and b).

The efficiency observed for Rp3-Db is higher than the transfection efficiency obtained for the dynein light chain Lc8 with the same DNA-binding sequence, as reported in our previous work (Toledo et al. 2012). The modified Lc8 harboring the DNA-binding domain presented a transfection efficiency of approximately 3 x 10^6 RLU/mg at a protein/pDNA molar ratio of 8,000. This result is 3.3 times lower than the result obtained for Rp3-Db at the same molar ratio. The molar ratio of 12,000 resulted in even higher transfection levels (Fig. 6b).

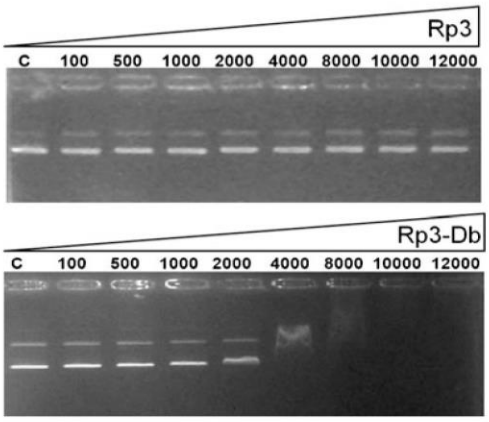
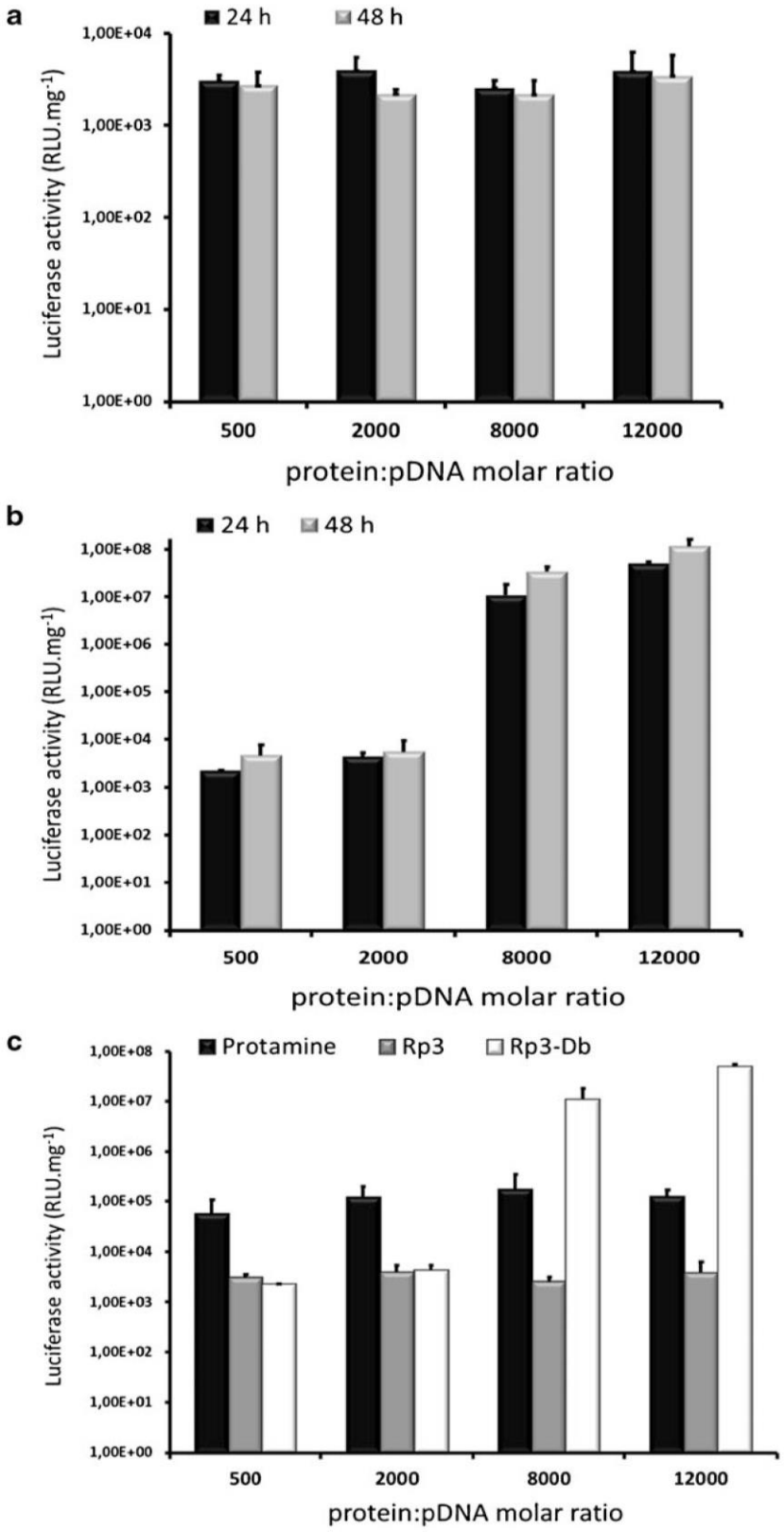


Fig. 5 Evaluation of the ability of Rp3 and Rp3-Db to interact and condense plasmidial DNA. Protein/pDNA complexes were formed at different protein/pDNA molar ratios ranging from 100 to 12,000. The addition of an N-terminal DNA binding domain to Rp3 confers it the ability to interact with DNA molecules as shifted bands can be observed at protein/pDNA molar ratios of 2,000 and above. No shifted bands can be observed for Rp3/pDNA complexes

Fig. 6 Gene reporter expression levels obtained for **a** Rp3/pDNA and **b** ▶ Rp3-Db/pDNA complexes after (*black squares*) 24 and (*white squares*) 48 h. **c** Comparative transfection efficiency observed for Rp3, Rp3-Db, and protamine after 24 h. All complexes were evaluated at protein/pDNA molar ratios of 500, 2,000, 8,000, and 12,000. Please note that *y* axis is in log scale in all plots


a
24 h
48 h
Luciferase activity (RLU.mg-1)
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
500
2000
8000
12000
protein:pDNA molar ratio
b
24 h
48 h
Luciferase activity (RLU.mg-1)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
500
2000
8000
12000
protein:pDNA molar ratio
c
Protamine
Rp3
Rp3-Db
Luciferase activity (RLU.mg-1)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
500
2000
8000
12000
protein:pDNA molar ratio

We also observed that the transfection efficiency of Rp3 without the DNA-binding domain (around 10^4 RLU/mg) is comparable to the efficiency reported for the Lc8 light chain without the DNA binding sequence (Toledo et al. 2012), and the efficiency is not dependent on the protein/pDNA molar ratio. This behavior is in agreement with the fact that Rp3 is not able to efficiently interact with and condense pDNA molecules, as shown by the electrophoretic mobility shift assays (Fig. 5).

Finally, we compared the transfection efficiencies of Rp3-Db and protamine, which is a highly positively charged polypeptide that has been well characterized as a non-viral delivery vector (Tsuchiya et al. 2006; Arangoa et al. 2003). Our transfection assays showed that Rp3-Db/pDNA complexes at molar ratios of 8,000 and 12,000 presented a Luciferase reporter gene expression that was 62- and 398-fold higher, respectively, when compared with protamine/pDNA complexes at the same molar ratio (Fig. 6c).

Discussion

Here, we present the cloning, expression, and purification of the human dynein light chain Rp3 and the first report of its low-resolution structural characterization. Our results suggest that recombinant human light chain Rp3 is highly dependent on the redox environment as a single dimer population is observed under reduced conditions and dimers, tetramers, and hexamers can be observed under non-reducing conditions. Additionally, recombinant human Rp3 does not occur as a folded monomer intermediate during the unfolding process by low pH, as previously reported for *D. melanogaster* Lc8 but remains as a dimer in solution at pH 4.0 followed by unfolding at lower pH values. The observed stability of the Rp3 dimer over a broad pH range is a valuable feature for a non-viral delivery vector, as pDNA/protein delivery complexes may be subjected to significant pH variations inside the cells, e.g., acidification in endocytic compartments (pH~5.0) during intracellular trafficking.

Our SAXS data analysis indicated an exposed Rp3 N terminus in the quaternary structure, i.e., the N terminus was not buried in the monomer–monomer interface or in the monomer fold. This feature led us to introduce the DNA-binding domain at the N terminus rather than the C terminus to ensure that the domain was exposed and able to interact with DNA molecules. Moreover, previous reports showed that the monomer–monomer interfaces of dynein light chains are involved in binding to the dynein intermediate chain (Makokha et al. 2002). Therefore, the addition of a DNA-binding at this site may compromise the dynein light chain–intermediate chain interaction in situ.

The physic-chemical and structural data obtained for human dynein light chain Rp3 allowed us to successfully generate, express, and purify its modified variant with an N-terminal DNA binding domain (Rp3-Db). The functionality of both Rp3 and Rp3-Db was further proved by dynein intermediate chain interaction assay and electrophoretic mobility shift assay which showed that both proteins were able to interact with dynein intermediate chain and only Rp3-Db was able to interact with pDNA, respectively. This information is of great relevance as we expected that once inside the cell, the pDNA/Rp3-Db complex interacts with the dynein motor complex to mediate a more rapid and safe transport of exogenous DNA material to the proximity of the cell nuclei.

Our transfection data showed that Rp3-Db delivery efficiency was 10^3- to 10^4-fold higher than that of the unmodified recombinant Rp3 and 400-times higher than protamine-mediated transfection. The higher delivery efficiency of Rp3-Db proves the feasibility of our approach to use modified light chains as delivery vectors based on the retrograde cellular transport mediated by dynein complex. We are now focusing our efforts on elucidating whether the protein/pDNA complex traffic inside the cell is dependent on the dynein motor complex/microtubule.

Finally, our strategy to evaluate dynein light chains as delivery vectors was based on several criteria: (1) these light chains exhibit different cargo binding affinities (Tai et al. 1998; Tai et al. 2001; Yeh et al. 2005; Benison et al. 2007); (2) TcTex and Rp3 compete for the same binding domain in the dynein intermediate chain N terminus, whereas Lc8 binds to a different region (Tai et al. 2001; Makokha et al. 2002); (3) light chains form complexes with intermediate chains with different affinities; and (4) the expression levels of light chains (including dynein heavy and intermediate chains) are tissue-dependent (Vaughan and Vallee 1995; King et al. 1998; Nurminsky et al. 1998; Chuang et al. 2001). These reports suggest that different dynein motor complexes may be found in different cell types with diverging cargo affinities. Therefore, the characterization of the main light chains (Lc8, Rp3, and TcTex) as non-viral delivery vectors allows us to evaluate the efficiency of different protein/pDNA complexes that may be related to the affinity of the light chain to the dynein motor complex leading to different delivery efficiency.

Another positive aspect of the strategy of modular proteins presented here is the feasibility of adding new functional domains to the dynein light chain. Our group is currently developing modified versions of the Rp3 light chain with an enhanced N-terminal DNA-binding domain (with 9 and 12 arginine residues) and a cell-penetrating peptide at the C terminus (an HIV-originated TAT peptide flanked by histidine residues). This approach may further enhance pDNA/protein complex internalization and endosomal escape favoring transfection efficiency and may contribute to a better understanding of the molecular mechanisms involved in intracellular trafficking.

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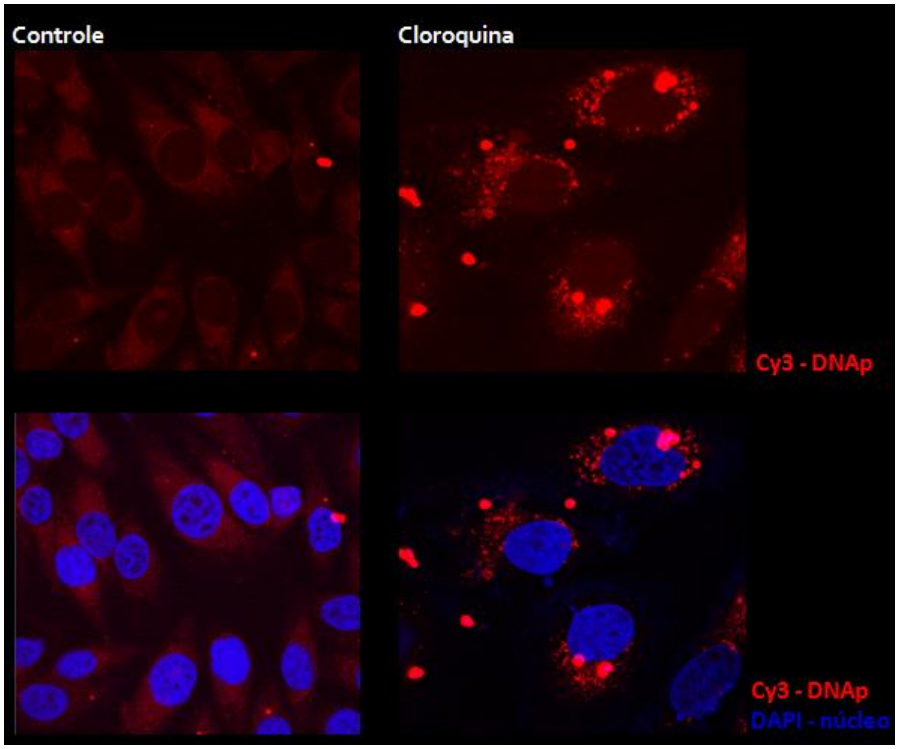
6. Resultados Complementares

6.1 Microscopia confocal de fluorescência

Adicionalmente aos resultados apresentados no artigo científico já publicado, intitulado *"Development of a recombinant fusion protein based on the dynein light chain LC8 for non-viral gene delivery",* foram realizados ensaios de microscopia confocal de fluorescência utilizando-se o vetor não-viral desenvolvido LD4 (cadeia leve Lc8 com o domínio de ligação ao DNA 4) complexado com DNA plasmidial na relação molar proteína:DNAp de 8000. Previamente ao processo de transfecção, as células foram tratadas com cloroquina, com ciliobrevina D ou com ambas as drogas (Figura 6.1). Tal abordagem visou averiguar se o vetor não-viral desenvolvido de fato recruta o transporte retrógrado mediado por Dineína no processo de entrega gênica.

PAINEL 1



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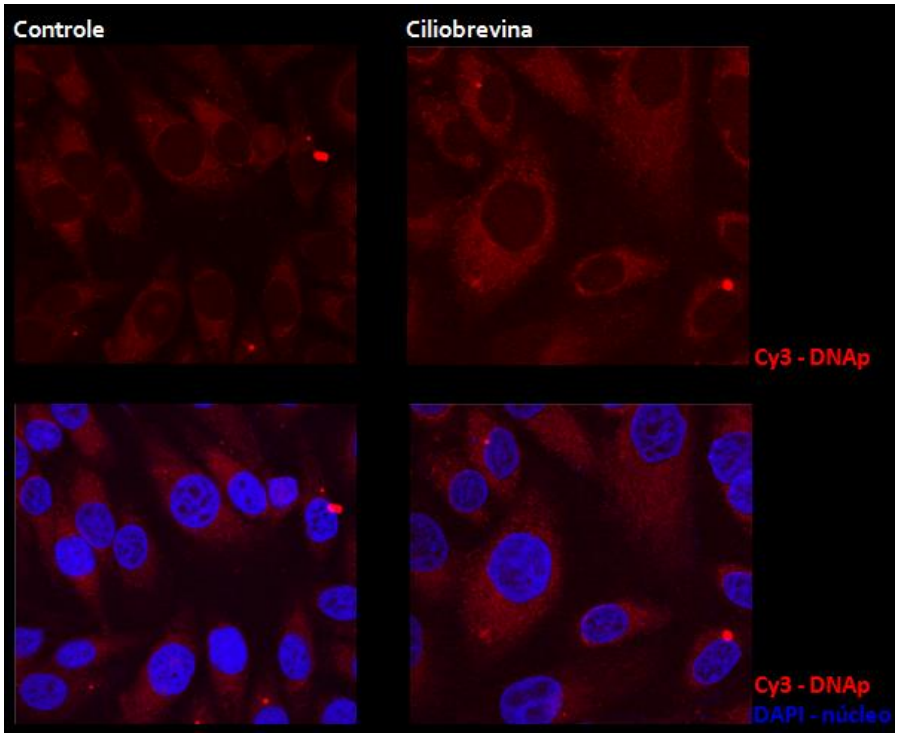

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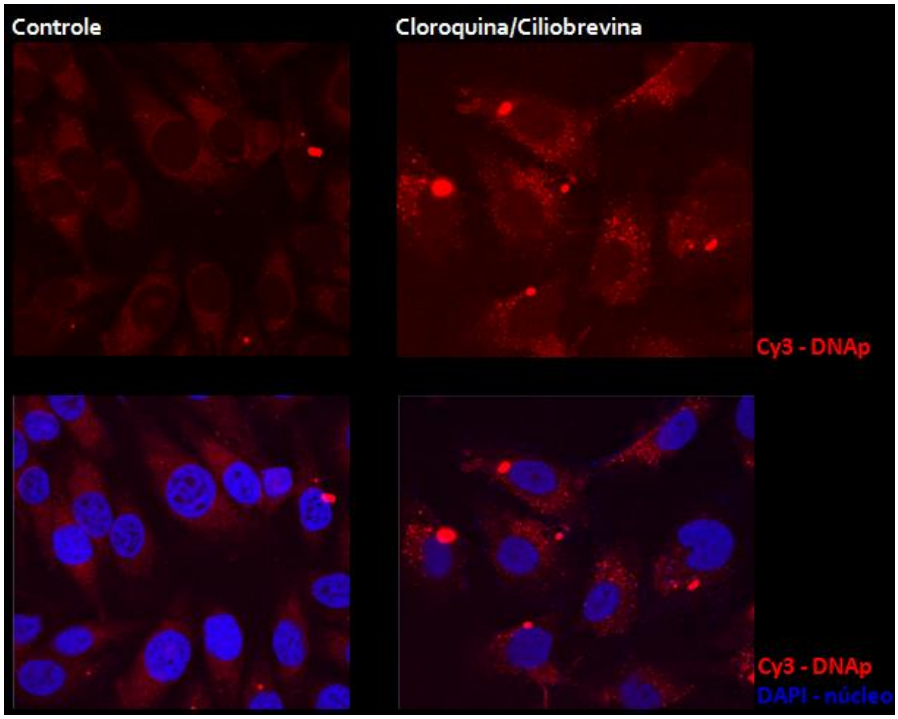

Figura 6.1 - Microscopia confocal de fluorescência. Células HeLa foram transfectadas com complexos de entrega LD4-DNAp (razão molar proteína:DNAp de 8000), com fixação 24 h após a transfecção. PAINEL 1: células controle e células tratadas com cloroquina. PAINEL 2: células controle e células tratadas com ciliobrevina. PAINEL 3: células controle e células tratadas com cloroquina e ciliobrevina. Núcleo marcado com DAPI e DNA plasmidial marcado com Cy3.

O vetor plasmidial foi marcado com o fluoróforo Cy3 de forma a acompanhar sua distribuição dentro da célula sob os diferentes tratamentos realizados. O processo de fixação foi realizado após 24 horas do processo de transfecção. Observa-se que o tratamento com cloroquina impede que significativa parte dos complexos de entrega (LD4-DNAp-Cy3) seja degrada na via endocítica uma vez que o sinal do DNA plasmidial é superior àquele observado na amostra controle. Nota-se também acúmulo do material genético exógeno na região perinuclear. O tratamento com ciliobrevina D resulta em um sinal do DNA plasmidial mais forte em comparação com o controle, muito embora sem acúmulo deste na região perinuclear. Tal observação é consonante com a paralisação do transporte retrógrado mediado por Dineína, cujo domínio de ATPase foi inibido pela droga. O uso concomitante de ciliobrevina D e cloroquina mostrou um aumento do sinal do material genético e um menor acúmulo perinuclear. Tais resultados fortalecem os indícios obtidos no presente estudo mostrando que os vetores não-virais desenvolvidos de fato se utilizam do transporte retrógrado mediado por Dineína para mediar o transporte do material genético exógeno em direção ao núcleo. Abordagens futuras realizando ensaios de microscopia em diversos intervalos de tempo após o processo de transfecção podem adicionar mais informações sobre o tráfego de moléculas plasmidiais mediado pelos vetores desenvolvidos.

6.2 Otimização do domínio peptídico de ligação ao DNA - Domínio D5

Outra abordagem adotada no presente estudo foi a expansão do domínio de ligação ao DNA 4. Tendo-se em vista que tal domínio mostrou ser mais eficiente em mediar a interação e condensação de moléculas plasmidiais, sua variante duplicada (domínio D5) foi produzida e fusionada ao N-terminal da cadeia leve de Dineína Lc8 (Lc8-D5) (Figura 6.2 A). O maior número de resíduos de arginina (R) no domínio de ligação ao DNA justifica-se pela possibilidade deste ser mais eficiente no processo de formação de complexos de entrega quando em contato com moléculas

plasmidiais. Tal característica possibilitaria a obtenção de complexos de entrega de alta eficiência utilizando razões molares de vetor protéico:DNA plasmidial menores. Esta redução tem considerações práticas quanto aos quadros de citotoxicidade, resposta imunológica e custos de produção dos vetores não-virais descritos no presente estudo.

A proteína recombinante Lc8-D5 foi produzida e purificada com sucesso utilizando-se os mesmos métodos descritos para os outros vetores não-virais protéicos produzidos no presente trabalho (Figura 6.2 B). Ensaios preliminares de transfecção demonstraram que a proteína Lc8-D5 é capaz de mediar a entrega de DNA plasmidial ao núcleo de células HeLa (Figura 6.2 C). Entretanto, estudos adicionais são necessários para averiguar se a duplicação do domínio de ligação ao DNA permitirá a utilização de menor quantidade do vetor protéico na formação de complexos de entrega.

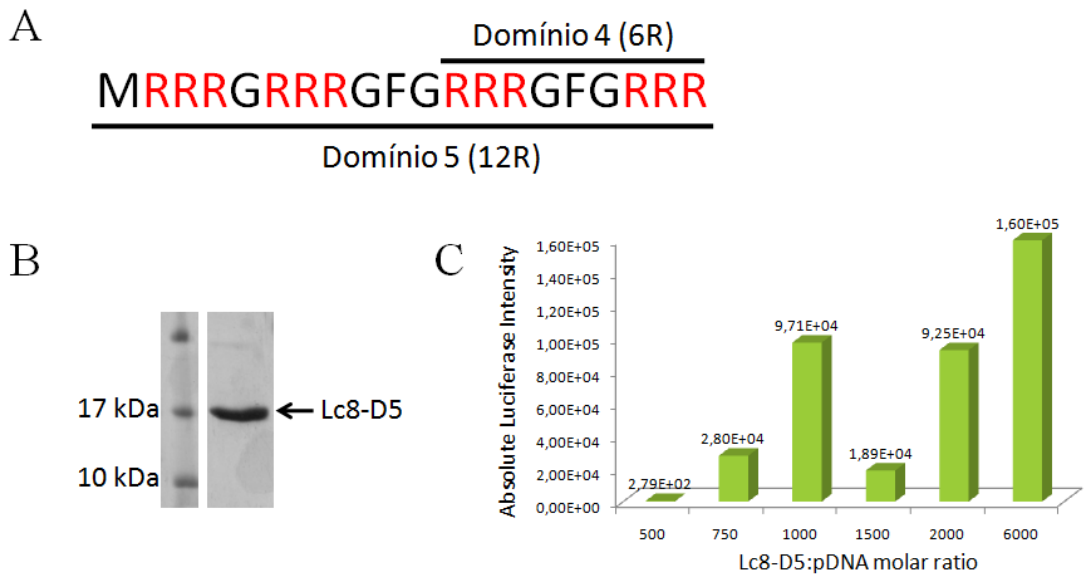

Figura 6.2 - A: Seqüência em aminoácidos do domínio de ligação ao DNA 5. **B:** Purificação da cadeia leve Lc8 fusionada ao domínio de ligação ao DNA 5. **C:** Ensaio preliminar de transfecção de células HeLa utilizando-se Lc8-D5 como vetor não-viral de entrega gênica. Relações molares proteína:DNAp de 500 a 6000 foram utilizadas.

6.3 Microscopia confocal "*time-lapse*" - Rp3-Db

O tráfego intracelular de complexos de entrega formados por DNA plasmidial e o vetor não-viral protéico Rp3-Db também foi avaliado utilizando-se microscopia confocal "*time-lapse*". Esta

técnica permite obter imagens de microscopia confocal do mesmo campo de análise em curtos intervalos de tempo, permitindo acompanhar os processos de internalização e tráfego dos complexos de entrega estudados por longos períodos. Utilizando-se DNA plasmidial marcado com o fluoróforo Cy3, complexos de entrega foram formados com a proteína Rp3-Db na razão molar proteína:DNA plasmidial de 8000 e utilizados na transfecção de células HeLa. Imagens foram coletadas por aproximadamente 12 horas utilizando-se o equipamento Nikon BioStation IM-Q (Nikon).

Ao longo das 12 horas de análise observa-se o acúmulo dos complexos de entrega na região perinuclear das células (Figura 6.3). Ressalta-se que, no tempo de "zero horas", complexos de entrega podem ser observados na região perinuclear de algumas células indicando que o processo de internalização e transporte ocorre em curto espaço de tempo. O ponto destacado nas imagens (seta) representa com mais clareza o processo de internalização dos complexos de entrega gênica e seu acúmulo perinuclear. Adicionalmente, pode ser observado que tais complexos, uma vez internalizados, migram de maneira linear em direção à um único ponto da região perinuclear. Tal observação é consistente com o tráfego celular retrógrado mediado por Dineína levando à um acúmulo destes complexos na região do centrossoma.

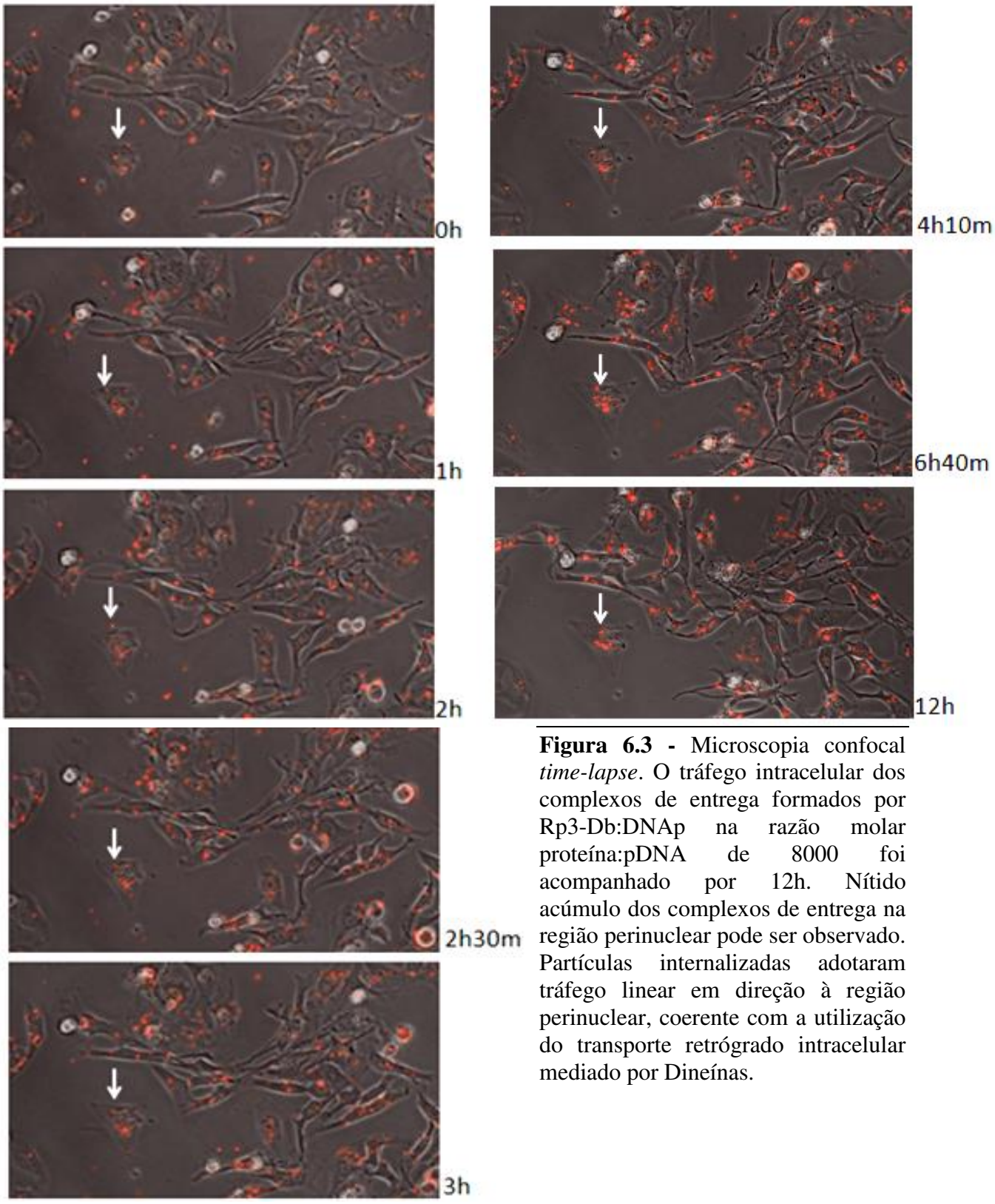

Figura 6.3 - Microscopia confocal *time-lapse*. O tráfego intracelular dos complexos de entrega formados por Rp3-Db:DNAp na razão molar proteína:pDNA de 8000 foi acompanhado por 12h. Nítido acúmulo dos complexos de entrega na região perinuclear pode ser observado. Partículas internalizadas adotaram tráfego linear em direção à região perinuclear, coerente com a utilização do transporte retrógrado intracelular mediado por Dineínas.

7. Conclusões e Perspectivas

O presente estudo relata a utilização de cadeias leves de Dineína modificadas, produzidas em *E. coli* como vetores não-virais de entrega gênica. A adição de um domínio peptídico catiônico de ligação ao DNA à região N-terminal das cadeias leves de Dineína Lc8 (LD4) e Rp3 (Rp3-Db) conferiu à estas a habilidade de interagir com moléculas de DNA plasmidial, compactando-o em partículas capazes de mediar a entrega gênica ao núcleo de células eucarióticas. A eficiência de transfecção observada para ambas as construções é superior àquela apresentada pelo peptídeo catiônico protamina. Adicionalmente, a combinação da proteína LD4 com o lipídio catiônico Lipofectamina resultou em complexos de transfecção ainda mais eficientes. A análise do efeito citotóxico dos vetores não-virais desenvolvidos no presente projeto mostrou que estes apresentam baixa toxicidade celular e, devido ao fato de se basearem em proteínas humanas, espera-se que apresentem baixa resposta imunológica. Ressalta-se também o relativo baixo custo de produção relacionado à produção das proteínas recombinantes desenvolvidas no presente projeto, característica de relevância no processo de desenvolvimento de vetores não-virais para terapia gênica.

A abordagem do presente estudo em utilizar cadeias leves de Dineína como vetores de entrega gênica baseia-se na possibilidade de recrutar o transporte retrógrado celular mediado pelo complexo motor Dineína, visando facilitar o transporte do material genético terapêutico da periferia celular para a região perinuclear. No intuito de averiguar se de fato a eficiência de entrega gênica observada decorre do recrutamento do transporte retrógrado celular, nossos estudos revelaram que: (1) ambas as proteínas LD4 e Rp3-Db, apesar de possuírem o domínio de ligação ao DNA em seu N-terminal, apresentam-se corretamente enoveladas e diméricas, seu estado oligomérico funcional; (2) as cadeias leves de Dineína modificadas LD4 e Rp3-Db são capazes de interagir com a com o

domínio N-terminal da cadeia intermediária de Dineína *in vitro*, demonstrando sua capacidade funcional; (3) a eficiência de entrega depende do funcionamento da rede de microtúbulos, utilizada pelo complexo motor Dineína no transporte celular; (4) análises de microscopia confocal mostram acúmulo perinuclear de partículas de entrega após 24 h de transfecção em células tratadas com cloroquina (que permite o escape endossomal dos complexos internalizados), tal acúmulo não é nitidamente observado quando o complexo motor é inibido pela ciliobrevina D e a utilização concomitante destas drogas resulta em maior sinal dos complexos de entrega no citoplasma, com acúmulo perinuclear não tão evidente.

No contexto destas observações, acredita-se que os complexos de entrega estudados cheguem à região perinuclear através de duas principais rotas: (1) uma vez internalizados em vesículas endossomais, parte dos complexos de entrega consegue escapar das vesículas e através do transporte retrógrado, mediado pelo complexo motor Dineína, chega à região perinuclear (2) vesículas endossomais contendo complexos de entrega seguem na via de degradação endocítica que por sua vez depende do transporte retrógrado celular mediado por Dineínas para a maturação das vesículas endossomais e sua fusão com vesículas lisossomais (Huotari & Helenius, 2011), aproximando-se da região perinuclear e quando da fusão com vesículas lisossomais e acidificação da cavidade luminal, parte dos complexos consegue escapar e seguir para a região perinuclear.

Portanto, a eficiência de entrega gênica dos vetores desenvolvidos provavelmente decorre da sua interação direta com o complexo motor Dineína bem como pelo escape de vesículas endossomais que foram transportadas para a proximidade da região nuclear.

A elevada eficiência de transfecção demonstrada pelos vetores não-virais baseados em cadeias leves de Dineína, apresentados no presente estudo, associada à sua baixa toxicidade celular e baixo custo de produção são características desejáveis e promissoras no desenvolvimento de vetores para terapia gênica. Contudo, ressalta-se que a degradação intracelular dos complexos de entrega

aprisionados na via endocítica permanece como um obstáculo à transfecções mais eficientes. A adição de domínios peptídicos membrano-ativos e/ou de clusters de resíduos de histidina aos vetores desenvolvidos, possibilitam facilitar o processo de internalização celular e escape endossomal, respectivamente, e estão sendo adotados pelo nosso grupo de pesquisa.

O presente estudo mostrou ser possível mimetizar partículas virais e recrutar o transporte celular retrógrado para facilitar o tráfego intracelular de material genético exógeno em direção ao núcleo. Tal abordagem possui alto potencial biotecnológico, devido às possíveis aplicações em terapia gênica e vacinação por DNA. Ainda assim, é uma abordagem pouco explorada pela comunidade científica. O presente trabalho espera demonstrar sua viabilidade, elucidar ainda que parcialmente seus mecanismos, e contribuir para futuras abordagens no desenvolvimento de vetores não-virais seguros e eficientes.

8. Referências

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9. Anexos

9.1 Manuscrito

"Expression and purification of a small TAT based peptide for gene delivery studies"

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Manuscrito em preparação para futura submissão à revista Protein Expression and Purification

Abstract

The TAT peptide has a high biotechnology potential due to its ability to transpose cellular membranes which allows its use as o molecular tool to mediate the cellular internalization of many molecules such as proteins, DNA and RNA. In the present manuscript, we report the cloning, optimization of the expression conditions, and purification of a TAT based small peptide aiming at gene delivery applications. By analyzing the cytotoxic effect of TAT peptide expression to the *E. coli* expression host cells, we were able to determine optimal conditions that allows satisfactory expression yields of the peptide. We were able to use a single Ni-NTA affinity chromatographic step to purify the recombinant peptide and confirmed its ability to interact and condense plasmidial DNA (pDNA). Transfection assays using cultured HeLa cells indicated that TAT:pDNA complexes were capable of delivering genetic material although at a relatively low transfection efficiency due to endosomal entrapment during intracellular trafficking. The described protocol allows the expression and purification of small TAT based peptides with satisfactory yield and purity and stands as an alternative to the costly chemical synthesis of peptides used in gene delivery studies.

1. Introduction

Cell penetrating peptides (CPPs) comprises a peptide group that has the remarkable ability to transpose cellular membranes with high efficiency. The biotechnological value of this property has gained the attention of the scientific community as it may be applied to enhance internalization of therapeutic molecules such as proteins, peptides, RNA and DNA molecules into human cells (Lo & Wang, 2008; Koren and Torchilin, 2012; Heitz *et al.*,2009; Deshayes *et al.*, 2005). The TAT peptide, derived from the HIV transactivator of transcription factor is a CPP extensively studied. It has a highly positive charge due to the presence of six arginine and two lysine residues in its sequence ($_{47}$YGRKKRRQRRR$_{58}$) with an efficient capacity to translocate across cellular membranes (Herce & Garcia, 2007). The mechanism by which the TAT peptide mediates cellular membrane transposition was proposed by Herce & Garcia (2007) which involves a strong interaction between the arginine residues and the phosphate group of the lipid bilayer that could form a hydrophilic pore, allowing the peptide to diffuse across the cellular membrane. Due its cell transposition ability, the applicability of the TAT peptide as a delivery tool for pharmacological compounds, such as DNA and RNA molecules, has been exploited as well as its association with other delivery vectors (Lo & Wang, 2008; Yamano *et al.*, 2011; Bullok *et al.*, 2002; Song *et al.*, 2010; Bolhassani *et al.*, 2011 and Brooks *et al.*, 2005). The modification of TAT peptides by introducing histidine residues at the N- and C-terminals has also been described in order to enhance the endosomal scape ability of TAT based delivery vectors exploiting the *proton sponge* effect triggered by histidine clusters (Lo & Wang, 2008).

Our research group is focused on the development and characterization of recombinant modular proteins for non-viral gene delivery (Toledo *et al.*, 2012; Toledo *et al.*, 2013) and is interested on the use of modified TAT peptides as fusion domains to enhance cellular uptake and intracellular trafficking. As previously demonstrated by Lo & Wang (2008), small changes on the

primary sequence of the TAT peptide may lead to dramatic differences on delivery efficiency and, thus, a better understanding on the mechanisms of intracellular trafficking of TAT based vectors is still necessary. However, heterologous expression of small cationic peptides in *E. coli* host is usually hampered by intracellular degradation of the expressed peptide and by its toxicity to the host cell, whereas chemical synthesis, most commonly used for TAT peptide production, is especially costly at many countries.

In this context, the development of simple cloning, expression and purification protocols for the production of TAT based peptides is important and may be of the interest of those working on the field of gene and drug delivery. In the present study, we report the development and evaluation of an expression (in *Escherichia coli* prokaryotic host) and purification protocol for a modified TAT peptide, flanked by histidine residues clusters.

2. Material and methods

2.1. Cloning of TAT peptide DNA sequence into the pET28a expression vector

The complementary oligonucleotide sequences corresponding to TAT peptide flanked by two clusters of histidines residues was synthesized and annealed (forward sequence: 5' AA TTC CAT CAT CAT CAT CAT TAT GGT CGT AAA AAA CGT CGT CAG CGT CGT CGT CAT CAT CAT CAT CAT TAA C 3', reverse sequence: 5' TC GAG TTA ATG ATG ATG ATG ATG ACG ACG ACG CTG ACG ACG TTT TTT ACG ACC ATA ATG ATG ATG ATG ATG G 3'). The final double strand oligonucleotide was cloned into the pET28a vector between *Eco*RI and *Xho*I restriction endonucleases sites. After electroporation in DH5-α *Escherichia coli* cells, clones were screened by single restriction enzyme digestion of purified plasmids using *Hind*III endonuclease.

Purified pET28 vector harboring the TAT peptide oligonucleotide sequence, obtained from positive clones, was electroporated in BL21(DE3) *E. coli* expression strain.

2.2. Expression and purification of recombinant TAT peptide

For the expression of TAT peptide, BL21(DE3) *E. coli* cells harboring TAT-pET28a vector were inoculated in 3.0 ml of LB growth medium supplemented with 0,2% glucose and 30 µg/ml kanamycin at 300 rpm and 37 ºC overnight. After growth, cells were inoculated in 1.0 L LB medium also supplemented with 0,2% glucose and 30 µg/ml kanamycin at 300 rpm and 37ºC until an optical density of 2.0. Peptide expression was induced using 0.4 mM IPTG for 90 min. Cells were pelleted by centrifugation at 6000*g* for 12 min.

Protein extraction was performed by resuspending the cells in buffer A (50 mM sodium phosphate pH 8.0 containing 1.0 M NaCl, 0.1 mM EDTA and 1 mM PMSF) followed by cell disruption by sonication. The cell extract was clarified by centrifugation at 16000*g* for 45 min at 5ºC. TAT peptide purification was performed in a single Ni-NTA affinity chromatography step using a 1.0 mL gravity column previously equilibrated with extraction buffer. TAT peptide elution was performed by a imidazole step gradient consisting of elution steps with 20, 50, 75, 100, 200 and 500 mM imidazole in extraction buffer. The concentration and purity of the peptide was evaluated by SDS-PAGE.

A further size exclusion chromatographic step was adopted to perform buffer exchange and to remove any contaminants. Superdex 200 GL10/300 or Superdex 75 GL10/300 prepacked column (GE Healthcare, Uppsala, Sweden) was used with an AKTA FPLC system to perform buffer exchange against PBS at pH 7.4. Further concentration of the peptide was performed using a Centricon® device with a MWCO of 3000 Da. Final peptide concentration was determined by optical absorbance at 280 nm.

2.3. Analysis of the toxicity of TAT peptide expression to the *E. coli* expression vector

To evaluate the toxicity of TAT peptide expression to *E. coli* BL21(DE3) cells, we monitored the growth of cells expressing the TAT peptide and cells harboring only the pET28 vector as a control. Previously grown cells (at an $O.D._{600}$ of 2.0) were inoculated (100 µL) in 100 mL of LB growth medium supplemented, when indicated, with 0.2% glucose, 30 µg/mL kanamycin and kept at 37 ºC and 300 rpm. At indicated optical densities, protein expression was induced by addition of 0.4 mM IPTG. The optical density at 600 nm was monitored for 12 hours after inoculation. Growth curves were analyzed in triplicates for each case.

Additionally, we performed a serial dilution of both BL21(DE3) *E. coli* strains (harboring TAT:pET28a vector and pET28a vector only). For both cultures, we started at a $O.D._{600}$ of 2.0 and dilutions were made up to 10^{-7}. Five microliters of each dilution were applied on a LB agar plate which was previously supplemented with 0,4 mM. Plates were incubated for 24h at 37ºC. Each experiment was performed in triplicate.

2.4. Detection of TAT peptide expression by *western* blot

The expression of TAT peptide was monitored under different conditions by *western* blot. We used an anti-His_6-tag antibody to detect TAT expression as it has a hexahistidine tag derived from the pET28a vector and is flanked by clusters of 5 histidine residues. Cells were harvested after 12 hours of growth analysis by centrifugation for 10 min at 15000*g*. Cells from each condition analyzed were individually resuspended in 100 µL deionyzed water and 100 µL of SDS-PAGE loading buffer was added. Samples were denaturated by boiling for 10 min and applied to a SDS-PAGE 15% matrix. Western blot was performed under standard conditions using a Semi-dry blotting System (Bio-Rad Laboratories Inc., USA) and primary antibody against histidine tag was used at 1:8000 dilution for 2 h. Secondary antibody linked to alkaline phosphatase was used at

1:8000 dilution for 2 h followed by membrane revelation with NBT/BCIP color development substrate (Promega).

2.5. Evaluation of recombinant TAT peptide as DNA delivery vector

The ability of recombinant TAT peptide to bind to and condensate plasmidial DNA was evaluated by eletrophoretic mobility shift assay using an 0,8% agarose matrix. pVAX-LUC plasmidial vector, described previously (Toledo *et al*., 2012) was used for shift and transfection assays. Purified TAT peptide was used after dialysis against PBS at pH 7.4 or 20 mM MES buffer at pH 6. TAT-pDNA complexes were assembled at different peptide:pDNA molar ratios ranging from 100 to 128000. They were formed in deionized water in a final volume of 50 µL and were incubate for 60 min at room temperature (~25ºC). Before loading into the agarose matrix, glycerol at a final concentration of 8,7% was added to each TAT-pDNA complex. Ethidium bromide was used for gel staining.

2.6. Transfection of HeLa cells using recombinant TAT peptide as delivery vector

HeLa cells (human epithelial carcinoma) were grown in F-12 medium (Ham Nutrient Mixture, Gibco, England) supplemented with 10% (v/v) FBS (fetal bovine serum) (Gibco, England). Cells were plated (5 x 10^4 cells per well) and incubated at 37ºC in humidified atmosphere with 5% CO_2. At cellular confluence of 70-80% cells were transfected with pDNA:TAT complexes at 1:140000 molar ratio. To evaluate the endosomal escape of the delivery complex, transfection assays were also performed with pre-treatment of the cells with 100 µM chloroquine. Cells were collected after 24 h post-transfection for determination of luciferase activity using the Luciferase Assay System (Promega, USA), following the manufacturer's instructions. Luminescence intensity was normalized against protein concentration in each transfection

sample, as determined by the Micro BCA Protein Assay Kit (Thermo Scientific, USA).

3. Results and discussion

3.1 Growth curves of TAT expressing cells shows its citotoxic effect

We have successfully cloned the TAT based peptide into the pET28a vector and selected positive clones by endonuclease digestion of purified plasmids (Figure 1). The cytotoxicity of the recombinant TAT peptide was first analysed by performing growth curves of *E. coli* BL21(DE3) expression strains harboring the pET28-TAT plasmid. Peptide expression was induced at different optical densities and *E. coli* cells harboring the empty pET28a vector was used as a control.

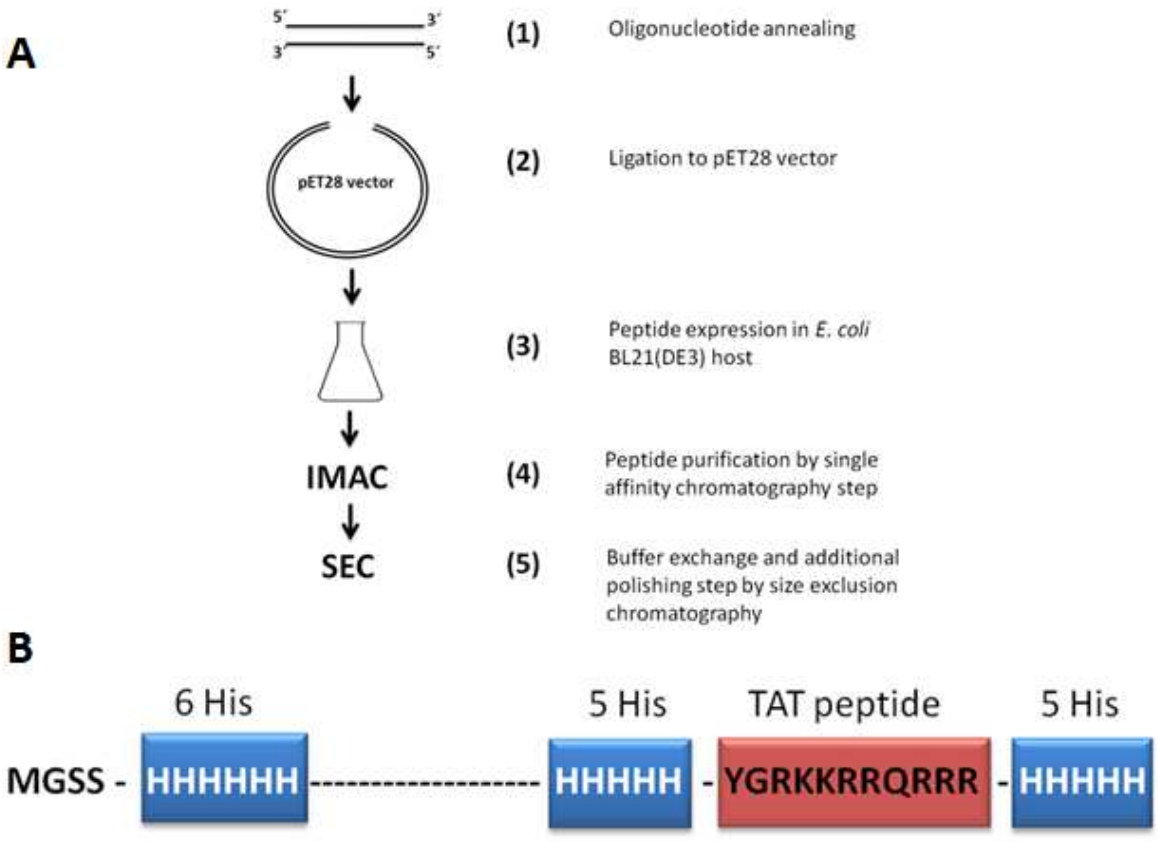

Figure 1 - A: Schematic representation of the cloning, expression and purification of the TAT based peptide developed in the present study. **B:** Representation of the TAT based peptide cloned in pET28 vector.

As seen on Figure 2, no difference in growth curves were observed when cells were grown without induction by IPTG. Addition of 0.2% glucose to inhibit basal expression of the cloned gene also did not affect the growth curves (Figure 2A).

However, when cells were induced at low optical density (OD = 0.2), a decrease in pET28-TAT harboring cell growth rate could be observed when comparing to the control cells (Figure 2B). Surprisingly, peptide expression induction at higher cell densities (OD = 1.3 and 2.2) did not present the same effect (Figure 2C and D) as no difference could be observed in the growth rate between TAT expressing and the control cells.

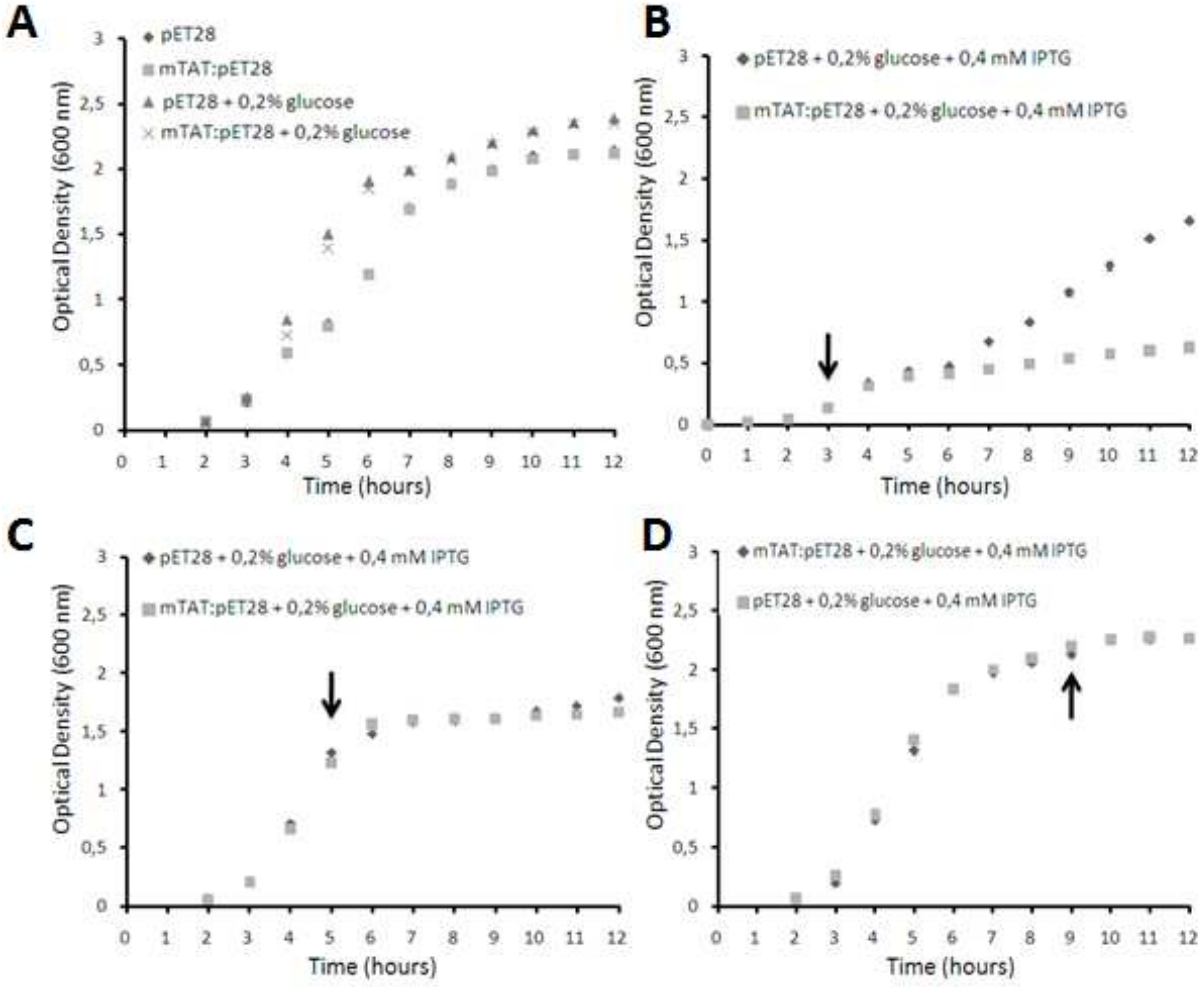

Figure 2 - Growth curves of pET28-TAT and pET28 (control) harboring BL21(DE3) cells measured at 600 nm. **A:** Growth curves in the presence or absence of 0.2% glucose. **B:** Induction of expression by 0,4 mM IPTG at low optical density (0.2). **C and D:** Induction of expression by 0.4 mM IPTG at optical densities of 1.3 and 2.2. Arrows indicate addition of 0.4 mM IPTG.

We monitored the TAT peptide expression by *western* blot to assure that the cell growth behavior was indeed correlated to the production of the peptide. Using anti His-tag antibody, we were able to visualize the expression of the TAT peptide and also the peptide produced by empty pET28a vector (Figure 3B). In the absence of induction by IPTG, no basal expression of TAT peptide could be detected by this technique, however we may consider that some expression might occur and could not be detected which would still affect cell growth, therefore we maintained the 0.2 % glucose supplementation of the growth medium. By adding 0.4 mM IPTG at optical densities of 1.3 (TAT+glc+IPTG^1) and 2.2 (TAT+glc+IPTG^2) it was possible to observe the expression of the recombinant TAT peptide whereas no clear bands could be observed in both controls (p28+glc+IPTG^1 and p28+glc+IPTG^2) (Figure 3A).

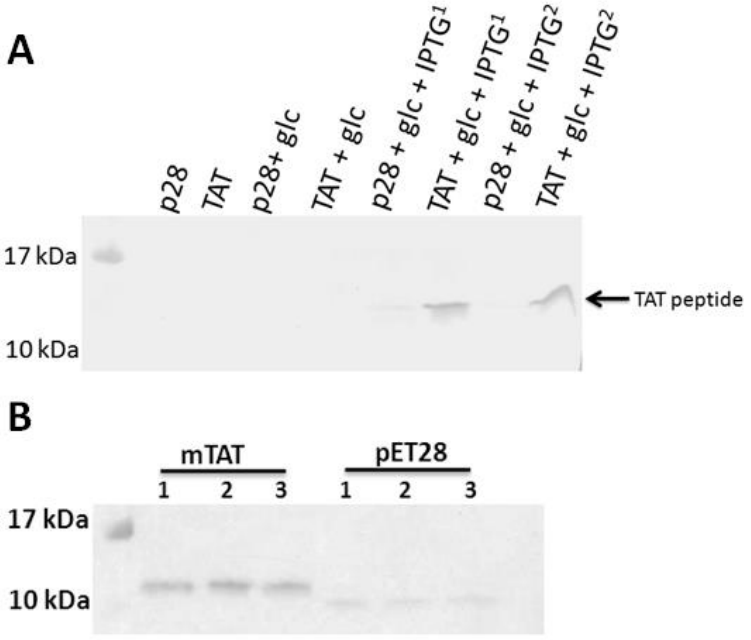

Figure 3 - TAT peptide expression analysis by *western* blot. **A:** Detection of TAT expression after 12h under the same conditions used to analyze cellular growth. glc indicates presence of 0.2% glucose in the medium.. IPTG^1 indicates addition of 0,4 mM IPTG when cells reached an OD_{600} of 1.3. IPTG^2 indicates addition of 0.4 mM IPTG when cells reached an OD_{600} of 2.2.

The same cytotoxic effect at low cell density was observed by serial dilution assays of cells expressing the TAT peptide (Figure 4). Interestingly, no differences in cell viability was observed between TAT expressing and control cells without pre-induction with 0.4 mM IPTG for 1 hour at 37ºC and 300 rpm before platting. Induction of expression lead to a decreased cell viability of TAT-pET28 harboring cells when compared to the control (Figure 4, indicated by arrow). This result is in agreement with the growth curves that showed, as it indicates that recombinant TAT peptide expression at low optical density decreases cell viability.

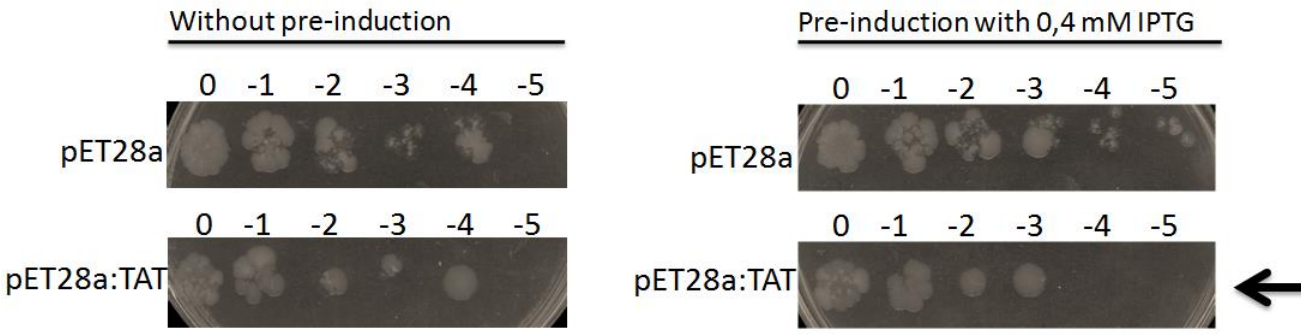

Figure 4 - Serial dilution analysis to evaluate the toxic effect of TAT peptide expression in *E. coli* expression host. Pre-induction of peptide expression for 1 h at 37ºC and 300 rpm using 0.4 mM IPTG before plating shows decreased cell viability of pET28a:TAT harboring cells in comparison to control cells (empty pET28a harboring cells).

3.2 Expression and purification of recombinant TAT peptide

Once we have investigated the optimal growth and induction conditions that would allow the production of the TAT peptide, maintaining cellular viability, we performed larger scale peptide expression and purification. As we observed that peptide expression induction at higher optical densities did not affect cellular viability we have grown cells in LB medium supplemented by 0.2 % glucose. Although western blot did not indicate basal expression of recombinant TAT peptide, we adopted this approach to rule out any effects caused by minimal amounts of TAT expression during

cellular growth. Expression was preformed growing cells to an O.D. of 2.0 at 37ºC and 300 rpm, followed by peptide expression induction by the addition of 0.4 mM IPTG and further incubation at the same conditions for 2 h. Despite the fact that we observed TAT expression after 12 h post-induction using *western* blot (Figure 3 A and B), larger scale expression assays for 12 h showed low expression yields wile induction for short periods (1-3 hours) presented better results. After cell harvesting and lysis, purification was successfully performed by using a single Ni-NTA affinity chromatography step (Figure 5).

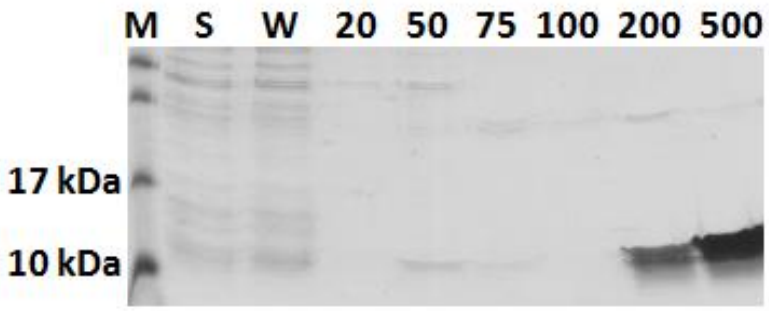

Figure 5 - 15% SDS-PAGE analysis of the Ni-NTA affinity chromatography purification step. M: molecular marker. S: soluble fraction. W: wash step. 20-500: Elution step with 20 to 500 mM Imidazole. Purified TAT based peptide is eluted with 200 and 500 mM Imidazole.

An additional size exclusion chromatographic step was finally performed in order to simultaneously remove trace impurities of TAT peptide samples eluted with 200 and 500 mM imidazole and to exchange the sample buffer to PBS, for further DNA interaction assays. The final yield of the recombinant TAT peptide purification process was calculated as 2 miligrams per liter of growth medium.

3.3 Recombinant TAT peptide is able to interact with and condense pDNA

In order to access the ability of the *E. coli* produced TAT peptide to interact with plasmidial DNA we performed an electrophoretic mobility shift assay (Figure 6). We observed that purified

TAT peptide, after buffer exchange to PBS by size exclusion chromatography, was able to interact with plasmidial DNA leading to its retarded migration in the agarose matrix at peptide:pDNA molar ratios from 500 to 16000 (Figure 6A). Additionally, we could observe that when the TAT peptide was dialyzed against 20 mM MES buffer at pH 6.0, the complexes formed could be observed migrating towards the negative electrode, indicating that TAT binding to the plasmidial molecule neutralized its negative charge forming positively charged complexes (Figure 6B).

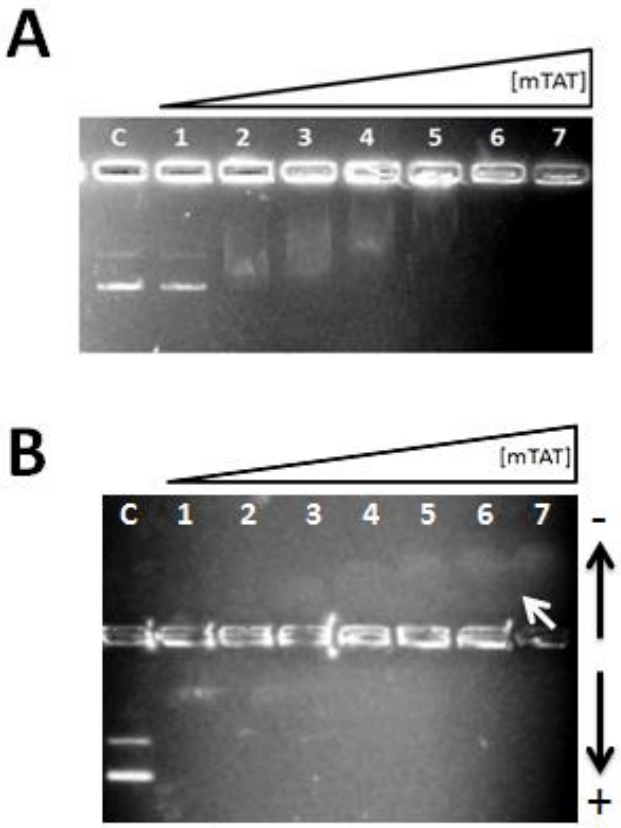

Figure 5 - Electrophoretic mobility shift assay to evaluate TAT peptide interaction with plasmidial DNA. **A**. C: control pDNA, 1-7: pDNA:peptide molar ratios of 1:100, 1:500, 1:2000, 1:4000, 1:8000, 1:16000 and 1:32000. **B:** TAT peptide:pDNA complexes formed with TAT dialyzed against 20 mM MES buffer at pH 6. C: control pDNA, 1-7: pDNA:peptide molar ratios of 1:2000, 1:4000, 1:8000, 1: 16000, 1:32000, 1:64000 and 1:128000. Arrow indicates positively charged complexes migrating towards the negative electrode.

3.4 Recombinant TAT peptide can mediate pDNA delivery to HeLa cells

Transfection of HeLa cells with TAT:pDNA complexes showed that recombinant TAT peptide can mediate delivery of plasmidial DNA to eukaryotic cell nucleus. However, the transfection efficiency obtained was lower than that mediated by protamine, a well characterized peptide used as non-viral delivery vector. As shown in Figure 6, chloroquine treatment of cells revealed that TAT:pDNA complexes are probably being trapped and degraded inside lysosomes. The low transfection efficiency mediated by TAT peptide indicate that further improvements in sequence or fusion with additional domains or proteins is necessary for the development of more efficient non-viral gene delivery vectors.

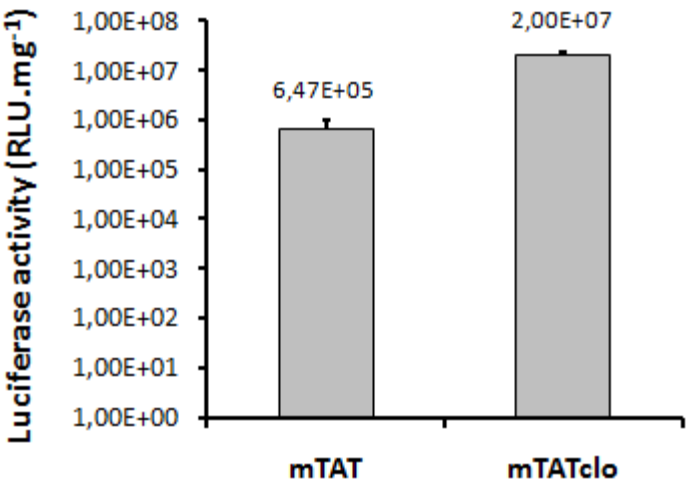

Figure 6 - Transfection of HeLa cells by TAT:pDNA complexes. Transfection efficiency is enhanced by previous treatment of the cells with chloroquine indicating that endosomal entrapment and degradation of TAT:pDNA complexes still occurs.

4 Conclusion

In the present work we report the optimization of growth and expression conditions to produce a small positively charged recombinant TAT peptide in *E. coli* prokaryotic expression host. By monitoring the growth of TAT expressing cells we were able to access the best optical density to

induce the peptide expression without causing decrease in cellular viability. Peptide recovery by a single affinity chromatography step was higher when its expression was induced by 1 to 3 hours, indicating that it may still affect cell viability, even at higher optical densities. The purified peptide was able to interact with plasmidial DNA and mediate its internalization into eukaryotic cells although a considerable fraction of the delivery complexes remained trapped in the endocytic pathway. The present protocol stands as an attractive alternative to TAT based peptide production as it allies high yield and purity with low cost of production with scale up possibilities in contrast with the costly chemical synthesis.

5. References

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9.2 Declaração da Comissão Interna de Biossegurança

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada *"Tráfego Intracelular de Vetores Não-Virais: Desenvolvimento de Proteínas de Fusão para Transporte de DNA Plasmidial Através da Interação com Proteínas Motoras"*

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio – Comissão Interna de Biossegurança , projeto No. 01/2010, Instituição: UNICAMP – CBMEG.

( ) CEUA – Comissão de Ética no Uso de Animais , projeto No. _____, Instituição: _____.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

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Aluno: Marcelo Augusto Szymanski de Toledo

Adriano Rodrigues Azzoni

Orientador: Adriano Rodrigues Azzoni

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Carimbo e assinatura