UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Affinity purification and delivery of a p53-encoding plasmid DNA for gene mediated cancer therapy

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Thesis for obtaining Doctor Degree in

Biochemistry

(3º cycle of studies)

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Covilhã, november of 2018

Dedicatory

To my family and closest friends…

Acknowledgements

I need to start this part by tell: “Professor, 2018 came too fast! Who will guess this in 2013?”. Having said that, I obviously need to start the acknowledgements by express my deepest gratitude to my supervisor Prof. Fani Sousa. Her guidance, knowledge, support, patience and precious revisions were crucial for the success of this work.

I also want to express my deepest gratitude to Professor João Queiroz for all the knowledge and wise opinions transmitted during the development of the research work.

I also gratefully acknowledge the financial support from the Fundação para a Ciência e Tecnologia (FCT), through the PhD fellowship (Ref SFRH/BD/96809/2013).

I would also like to acknowledge to the Health Sciences Research Centre from the University of Beira Interior for give me the opportunity to develop this study on their facilities. Moreover, I would like to express my gratitude to all the people from the Health Sciences Research Centre and Biopharmaceuticals and Biomaterials group, who in one way or another helped me in the development of my work. A special thanks to my lab colleagues and, in particular to Ângela Sousa for their help and support in my panic/crazy times! The peace that you gave me in that times was priceless!

I want to acknowledge to Professor Ana Cecilia Roque and their research group for so well received me during my stay at UCIBIO. It was an experience that I will always remember with affection.

I am very grateful to my friends! Guys, I know that I’m not a person who shows as much affection as you want but, I really love you all! Thank you for being my rock especially during the times when trust and willpower were leaving me.

Finalmente, não poderia acabar esta seção sem agradecer aos meus pais. Todo o vosso esforço culminou naquilo que sou e naquilo que alcancei até hoje! OBRIGADA! Sisters, you know, you are boring and annoying but I like you too…And never forget: We are unstoppable!

In fact, I will never be able to express in words the deep gratitude towards all of those persons that were with me in this journey! THANK YOU ALL!

List of papers

Papers included in the thesis resulting from this Doctoral work:

I. **p53 as the Focus of Gene Therapy: Past, Present and Future**
Valente, J. F. A., Queiroz J. A., Sousa F.
Current drug targets (2018), *In press*. DOI: 10.2174/1389450119666180115165447

II. **Dilemma on pDNA purification: binding capacity vs selectivity**
Valente, J. F.A., Queiroz J. A., Sousa F.
Submitted for publication (2018)

III. **Selective purification of supercoiled p53-encoding pDNA with L-methionine-agarose matrix**
Valente, J. F. A., Sousa, A., Queiroz, J. A., Sousa, F.
Analytical Biochemistry 459 (2014): 61-69. DOI: 10.1016/j.ab.2014.05.011

IV. **DoE to improve supercoiled p53-pDNA purification by O-phospho-L-tyrosine affinity chromatography**
Valente, J. F. A., Sousa, A., Queiroz, J. A., Sousa, F.
Submitted for publication (2018)

V. **Macroporosity in affinity chromatography: supercoiled p53 encoding plasmid isolation**
Valente, J. F. A., Sousa, A., Queiroz, J. A., Sousa, F.
(Submitted for publication 2018)

VI. **The biological performance of purified supercoiled p53 plasmid DNA in different cancer cell lines**
Valente, J. F. A., Sousa, A., Gaspar V.M., Queiroz, J. A., Sousa, F.
Submitted for publication (2018)

VII. **Plasmid DNA nano-complexation with Chitosan and Polyethyleneimine**
Valente, J. F. A., Sousa, A., Pereira P., Queiroz, J. A., Sousa, F.
(In Preparation)

Papers not included in the thesis:

I. **Affinity analysis and application of dipeptides derived from l-tyrosine in plasmid purification**
Ferreira, S., Carvalho, J., Valente, J. F. A., Corvo, M. C., Cabrita, E. J., Sousa, F., Queiroz, J. A., Cruz, C.
Journal of Chromatography B 1006 (2015): 47-58. DOI: 10.1016/j.jchomb.2012.10.025

II. **Microencapsulated Chitosan-Dextran Sulfate Nanoparticles for Controlled Delivery of Bioactive Molecules and Cells in Bone Regeneration**
Valente, J. F. A., Gaspar, V. M., Antunes, B. P., Coutinho, P., Correia, I. J.
Polymer 54.1 (2013): 5-15. DOI: 10.1016/j.polymer.2012.10.032

III. **Poly (ester amide) s based on (L)-lactic acid oligomers and α-amino acids: influence of the α-amino acid side chain in the poly (ester amide) s properties**
Fonseca, A. C., Coelho, J. F., Valente, J. F. A., Correia, T. R., Correia, I. J., Gil, M. H., Simões, P. N.
Journal of Biomaterials Science, Polymer Edition 24.12 (2013): 1391-1409. DOI: 10.1080/09205063.2012.762293

IV. **Alginate based scaffolds for bone tissue engineering**
Valente, J. F. A., Valente, T. A. M., Alves, P., Ferreira, P., Silva, A., Correia, I. J.
Materials science and engineering: C 32.8 (2012): 2596-2603. DOI: 10.1016/j.msec.2012.08.001

List of Scientific Communications

Oral scientific communications of this Doctoral work:

I. **A library of amino acids to screen adsorbents suitable for sc p53 encoding plasmid isolation**
Valente J.F.A., Pinto J.M., Azevedo G.M., Pina A.S., Queiroz J.A., Roque A.C.A., Sousa F.
II International Congress in Health Sciences Research: Towards Innovation and entrepreneurship (2017) Covilhã, Portugal

II. **Purification and delivery of the p53 pDNA genome guardian**
Valente, J. F. A., Sousa, A., Gaspar V.M., Queiroz, J. A., Sousa, F.
XII ANNUAL CICS-UBI SYMPOSIUM (2017) Covilhã, Portugal

III. **p53 supercoiled encoding plasmid in different cancer cell lines – Transfection and Expression effects**
Valente, J. F. A., Sousa, A., Gaspar V.M., Queiroz, J. A., Sousa, F.
Bio.Iberoamerica (2016) Salamanca, Spain

IV. **Superporous affinity chromatography for the supercoiled p53 encoding plasmid isolation**
Valente, J. F. A., Sousa, A., Cruz C., Queiroz, J. A., Sousa, F.
ISPPP (2016) Salzburg, Austria

V. **Selective purification of supercoiled p53-encoding pDNA with L-methionine-agarose matrix**
Valente, J. F. A., Sousa, A., Queiroz, J. A., Sousa, F
IX ANNUAL CICS-UBI SYMPOSIUM (2014) Covilhã, Portugal

Poster presentations of this Doctoral work:

I. **Could it be possible to quickly determine the best ligands?**
Pinto J.M., Valente J.F.A., Pereira P., Pina A.S., Queiroz J.A., Roque A.C.A., Sousa F.
II International Congress in Health Sciences Research: Towards Innovation and entrepreneurship (2017) Covilhã, Portugal

II. **Recovery of recombinant microRNAs with modified magnetic nanoparticles**
Pinto J.M., Figueiró G.A., Valente J.F.A., Pina A.S., Pereira P., Queiroz J.A., Roque A.C.A., Sousa F.
XII ANNUAL CICS-UBI SYMPOSIUM (2017) Covilhã, Portugal

III. **Supercoiled p53-encoding plasmid purification by amino acids-based affinity chromatography – A comparison**
Valente, J. F. A., Sousa, A., Queiroz, J. A., Sousa, F.
9º ENCONTRO NACIONAL DE CROMATOGRAFIA / XVI COLACRO (2016) Covilhã, Portugal

IV. **Novel superporous arginine matrix for supercoiled p53 encoding plasmid isolation**
Valente, J. F. A., Sousa, A., Cruz C., Queiroz, J. A., Sousa, F.
Microbiotec (2015) Évora, Portugal

V. **Purification of the supercoiled p53-encoding plasmid by a L-methionine-agarose matrix**
Valente, J. F. A., Sousa, A., Queiroz, J. A., Sousa, F.
10th ESBES-IFIBiop (2014) Lille, France

VI. **F. Sousa, p53 Supercoiled plasmid purification by l-methionine agarose matrix**
Valente, J. F. A., Correia I.J., Sousa, F
19th International Symposium on Separation Sciences (2013) Poreč, Croatia

Resumo alargado

Introdução

O cancro é uma das principais causas de morte em todo o mundo fazendo com que exista bastante investigação nesta área na tentativa de melhor compreender a doença e de desenvolver estratégias terapêuticas mais eficazes e seguras. Para o tratamento desta doença, a terapia génica e a vacinação com DNA foram propostas como alternativas às formas mais comuns de tratamento. Entre as várias alterações genéticas que podem ser responsáveis pelo processo oncogénico, estão incluídas as alterações relacionadas com a p53, nomeadamente em relação à sua expressão ou atividade. A p53 foi no passado considerada um oncogene no entanto, mais recentemente, provou-se que esta proteína é na realidade um supressor de tumor, muito poderoso envolvido em processos como a apoptose e a senescência. Esta "guardiã do genoma" atua sempre que as células são expostas a condições de stress, e alterações na estabilidade desta proteína podem resultar em instabilidade genómica. A p53 tem suscitado interesse em muitos grupos de investigação uma vez que está descrito que em 50% dos casos de cancro são identificadas mutações no gene que codifica a p53, enquanto os restantes 50% possuem componentes defeituosos na pós-tradução ou alterações nas vias de sinalização da p53. Diversos estudos demonstraram que a transfeção de células cancerígenas com o plasmídeo que codifica para a p53 pode direcionar as células para a apoptose e/ou para uma paragem no seu crescimento, sugerindo que uma abordagem relacionada com a terapia génica no tratamento do cancro pode promover o restabelecimento da função normal de p53. Portanto, é muito importante restabelecer a expressão e atividade da p53 em células cancerígenas.

Neste sentido, a terapia génica tem já usado a possibilidade de induzir a expressão de p53, associando o gene que a codifica a vetores virais ou não-virais, nomeadamente com recurso ao DNA plasmídico (pDNA), como opção terapêutica promissora e alguns exemplos práticos já foram estudados e aplicados com sucesso. A utilização de vetores não-virais apresenta vantagens em relação ao uso de vetores virais, já que o processo de produção é mais simples, economicamente vantajoso, sendo os vetores menos imunogénicos e mais seguros. Recentemente, a isoforma superenrolada (sc) de um plasmídeo que codifica a p53 provou ser mais eficiente na transfeção celular e na expressão proteica do que a conformação circular aberta, e de facto esta isoforma tem-se destacado das outras isoformas por possuir uma estrutura extremamente compacta e funcional. Assim, o pDNA sc é considerado mais eficiente para induzir a expressão do produto alvo, quando comparado a outras variantes conformacionais, como o pDNA circular aberto (oc) e linear.

A biossíntese recombinante de pDNA permite a produção de um extrato enriquecido com pDNA sc. No entanto este extrato é também composto por proteínas hospedeiras, RNA e outras estruturas de DNA, o que torna o processo de isolamento da isoforma sc do pDNA um requisito

imperativo. Para atingir este objetivo, a cromatografia de afinidade usando aminoácidos como ligandos (ex.: arginina, histidina e lisina) tem vindo a ser utilizada com sucesso. No entanto, é essencial desenvolver e estudar novas matrizes com maior especificidade e robustez, permitindo maiores graus de pureza e rendimento do pDNA sc obtido. Desta forma, este trabalho prevê a produção e otimização de novas matrizes de afinidade que permitam promover interações mais específicas entre os ligandos e o pDNA sc de forma a estabelecer processos que nos permitam elevados rendimentos e também amostras com graus de pureza que se enquadrem dentro das especificações emitidas pelas entidades regulamentares tais como a *Food and Drug Administration* (FDA) e a *European Medicines Agency* (EMA).

Após a obtenção de amostras altamente puras do vetor que codifica para a p53 é importante garantir uma entrega eficiente deste plasmídeo. Para tal neste trabalho foram também testadas nanopartículas tendo como finalidade não só a proteção do material genético que se pretende entregar (a isoforma sc do pDNA que codifica para a p53) mas também garantir que a máxima quantidade de pDNA chegava numa primeira fase ao interior da célula e subsequentemente ao interior do seu núcleo.

Sendo assim, e como previamente referido, todos os trabalhos realizados ao longo deste projeto focam-se no estudo e desenvolvimento de metodologias que poderão contribuir para a potencial aplicação de uma estratégia de terapia génica para re-estabelecimento da função da p53 em células tumorias. O estudo envolve assim, as diferentes etapas de um processo biotecnológico, desde o processo de purificação do pDNA que codifica para a p53, até ao seu processo de entrega na célula e avaliação da expressão da proteína e do seu efeito na apoptose das células.

Descrição do trabalho realizado

Para a realização deste trabalho, e no sentido de alcançar a biossíntese de pDNA, foi utilizado como hospedeiro recombinante a bactéria *Escherichia coli* modificada com o plasmídeo que codifica para a p53 com o objetivo de se obterem extratos ricos neste pDNA. Após lise destas células foram obtidos extratos mais ou menos complexos, de forma a isolar a isoforma sc enrolada do pDNA que codifica para a p53. Estes extratos foram posteriormente purificados usando vários suportes cromatográficos de afinidade que utilizavam diferentes aminoácidos como ligandos, tais como a L-metionina, L-tirosina e arginina, para a recuperação da isoforma sc. Posteriormente, e para compreender biologicamente a atividade e o efeito terapêutico desta isoforma sc, diferentes linhas celulares (HeLa, A549 e Fibroblastos dérmicos humanos) foram transfetadas com pDNA purificado com a metodologia desenvolvida e com kits disponíveis comercialmente, para posterior avaliação *in vitro*. Nesta vertente, foram realizados testes nos diferentes modelos celulares, para avaliar a citotoxicidade, a expressão do gene p53 e o efeito

apoptótico resultante. Os resultados proporcionaram informações relevantes sobre a potencial aplicação desta isoforma sc do pDNA que codifica para a p53 em terapia genética aplicada ao tratamento de cancro.

Principais resultados alcançados

Tendo em conta uma perspetiva geral dos principais resultados obtidos durante este trabalho pode dizer-se que três matrizes cromatográficas foram utilizadas com sucesso para o isolamento da isoforma sc do pDNA que codifica para a p53. De entre as matrizes estudadas a L-metionina e a O-Phospho-L-tirosina, ambas comerciais, demonstraram seletividade mesmo quando diferentes ácidos nucleicos faziam parte da amostra aplicada. As amostras de sc recolhidas através da purificação com ambas as matrizes apresentaram uma pureza concordante com todas as especificações das agências reguladoras para este tipo de amostra. Contudo, foi a amostra proveniente da purificação com O-Phospho-L-tirosina aquela que apresentou melhor grau de pureza assim como percentagem de recuperação. Possivelmente estes bons resultados podem estar relacionados com o uso de ferramentas de desenho experimental (DoE) aplicado apenas para a otimização da estratégia de purificação com esta matriz. Neste trabalho, e com o modelo escolhido, pretendeu-se otimizar e maximizar as respostas "grau de pureza" e "percentagem de recuperação", tendo sido possível estabelecer as melhores condições experimentais para conseguir um melhor desempenho do suporte cromatográfico de O-Phospho-L-tirosina quando comparada com a L-metionina.

Numa abordagem diferente, foi também utilizado um suporte macroporoso onde foi imobilizada arginina tendo, mais uma vez, como objetivo o isolamento da isoforma sc do pDNA que codifica para a p53. Neste trabalho foi aplicada uma amostra menos complexa, contendo apenas pDNA sc+oc, sendo que mais uma vez a isoforma sc foi isolada com sucesso. A utilização de uma matriz macroporosa teve como principal objetivo explorar um possível aumento da capacidade de ligação do suporte, de forma a aumentar a quantidade de amostra purificada por ensaio, aumentando a sustentabilidade/rentabilidade do processo cromatográfico. Neste sentido, comparou-se a capacidade dinâmica de ligação desta matriz com um suporte de agarose e um monólito que apresentavam também a arginina como ligando. Através desta comparação verificou-se que a utilização do suporte macroporoso promove um aumento superior a 50 % na capacidade de ligação do pDNA quando comparada com a sua homóloga comercial, mas em suporte de agarose não macroporoso. Estes resultados podem ser de extrema relevância nomeadamente quando se pensa na industrialização do processo cromatográfico.

Quando uma amostra de pDNA superenrolado que codifica para a p53 foi biologicamente avaliada e comparada com uma amostra de pDNA que contém as duas isoformas predominantes (sc+oc) verificou-se que a amostra purificada demonstrou maior resposta biológica,

nomeadamente ao nível da eficiência de transfeção, expressão da proteína P53 e indução de apoptose celular. Foi também interessante observar que esta isoforma não atua de igual forma em células tumorais e não tumorais e também, que existem variações na sua atuação quando diferentes células tumorais são estudadas. Neste caso, as células HeLa foram as que demostraram maior sensibilidade à isoforma sc do plasmídeo que codifica para a p53, já que foi nestas células que se observaram maiores níveis de transfeção, expressão de proteína e apoptose.

Finalmente, foram produzidos dois vetores catiónicos, formados através da complexação entre polietilenoimina (PEI) e quitosano (CH) com uma amostra nativa (sc+oc) de diferentes plasmídeos. Através dos resultados obtidos foi possível verificar que as nanopartículas de pDNA/PEI demonstraram algum efeito citotóxico em células não tumorais, o que poderá ser uma enorme desvantagem para a sua aplicação *in vivo*. Relativamente à entrada das nanopartículas no núcleo, foi demonstrado que poliplexos produzidos através da complexação com o pDNA mais pequeno apresentaram maior facilidade de entrada no núcleo das células estudadas. Por fim, foi avaliada a capacidade dos poliplexos estudados promoverem expressão da proteína P53 em células cancerígenas Hela. A partir dos resultados obtidos, observou-se que os níveis de P53 aumentaram 54,2% quando as células foram transfetadas com as nanopartículas de CH e 32% quando as células foram transfetadas com nanopartículas de PEI.

No geral, o trabalho científico realizado no âmbito desta tese pretende levar a comunidade científica a dar cada vez mais relevância ao uso de vetores não virais e em particular da aplicação da isoforma superenrolada do pDNA em terapia génica, de forma a promover o restabelecimento dos níveis da P53, como forma de ajudar na terapia de cancro. Para isso torna-se crucial desenvolver e compreender cada vez melhor os processos biotecnológicos que podem conduzir à obtenção deste biofármaco, garantindo a sua estabilidade e atividade.

Palavras-chave: Cromatografia de afinidade, Expressão da proteína P53,

Isoforma Superenrolada, Plasmídeo que codifica para a p53, Supressor de tumor, Terapia génica, Vetores não virais

Abstract

Cancer is still one of the leading causes of death worldwide. In order to treat this scourge, gene therapy and DNA vaccination have been proposed as an alternative to the common treatments. Among the several gene abnormalities that could be responsible for the oncogenic process, the ones presented in p53 stands up. p53 is one of the most important tumour suppressor gene being considered the "genome guardian" since when occurs exposure to stressful stimuli it is activated through post-transcriptional modifications increasing its stability and activity. This gene is directly and indirectly implicated in different cellular functions, including DNA damage repair, cell cycle arrest in G1/S and apoptosis. Several studies shown that transfection of cancer cells with wild-type p53-expressing plasmids could directly drive cells into apoptosis and/or growth arrest, suggesting that a gene therapy approach for cancer treatment can be related to the re-establishment of the normal p53 function. Recently, the supercoiled (sc) conformation of a p53-encoding plasmid proved to be more efficient in cell transfection and protein expression than open circular conformation. Aiming to successfully isolate this bioactive isoform, several chromatographic techniques have been used, namely amino acids-based affinity chromatography.

Concerning this chromatographic approach, in this doctoral work different amino acids like, L-methionine, L-tyrosine, and arginine, were used to isolate the sc p53 encoding plasmid. From this work, it was achieved a better recovery yield and purity levels for O-Phospho-L-tyrosine when compared with L-methionine agarose matrix. Regarding the macroporous arginine resin, it was possible to recover the sc p53 encoding pDNA with high purity, and an increase of more than 50% in the dynamic binding capacity was achieved, when comparing with their homologous commercial agarose matrix.

To understand the activity and the therapeutic effect induced by this sc isoform, different cell lines (HeLa, A549 and human dermal fibroblasts) were transfected with the pDNA purified either by the affinity purification strategy or with a commercial kit, for further *in vitro* evaluation. In particular, the cytotoxicity, the expression of the p53 transgene and the resulting apoptotic effect were evaluated in these *in vitro* cancer models. The results brought relevant information concerning the potential application of a sc p53 encoding plasmid in cancer gene therapy.

To eliminate different cancer types, treatments must be applied systematically and therefore, must be targeted to cancer cells. Concerning this and, to enable an easy and safe systemic therapy, stable and non-viral gene vectors have been developed to encapsulate and deliver foreign genetic materials in specific cell types, such as cancerous cells. The use of non-viral vectors usually promotes low immune response, they are easily prepared, have a low production cost and also, they can be easily produced at large scale. Other important characteristic about these vectors is the ability to transfer different and large transgenes being also able to be

stored for long periods due to their stability. Regarding this, in this doctoral work, chitosan (CH) and polyethyleneimine (PEI) were complexed with different plasmids in order to search for the suitable non-viral nanocomplex combination to be applied for gene therapy. Through the obtained results it was found that p53-encoding pDNA/PEI polyplexes demonstrate some toxicity in normal cells which could be a handicap for future therapeutic application of this nanocarriers. Also, from the track of the nanocarriers inside the cells, it was achieved a better transfection efficiency for the carriers delivering the smaller pDNA. The ability of the polyplexes to promote P53 protein expression was also evaluated using HeLa cancer cells and an increase of 54.2% and 32% of the P53 levels was achieved when CH and PEI nanocarriers were respectively applied.

Overall, the scientific work performed in this thesis hopes to lead the scientific community to give more and more relevance to the use of the supercoiled pDNA isoform for gene therapy involving the reestablishment and restoration of the levels of p53. Moreover, it has been shown that the use of chromatography using specific amino acid ligands is a crucial factor in the final quality of the recovered sc pDNA sample, being this a predominant parameter for the accomplishment of the desired therapeutic results. Finally, the effort in the search and development of suitable non-viral vectors should stand up since it can guarantee the stability and activity of the sc p53 encoding plasmid during the delivery process.

Keywords: Affinity chromatography, Gene therapy, Non-viral-vector, p53 encoding plasmid, P53 protein expression, tumour suppressor, Supercoiled DNA

Thesis Overview

The present thesis has his structure dived in 4 main chapters. The **Chapter I** was created with the purpose of explaining the main goals to be achieved with this doctoral work. Then, the **Chapter II** presents a bibliographical review of the main subjects of the work performed, being divided in two different review articles:

Paper I - p53 as the Focus of Gene Therapy: Past, Present and Future

This review article presents the biochemistry of the p53 protein and also summarizes the different methods used to deliver and/or target the p53 in therapeutic approaches, indicating the main results obtained with the different strategies applied. The article also describes the ongoing approaches focusing on the combinatorial therapeutics, conjugating gene therapy vectors with chemo or radiotherapy.

Paper II- Dilemma on pDNA purification: binding capacity vs selectivity

With this literature review it was created a description of the different approaches used over the time to purify plasmid DNA, focusing not only on the different kind of supports used but also in the different ligands applied for the isolation of this biomolecule. Overall, it was presented a critical discussion relying on the relevance of the binding capacity versus selectivity of the supports applied until now.

The **Chapter III** presents the results obtained during the PhD course and have the purpose of showing the practical achievements of the goals purposed in **Chapter I**. This chapter was divided in some original research papers organized as follows:

Paper III - Selective purification of supercoiled p53-encoding pDNA with L-methionine-agarose matrix

In this research work it was described for the first time the use of a new matrix of L-methionine-agarose to efficiently purify the supercoiled p53-encoding plasmid. The binding/elution conditions, such as salt concentration and temperature, were manipulated and combined to reach the best strategy. After accomplishing the purification process, several tests to assess

the quality of the supercoiled plasmid were performed, revealing that the final sc p53 encoding sample fulfil all the requirements of the regulatory agencies.

Paper IV - DoE to improve supercoiled p53-pDNA purification by O-phospho-L-tyrosine affinity chromatography

The O-Phospho-L-tyrosine chromatographic matrix was explored in this work to purify the supercoiled p53-encoding plasmid. A Composite Central Face (CCF) design was applied in this purification process in order to quickly determine the optimal chromatographic performance reaching the required purity degree and maximizing the recovery yield of the supercoiled plasmid DNA. Thus, the sc p53 encoding plasmid isolation was successfully achieved fulfilling the requirements of the regulatory agencies. This approach enables a faster establishment of the best purification conditions, which allows a reduction of the costs associated to the random experiment approach in the search for the suitable isolation conditions.

Paper V - Macroporosity in affinity chromatography: supercoiled p53 encoding plasmid isolation

In this paper, arginine was immobilized into macroporous beads and then the support was morphological and chemically characterized (through scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX) and Fourier transformed infrared spectroscopy (FTIR)). For the specific purification of the sc pDNA, different parameters were evaluated, namely the buffer composition, salt concentration, pH and temperature, in order to achieve the best binding/elution conditions. The dynamic binding capacity (DBC) of the macroporous arginine matrix was also evaluated and the results revealed an improvement in comparison with the arginine-agarose based matrix.

Paper VI - The biological performance of purified supercoiled p53 plasmid DNA in different cancer cell lines

In this study, an affinity chromatography platform based on the L-methionine agarose matrix was used to purify the non-viral supercoiled topoisoform of plasmid DNA biopharmaceutics encoding p53. This purified biopharmaceutical was complexed with liposomes to comprehensively analyse the *in vitro* performance and therapeutic potential in different cancer and normal cell models, including those of lung, cervix and fibroblasts. The obtained results showed a higher apoptosis of HeLa cells, indicating that p53-based transgene therapy may be

particularly effective in this cancer. Overall, the findings emphasize the potential of sc pDNA non-viral gene therapy and provide important insights into the therapeutic potential of this approach.

Paper VII - Plasmid DNA nano-complexation with Chitosan and Polyethyleneimine

This study explored and compared nanocarriers based on the complexation of plasmid DNA with chitosan or polyethyleneimine polymers in order to search for a suitable vector in terms of encapsulation efficiency, zeta potential and size. Then, the cytotoxic effect of the best ratios was accessed and, for p53 encoding plasmid nanoparticles, the ability to promote protein expression was evaluated.

Finally, the **Chapter IV** summarizes the concluding remarks obtained during this research work, regarding the application and characterization of some chromatographic supports for p53 encoding pDNA purification by amino acids affinity chromatography. Also, conclusions from the biological performance in cancer/non-cancer cells after transfection with the sc p53 encoding plasmid purified with commercially/non-commercially available methods are included. Then, some future trends were also mentioned as future works to be developed in this research field.

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Chapter I

Global Aims

This doctoral project is focused on the use of recent platforms based on amino acids-affinity chromatography to efficiently purify the sc p53 encoding pDNA. After the purification of the plasmids it must be assured their protection and delivery to cancer cells in order to assess the biological activity of this sc isoform and evaluate the real relevance of using the sc p53 encoding gene. To accomplish all these goals, several chromatographic supports were used to find a suitable matrix for the purification of the pDNA and also, non-viral polymeric nano vectors were developed and compared with a commercial system to encapsulate the target pDNA and evaluate the efficacy of these methodologies for sc p53 encoding pDNA delivery.

To achieve the aim purposed, this doctoral research work had been developed in accordance to the following tasks:

1. Screening, production and development of new amino acids affinity-based matrices for the sc p53 encoding pDNA recovery

To accomplish this task, several raw materials were used as chromatographic supports (e.g. agarose and macroporous resin) as well as different amino acids were used as ligands (L-metionine, L-tyrosine and L-arginine), to be evaluated for pDNA purification.

2. Biological assessment of pure (sc) vs a native (sc+oc) p53 encoding plasmid sample

Different cell lines were transfected using a commercial system carrying the sc or sc+oc p53 encoding gene and parameters such as transfection rate, protein expression and apoptotic behaviour were evaluated.

3. Production, optimization and biologically assessment of polymeric p53 pDNA nanocarriers

Nanocarriers made with a natural (chitosan) or a synthetic polymer (polyethyleneimine) were optimized and screened in order to find the suitable nanosystems for the studied pDNA delivery. The screening involved the characterization of several parameters such as zeta potential, dimension, polydisperse index, encapsulation efficiency, transfection rate and protein expression ability, to select the best conditions and delivery systems.

Chapter II

Paper I

P53 as the Focus of Gene Therapy: Past, Present and Future

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Current Drug Targets, **2018**, *19*, **000-000**

REVIEW ARTICLE

p53 as the Focus of Gene Therapy: Past, Present and Future

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ARTICLE HISTORY

Received: September 18, 2017
Revised: November 16, 2017
Accepted: January 10, 2018

DOI:
10.2174/1389450119666180115165447

Abstract: ***Background*:** Several gene deviations can be responsible for triggering oncogenic processes. However, mutations in tumour suppressor genes are usually more associated to malignant diseases, with p53 being one of the most affected and studied element. p53 is implicated in a number of known cellular functions, including DNA damage repair, cell cycle arrest in G1/S and G2/M and apoptosis, being an interesting target for cancer treatment.

***Objective*:** Considering these facts, the development of gene therapy approaches focused on p53 expression and regulation seems to be a promising strategy for cancer therapy.

***Results*:** Several studies have shown that transfection of cancer cells with wild-type p53 expressing plasmids could directly drive cells into apoptosis and/or growth arrest, suggesting that a gene therapy approach for cancer treatment can be based on the re-establishment of the normal p53 expression levels and function. Up until now, several clinical research studies using viral and non-viral vectors delivering p53 genes, isolated or combined with other therapeutic agents, have been accomplished and there are already in the market, therapies based on the use of this gene.

***Conclusion*:** This review summarizes the different methods used to deliver and/or target the p53 as well as the main results of therapeutic effect obtained with the different strategies applied. Finally, the ongoing approaches are described, also focusing on the combinatorial therapeutics to show increased therapeutic potential of combining gene therapy vectors with chemo or radiotherapy.

Keywords: p53, apoptosis, non-viral vectors, viral vectors, gene therapy.

1. INTRODUCTION

Cancer results from the accumulation of mutations that can occur during decades and have the ability to inactivate tumour suppressor genes and activate oncogenes. Tumour suppressor genes are responsible for the inhibition of cell growth and/or induction of cellular apoptosis to prevent cancer formation. Amongst all the tumour suppressor genes, one of the most important is the p53, which acts as a sensor of DNA damage and other cellular and metabolic stresses including hypoxia, oncogenetic activation and nutrient deprivation. In response to stress, p53 can induce cell cycle arrest and subsequent DNA repair, senescence or apoptosis, depending on the damage and cellular context (Fig. **1**) [1].

Also, if the p53 protein is dysfunctional, it could result in genomic instability which is a hallmark of cancer [2]. One of the most important reasons for deep attention that is paid to p53 by the research groups dedicated to cancer investigation is the fact that 50% of the patients with cancer contain various inactivating mutations in their p53-encoding gene, while the other 50% possess defective components in post-translational modification of p53 protein or alterations in p53 signaling pathway [3]. Therefore, it is very important to re-establish the p53 expression and activity in cancerous cells.

To perform this, gene therapy has been considered as a promising therapeutic option and some practical examples were already studied and successfully applied.

Gene therapy comprises the replacement or addition of a correct copy of the abnormal gene, with the purpose of restoring the genetic information, thus reverting the associated disease. This genetic-based therapeutic approach intends to increase the quality of life and also the life expectancy of the treated individual [4, 5].

In what concerns the delivery of the therapeutic gene, it could be done directly on the patient, however, if nucleic acids are delivered in a free form, they can suffer from rapid extra and intracellular enzymatic degradation, which drastically reduces the gene available and the expression of the target protein. Moreover, due to the high molecular weight and anionic nature, gene-based products are usually difficult to achieve cellular uptake. Finally, even the small amount of gene reaching the cells can suffer degradation in the endosomal/lysosomal compartments [6]. Regarding that and, to overcome the barriers associated with the administration of

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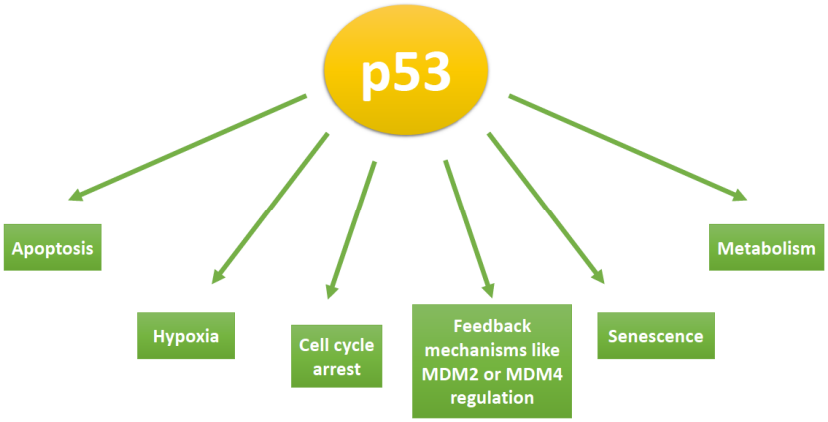

Fig. (1). Cellular processes regulated by p53.

genetic material, the main strategy under study is the association of the genetic material with suitable delivery systems. In general, these delivery systems should be characterized in terms of safety (biocompatibility and biodegradability), resistance to degradation, capability to access the appropriate cellular compartment, specificity towards the target sites and ability to selectively modulate the expression of the target gene or to express the protein for a desired period of time [6, 7].

Up until now, several clinical research studies using viral vectors for the delivery of p53 have been conducted and there are already available in the market some p53-viral vectors-based products, such as Advexin and Gendicin. However, the application of viral vectors can induce high immunogenicity and, can promote high rate of pre-existing immunity which limits their clinical use, increasing the need to create systems with the same efficiency but without these deleterious effects [8, 9]. Regarding that, non-viral vectors can represent a good alternative to the viral systems. Actually, non-viral vectors present many advantages when compared with the viral ones, mainly due to the absence of viral components, the lack of immunogenicity, the lower production costs and easier manufacturing processes.

In general, viral and non-viral strategies proved to be promising in the reestablishment of the p53 levels in cancer cells, being the viral vectors the ones that are used in more clinical studies. However, researchers around the world have started to focus on non-viral vectors and in the specific delivery to target cells.

2. P53 PROTEIN - AN INTRODUCTION TO P53 BIOLOGICAL ACTIVITY

p53 was regarded as an oncogene in the past, however, more recently, it has been proved repeatedly that p53 is, in fact, a very powerful tumour suppressor involved in apoptotic and senescent processes [10, 11]. The p53 protein is considered the "genome guardian" since when the cells are exposed to stressful stimuli, p53 is activated through post-transcriptional modifications increasing its stability and activity [12].

P53 is a protein that contains 393 amino acids and, is structurally and functionally composed of four different domains: an acidic amino-terminal domain used in the transcriptional activation, a DNA-binding domain in the central space, a tetramerization domain and finally, a C-terminal regulatory domain rich in basic amino acids which play an important role in the regulation of the core DNA-binding domain (Fig. **2** outlines the structure of p53). Regarding these domains, the N-terminus mainly interacts with transcriptional factors, like TFIID (TBP, TAFs) which is extremely important in the regulation of gene expression and working in the transcriptional machinery. Another protein that binds in this terminal is MDM2 (murine double minute 2) which is responsible for the negative regulation of the transcriptional activity of p53 [13]. The tetrameric part is, in fact, a dimer of a dimer bond to four repeats of a DNA sequence [14]. The C-terminal domain is responsible for the p53 binding to specific DNA sequences in the central domain. Particular changes in the C-terminal of this domain, like deletion or phosphorylation, have been described as being responsible for the activation of the sequence-specific DNA-binding by the central core domain [15]. The DNA-binding region is the central core of p53 and is the place where most of the p53 missense mutations were found [16]. It is made up of immunoglobin like β-sandwich of two antiparallel β-sheets, providing a scaffold for a flexible DNA-binding surface. This surface is made of two large loops stabilized by a zinc atom and a loop-sheet-helix motif. The zinc binding is extremely important for the correct folding and requires the reduction of thiol groups on cysteines [17].

p53 also plays a critical role in DNA repair and, in the control of cell cycle progression, being particularly responsible for the regulation of the cyclin-dependent kinase (CDK) p21 gene expression. The p21 binds to different CDK/cyclin complexes promoting their inhibition, and consequently, blocking the cell cycle progression [18, 19]. In addition, p53 also directly influences the transcription of CDKs, responsible for the phosphorylation of tumour suppressors of the Rb family [20]. Thus, low levels of these CDKs will not allow the activation of pRb, and consequently, induce cell cycle arrest. In G2/M checkpoint the p53 has also an important role since it interferes with the expression of several intertwined targets, including Cdc25C, 14-3-3δ and GADD45. The Cdc25C is a mitosis-promoting phosphatase that dephosphorylates and activates cyclin B1/cdc2 complex. In case of DNA damage, Cdc25C is inhibited, resulting in the G2/M arrest [21].

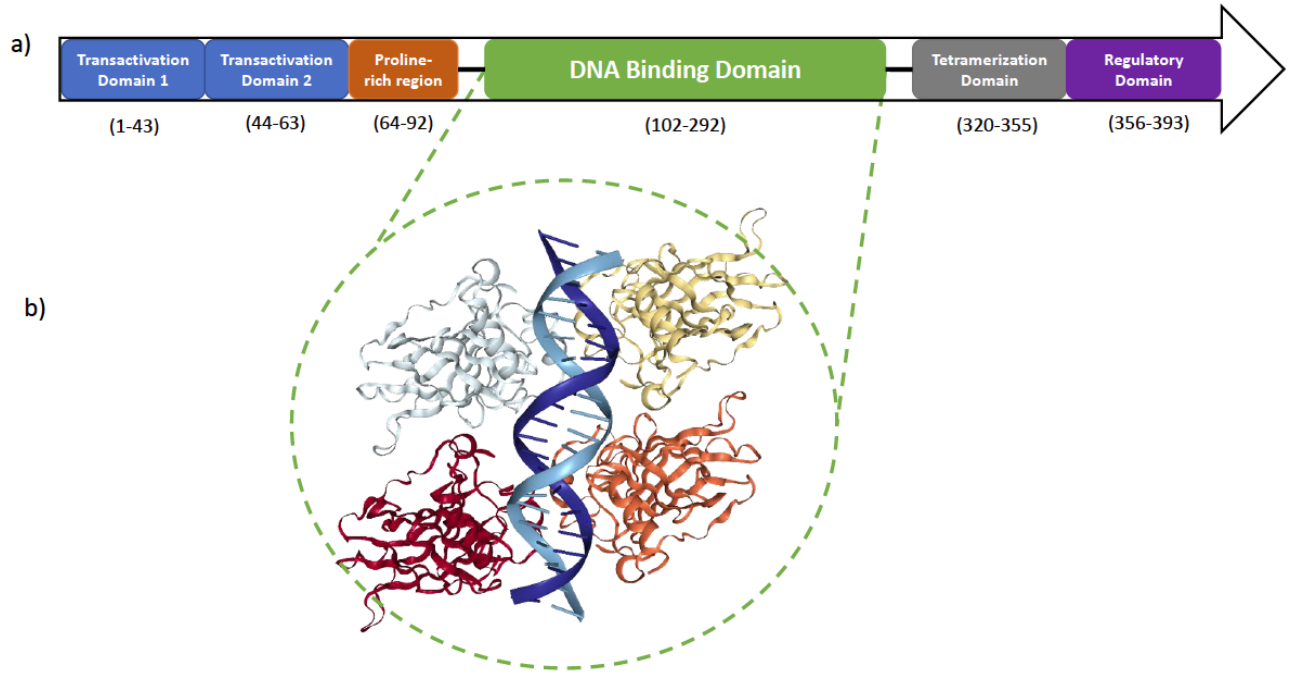

Fig. (2). p53 based structure is made of 393 amino acids organized in different domains: transactivation domain 1 and 2, a proline-rich region, DNA binding domain, tetramerization domain and also the regulatory domain. The interactions between this protein and DNA is performed through the DNA binding domain.

In the absence of stress or damage, the p53 is present in very low concentrations and is highly unstable. This is mainly due to the interaction with other proteins like the MDM2 that is one of the most important regulators of the p53 activity and stability. Usually MDM2 levels are very high in human cancers. MDM2 is an E3 ubiquitin ligase that binds to the N-terminus of p53 through a hydrophobic pocket domain, transferring mono-ubiquitin tags onto lysine residues [22]. Hence, when cells are in a non-stress environment, p53 function is kept in a basal state which is achieved due to the constant action of this MDM2-p53 loop that is responsible for the elimination of the p53 in excess [22]. The degradation of p53 is dependent on MDM2 levels, and a reduced binding of MDM2 to p53 reduces the E3 activity, leading to an inhibition of ubiquitylation function. Regarding this regulatory effect of MDM2 in p53 levels and activity, it could be important to target this interaction as a way to develop a therapeutic strategy [2].

MDMX, also known as MDM4, is a protein from MDM2 family that also binds to the N-terminal of p53 and was found to be related to the inhibition of p53 since it contributes to p53 degradation [23]. Similarly, to MDM2, MDMX is an important negative regulator of p53, and this negative regulation is one mechanism by which MDMX acts as an oncogene to transform cells when overexpressed. MDM4 has also another important role, which is the MDM2 stabilization, and is important to be referred that it is overexpressed in several types of cancers that retained wild-type p53 including gliomas, a number of pre-B acute lymphoblastic leukemias, tumor cell lines, and some primary tumors including breast tumors, head and neck squamous cell carcinomas, and retinoblastomas [24].

3. P53 MUTATIONS

Up until now, more than 2000 different mutants of the p53 have been found, acting in different ways. The mutations found are sporadic, germline, gain-of-function, oncogenic, rebel-angel, Yin and Yang, prion-like, metastasis-inducer, mediator of chemoresistance and modifier of stemness [25].

The somatic mutations in p53 have been reported in almost all the types of human cancers with different prevalence depending on the cancer type. Concerning that, in cancers from the aero-digestive tract (like oral, esophageal or bronchial cancers) the p53 is mutated in 75% of these cases, namely in smokers who are exposed to mutagens. In the case of the cancers in the low digestive tract (such as colon cancer) the p53 mutations are less prevalent being detected in 25% of all the cases. Cancers like cervix, testicular cancers, neuroblastoma and malignant melanomas, present a very low prevalence of p53 mutations (less than 5%). Nevertheless, in these cases, the p53 pathway can be functionally inactivated by viral or cellular oncogenes. An example is cervical cancer where the viral antigen E6 of the oncogenic types of Human Papilloma Virus binds to the wild-type p53 protein and induces its rapid degradation, thus effectively bypassing the need of an inactivating mutation to remove p53 function [26].

Usually, the majority of the tumour suppressor genes like RB, APC, or BRCA1 are inactivated by deletions or truncating mutations during the cancer progression. With regard to p53 mutations in human cancers, they are based on missense mutations in which a single nucleotide is substituted by another [27]. These mutations in p53 are very different and can

be located in different places of its sequence, inducing alterations on its thermodynamic stability. The majority of these mutations are responsible for the loss of p53 ability to bind DNA in a sequence-specific manner and activate transcription of canonical p53 target genes. The higher predominance of mutations in the p53 gene occurs between exons 4-9 that are responsible for encoding the DNA-binding domain of the protein. Among these mutations, 30% fall within 6 "hotspots" residues like R175, G245, R248, R249, R273, and R282 that are very frequent in almost every kind of cancers [28]. The existence of these hotspots could be explained by the susceptibility of particular codons to carcinogen-induced alterations and by positive selection of mutations that render the cell with growth and survival advantages [29]. Furthermore, the loss of function promoted by the mutations in p53 could be responsible for promoting tumour development. In case of a heterozygous situation (where both wildtype and mutant alleles exist) mutant p53 can antagonise wild-type p53 tumour suppressor functions in a dominant negative manner. Also, the inactivation of the wild-type p53 by the mutant p53 in a dominant negative mechanism stems from the fact that the transcriptional activity of wild-type p53 relies on the formation of tetramers, whose DNA binding function may be interfered by mutant p53 [29]. It is also important to consider that during cancer progression p53 mutations are frequently followed by loss of heterozygosity. For example, this kind of loss in the short arm of the chromosome 17, where p53 is located, implies a selective force driving the inactivation of the remaining wild-type allele, suggesting that the dominant negative activity of mutant p53 is not sufficient to completely inactivate wild-type p53 [30]. Moreover, different mutants of p53 isoforms can promote oncogenic activity by a gain-of-function mechanism where there is an acquisition of oncogenic properties by the mutant protein, compared with the mere inactivation of the protein. Regarding all above, dominant negative effect and gain-of-function can promote a selective selection of missense mutations in p53 during tumorigenesis [31].

4. P53-BASED THERAPIES

Nowadays, the most commonly used therapies for cancer treatment are based in chemo or radiotherapy. However, these treatments are usually ineffective to eradicate the tumour and can be extremely influenced by the presence of the wild-type p53, being extremely important to guarantee the regular behaviour of this tumour suppressor gene [32]. The resistance of cancer cells to drugs is a huge drawback in the cancer treatment, being mainly associated to the problems involving drug uptake or export, the prodrug activation or drug inactivation, changes in molecular targets, as well as alterations in DNA repair or modifications in the pro- and anti-apoptotic balance [33].

The p53 direct or indirect influence in cell resistance is dependent on several parameters including the way of action of the drug, the genetic alteration during carcinogenesis and also the cancer cell type. Furthermore, the mutations in p53 promote changes in the proapoptotic balance which by itself promote cancer cell resistance. In order to target tumours that overexpress the mutant p53, specific drugs such as PRIMA-1 (Proline-rich membrane anchor) and PRIMA-1Met have been applied [34]. The mechanism of action of these drugs is not completely understood however is thought that they upregulate p53 target genes such as BAX (Bcl-2-associated X protein), PUMA (p53 upregulated modulator of apoptosis) and NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) rescuing the activity of numerous p53 mutant species [35].

In fact, the influence of p53 in chemotherapy is so important that there are drugs, such as paclitaxel or vincristine, that are responsible for stabilizing wild-type p53 through the inhibition of the transcription associated with the mitotic arrest, preventing the p53 degradation and also affecting the microtubule-mediated transport of p53 [36]. In addition, the drugs used in chemotherapy have similar signaling cascades to the ones involved in p53 activation by DNA damage, and wild-type p53 is required for cell death in tumours after drug exposure [32].

The behaviour of p53 can also be balanced by the regulation of other biomolecules that directly influence its performance. An easy and effective example of this is the good results achieved in cell division control and in the adjustment of p53 levels when drugs that regulate p53-MDM2 protein interaction are applied. Three of the most important molecules responsible for blocking the interaction between these two proteins are small molecules such as nutlins, benzodiazepinediones and spiro-oxindoles [37-39]. Basically, the way of action of these drugs consists in mimicking and inhibit the p53 binding pockets with MDM2 inducing the accumulation of p53 restoring it transcriptional activity followed by apoptosis in MDM2 overexpressing tumour cells [35].

Studies with the application of ionizing radiation showed that the p53 behaviour affects the radiosensitivity of the cells so, concerning that, several studies have been performed using synergistically radiotherapy with gene therapy. For example, Lowe and colleagues (1994) showed that wild-type p53 mouse embryo fibroblasts transformed with the oncogene Hras responded to DNA-damage inducing apoptosis when a 7 gray absorbed dose of ionizing radiation was applied [40]. Koom and collaborators (2012) also studied the effect of the delivery of p53 using an adenoviral vector combined with radiotherapy in hepatocellular carcinoma cells. They showed, that p53 gene transfer using an adenoviral vector would enhance the cellular response to radiotherapy by inhibiting the p53-MDM2 interaction [41]. In addition, non-viral vectors like liposomes containing p53-encoding gene had been used in combination with radiation enabling complete tumour regression and inhibition of their long-term recurrence [42, 43].

The under development therapies to fight the proliferation of cancer cells and progression of tumours, involving the p53 or the p53 pathway as a leading element, still present a lot of problems that limit the generalized clinical application. Regarding that, there are a lot of questions concerning the effectiveness of the therapies involving the p53 that remained to be answered. Although, across the years, the scientists have improved information in this field and have specified and improved therapies trying to make them more personalized, they have studied each cancer particularly in order to find best ways to fight it.

4.1. p53 Reestablishment

Regarding the extreme importance of the expression, integrity and regulation of p53 in the organism for tumour control and to avoid therapy resistance, different therapeutics have been investigated, expecting the targeting of this biomolecule. Gene therapy to restore the p53 levels, inhibition of p53-MDM2 interaction, restoration of mutant p53 to wild-type p53, targeting the p53 family proteins, elimination of mutant p53 and also the development of p53 vaccines are some examples of these alternative strategies [44].

There are several problems concerning the nucleic acids delivery into the cell. Regarding that, one of the main concerns is related to physiological barriers that can change the cellular biodistribution and the intracellular bioavailability. When naked DNA is delivered, it only has the ability to resist degradation during 5 minutes due to its hydrophilic nature and high molecular weight. DNA itself presents a low cellular uptake. Moreover, the DNA that can actually enter into the cell is internalized in vesicles (like endosomes) that can digest DNA, limiting the access to cytoplasmic or nuclear targets. This also represents an important barrier in the gene-based drug efficiency [45].

Gene expression is highly dependent on the access of DNA to the nuclear compartment, which implies successful trafficking to the nucleus and penetration of the nuclear membrane. The nuclear membrane is not highly permeable however, it contains nuclear pore complexes with approximately 25 nm, which are responsible for the export and import of specific molecules to the nucleus. Due to these characteristics, molecules smaller than 40 kDa or particles with a threshold of 25 nm can be diffused through the nuclear pores, whereas larger molecules cannot access to the nucleus.

To overcome these barriers, several delivery systems for p53 gene-based therapeutics have been already successfully developed using different protocols and vectors, including adenovirus, retrovirus, vaccine-derived vectors and different types of nanoparticles modified or non-modified with specific ligands [6].

4.2. Strategies, Advantages and Constraints in the Production of a Clinical Grade Purity p53 Encoding DNA Plasmid

To accomplish the preparation of the genetic material complying with the quality standards for therapeutic application, some biotechnological processes have been developed, based on the design and construction of p53-encoding vectors that can be produced in large scale using recombinant hosts (Fig. **4**). In order to produce the p53 encoding plasmid, a recombinant *Escherichia coli* (*E. coli*) host can be used and grown through suitable fermentation conditions. The biosynthesis of pDNA in these cells enables the production of extracts rich in supercoiled (sc) pDNA, which is advantageous since this pDNA conformation has proved to be more efficient for cells transfection and gene expression than other isoforms, such as the open circular pDNA [46]. However, when alkaline lysis is performed to recover sc pDNA from the recombinant host, high concentrations of other impurities are also released, that must be removed in order to have a final plasmid product with high purity and activity, fulfilling the quality parameters established by the regulatory agencies, such as Food and Drug Administration (FDA), U.S.A.. Concerning that, several specifications are documented, namely regarding the product appearance (clear, colourless solution), the plasmid homogeneity (≈ 97% sc), and impurities levels (RNA and proteins should be undetectable, the amount of genomic DNA must be lower than 2 ng/μg of pDNA and the level of endotoxins should not be higher than 0.1 EU/μg of pDNA) [47, 48]. To guarantee the high level of purity for pDNA, different chromatographic methodologies have been investigated namely, size exclusion, anion exchange, hydrophobic interaction, reversed phase, thiophilic adsorption and, affinity chromatography [49].

Regarding the methods previously mentioned, the affinity approach has been one of the most exploited in the last years, as it has been demonstrated that these methods can specifically purify the most biologically active plasmid conformation. Actually, different studies already proved that a purified sc isoform of p53-encoding pDNA is the most efficient and effective isoform at inducing transgene expression [50]. As an example, in a previous study, Gaspar and colleagues obtained highly pure biopharmaceutical formulations of a p53-encoding vector, by using a strategy where the pcDNA3–FLAG–p53 plasmid was first amplified in a bacterial cell culture of *E. coli* DH5α. Then the authors used L-arginine as a specific ligand for affinity chromatography, and successfully isolated the sc isoform of different plasmids namely the pcDNA3-FLAG-p53 [51]. Completed the plasmid production and purification, the next concern is related with the delivery of the vector to the cells or tissues.

4.3. Viral *vs* Non-viral Vectors

4.3.1. Viral Vectors

Since p53 is lost or mutated in a wide number of cancers, it seems reasonable to try the re-establishment of the p53 expression and function through the replacement of the mutant form by a functional wild-type copy of the gene. One of the strategies used to deliver the correct genetic information is based on the use of suitable viral vectors, like retrovirus or adenovirus. However, the most used viral vectors at the moment are the adenoviral vectors (Adp53) and oncolytic adenoviruses (CRAdp) [26, 52]. The procedures already established attempt to regulate p53 levels in cancer cells. Actually, the activation of p53 can also play an important role in chemosensitizer or in chemoprotective mechanisms, depending on the cellular context. Regarding this, several viral vectors have been produced in order to promote a gene replacement therapy for p53.

In October 2003, the State Food and Drug Administration (SFDA) of China approved type 5 Ad bearing the human wild-type p53 gene (Ad-p53) for the treatment of head and neck cancer [53]. Ad-p53 (Gendicine®, Shenzhen SiBiono GeneTech, Shenzhen, China - now incorporated into Benda Pharmaceutical, Wuhan, China) has been initially developed by Introgen Therapeutics (Advexin®, Austin, TX, USA) for head, neck, and lung cancer treatment. While Introgen was working to obtain US FDA approval, SiBiono successfully completed clinical studies and launched the product. In November 2005, SFDA also approved type 5 Ad defective of

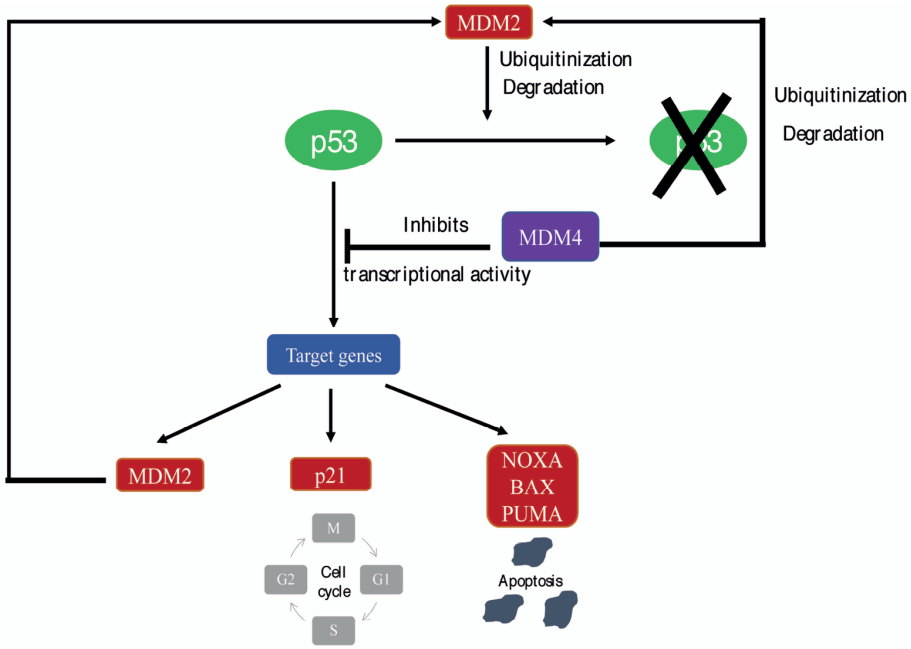

Fig. (3). Autoregulatory feedback loop between p53 and MDM2/MDM4.

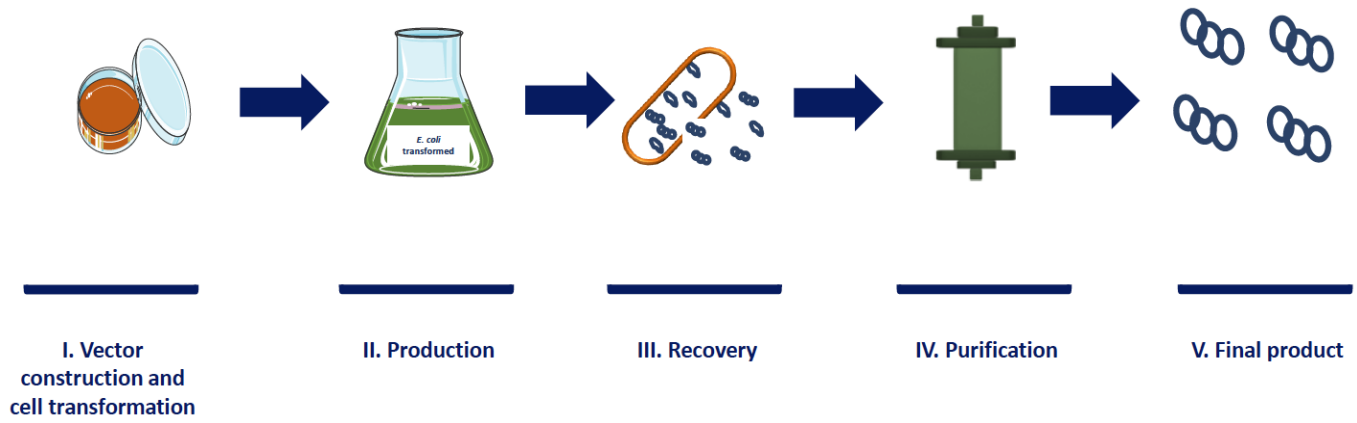

Fig. (4). Schematic representation of the process steps involved in sc pDNA production and purification using a chromatographic affinity strategy. (I) *E. coli* transformed with a p53-encoding plasmid; (II) Bacterial cell growth; (III) Alkalyne lysis; (IV) Chromatographic matrix; (V) sc pDNA.

the E1B-55 kDa molecule for head and neck cancer treatment [54]. While ONYX-015 had been investigated for the clinical efficacy, a Chinese Bioventure company, Shanghai Sunway Biotech (Shanghai, China), independently developed E1B-55 kDa-deleted Ad, completed their clinical studies and commercialized the medicine (Oncorine®) in 2006. Sunway Biotech has also acquired the exclusive license of ONYX-015 in the world.

The delivery vectors used in these products are based on adenovirus. Adenovirus is a double-stranded DNA virus characterized by the high transduction efficiency in several cell types, and oppositely to retrovirus, it can deliver the genetic material either to proliferating or not proliferating cells. Adenoviruses present low toxicity profile in humans and, since they do not integrate the genome, there is no risk of insertional mutagenesis, that can be seen as an advantage in comparison to other virus-based vectors [55]. The different mechanisms of action of the adenovirus with or without E1B deletion (this protein is responsible for bind and neutralizes p53 biological activity), in cells with wild-type or mutant p53 are represented in (Fig. **5**).

Among the strategies used until now, the Ad-p53 acts as a replacement therapy, aiming to promote the delivery of the p53 gene directly into the cancer cells to suppress the tumour growth. The main goal is promoting the expression of exogenous p53 in order to induce cell death and cell-cycle arrest in different tumour cells. Several preclinical and clinical studies proved its efficiency in the regression of different tumours such as head and neck, lung, colorectal, ovarian, bladder, prostate and esophageal (Table **1**) [56-62].

4.3.1.1. Retrovirus

While some viruses go through the cell machinery to maintain their life cycle, using exclusively the cytoplasm of the host cell, others have adapted to use the unique features offered by the nucleus. These include viruses from the

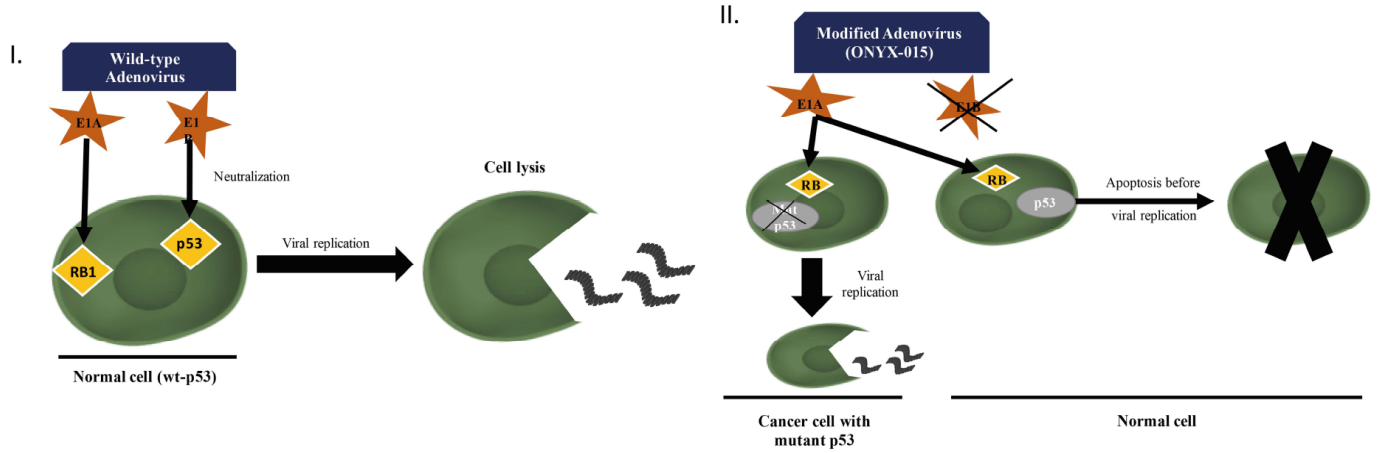

Fig. (5). Specific killing of cells using wild-type adenovirus (**a**) in normal cells which leads to cell lysis or, using E1B deleted adenovirus (ONYX-015) (**b**) in cells with mutant and wild-type p53 [26].

Table 1. Clinical trials approved worldwide using viral vectors to promote the delivery of p53.

Virus Type/ Product Name	Complementary Therapy	Type of Cancer	Phase of the Clinical Trial	Status	Year of the Last/Final Update	Clinical-trial.Gov/ref
Ad5CMV	-	Hepatocellular Carcinoma	I	Terminated	2003	NCT00003147
	-	Squamous cell cancer of the head and neck	III	Unknown	2008	NCT00041613
	-	Recurrent gliomas	I	Completed	2009	NCT00004041
	Radiation	Lung	I	Completed	2010	NCT00004225
	-	Oral Premalignancies	I	Terminated Sponsor withdrawal prior to study completion.	2011	NCT00410865
	Cisplatin and radiation	Mouth or Throat	II	This study has been terminated. Terminated for poor accrual	2012	NCT00017173
	-	Advanced Bladder	I	Completed	2013	NCT00003167
	Autologous Ex-panded T Lympho-cytes (CD3+, CD4+, and CD8+)	Lung	II	Terminated	2013	NCT00776295
	Standard chemo-therapy	Breast Cancer	I	Completed	2013	NCT00004038
	-	Oral Cavity or Pharynx	I/II	Completed	2013	NCT00064103
	-	Ovarian	I	Completed	2013	NCT00003588
	Docetaxel Doxoru-bicin hydrochloride	Breast	II	Completed	2013	NCT00044993
	-	Head and Neck	II	Unknown	2013	NCT00003257
	Paclitaxel and All-trans-Retinoic Acid	Small Cell Lung Cancer	II	Ongoing, but not recruiting	2016	NCT00617409
	Reload	Colorectal	I	Recruiting	2016	NCT02842125
	1-methyl-d-tryptophan	Metastatic Breast	II	Ongoing, but not recruiting	2016	NCT01042535

(Table 1) contd….

Virus Type/ Product Name	Complementary Therapy	Type of Cancer	Phase of the Clinical Trial	Status	Year of the Last/Final Update	Clinical-trial.Gov/ref
p53MVA	Gemcitabine	Recurrent Ovarian Epithelial Cancer Recurrent Fallopian Tube Carcinoma Recurrent Primary Peritoneal Carcinoma	I	Active, not recruiting	2017	NCT02275039
	Pembrolizumab	Solid Tumors That Have Failed Prior Therapy	I	Recruiting	2017	NCT02432963
Ad-p53	Paclitaxel and All-trans-Retinoic Acid	Small Cell Lung Cancer	II	Ongoing, but not recruiting	2016	NCT00617409
	Reload	Colorectal	I	Recruiting	2016	NCT02842125
	1-methyl-d-tryptophan	Metastatic Breast	II	Ongoing, but not recruiting	2016	NCT01042535
rAd-p53	-	Brain	I	Completed	2009	NCT00004080
	Standard chemotherapy (not specify)	Advanced Oral and Maxillofacial Malignant Tumors	IV	Unknown	2012	NCT00902083
	Radioactive iodine pre-surgery	Malignant Thyroid	IV	Unknown	2012	NCT00902122
	Standard chemotherapy	Advanced Oral and Maxillofacial Malignant	IV	Unknown	2012	NCT00902083
	Standard chemo and radiotherapy	Head and Neck Malignant Tumors	IV	Unknown	2012	NCT00894153
	-	Non-small-cell Carcinoma	II	Unknown	2012	NCT01574729
	Cisplatin	Advanced Head and Neck Cancer	II	Not yet recruiting	2015	NCT02429037
	Cisplatin	Malignant Pleural Effusion	II	Not yet recruiting	2015	NCT02429726
	Transcatheter embolization	Hepatocellular Carcinoma	II	Not yet recruiting	2015	NCT02561546
	Cisplatin Radiation	Advanced Head and Neck Cancer	II	Not yet recruiting	2015	NCT02429037
	TACE	Advanced Hepatocellular	II	Recruiting	2015	NCT02418988
	-	Ovarian, Fallopian Tube, or Peritoneal	I	Completed	2016	NCT00002960

family *Retroviridae*, which differ from other animal viruses by requiring integration into host chromosomes as an obligate step of their life cycles [63]. Among the most used retrovirus, there are the immunodeficiency virus (HIV) and human T-cell leukaemia virus (HTLV), responsible for acquired immunodeficiency syndrome (AIDS) and adult T-cell leukaemia (ATL), respectively. Murine leukaemia viruses (MLVs) are also well-studied since they are used with animal models for comparison studies of several human diseases (leukaemia, immunodeficiency, and neuropathogenic diseases) and also as gene transfer systems [64].

In 1993, it was for the first time proved that a retrovirus-mediated transduction of wild-type p53 was able to induce programmed cell death in multicellular tumour spheroids of human non-small cell lung cancer cell lines. These results suggested that retroviral vectors can penetrate into multiple

cell layers of three-dimensional tumour masses and induce the therapeutic effects [65].

However, the use of retrovirus remains a threat due to the potential for oncogene activation as a result of retroviral insertion into the host genome. Hence, alternatives that minimize this problem have been explored including the adenoviral vectors gene delivery [66].

4.3.1.2. Ad5CMV-p53

Restoration of wild-type p53 function in tumour cells can be achieved by the introduction of an intact complementary DNA copy of the p53 gene using a suitable viral vector, in most cases an Adp53. The use of this gene replacement therapy to transfer the p53 gene directly into cells has been demonstrated to suppress tumour growth because ectopic expression of exogenous p53 gene efficiently induces cell death and cell-cycle arrest in a variety of p53-inactivated tumour cells, with evidence for bystander effects in some cases. These viruses are engineered to lack certain early proteins and are thus replication defective. With this method, several patients have received Adp53-based gene therapies in clinical trials, mostly in the USA and in China [54].

As previously mentioned, Gendicine is the world's first Adp53-based gene therapy product approved by a government agency for clinical use. It consists in a recombinant human serotype 5 adenovirus in which the E1 region is replaced by a human wild-type p53 expression cassette. The mechanism of action is based on the activation of apoptotic pathways that stimulate immune response mediators, such as natural killer cells, inhibiting DNA repair and anti-apoptotic functions, and also blocking the transcription of survival signals [67].

In 2003, Gendicine was used in Phase I clinical trial for the treatment of 12 patients with advanced laryngeal cancer, with an average clinical course of 41 months. The results obtained demonstrated that there was no patient relapse for more than 5 years after Gendicine treatment which contrasts with the 30% 3-year relapse rate for patients with advanced laryngeal cancer receiving surgery alone [52]. For a Phase II clinical trial, patients with non-small-cell lung cancer (NSCLC) were injected with adenovirus p53 combined with docetaxel and the effects of this therapy were compared with those of standard single-agent docetaxel. The main results of this study were that direct intratracheal injection of Adp53 through the cricothyroid membrane into endobronchial NSCLC is safe, with acceptable levels of toxicity. After a mean follow-up of 12 months, the median survival time was 7.7 months for patients in the docetaxel plus Adp53 group and 5.9 months for the group that received only the docetaxel [68]. Another case report described the case of a patient with recurrent hepatocellular carcinoma (HCC) that after been submitted to a combined therapy of Gendicin with transcatheter arterial chemoembolization presented normal liver function and was in good clinical health with no recurrence [55].

Regarding another famous Adp53 vector, Advexin, it has not yet been approved by the Food and Drug Administration and the company developing it has closed, although some remarkable clinical cases have been reported [52]. In general, clinical trials have proved the ability of p53 to be successfully applied in the cancer treatment. For example, Shimada and collaborators drove a phase I/II study where Advexin was administrated to several patients with advanced esophageal squamous cell carcinoma and chemo radiation-resistance. From this study, only one patient demonstrated the progression of the disease condition, while the others revealed a stabilization on the disease [69]. This drug was also administrated in another Phase II trial, to thirteen patients with squamous cell carcinoma of oral cavity being the estimate 1-year progression-free survival of 92%. Additional Phase III clinical trial is warranted to enhance our understanding of the efficacy of Advexin in increasing the survival rates of patients with various malignancies [70].

Different research studies have also proved that the risk of insertion of the p53-encoding gene into the host cell genome is extremely low, and it is not increased even when cells are exposed to conventional treatments used in cancer therapy.

4.3.1.3. CRAp53

One of the major problems of using Adp53 is the low transduction rate, which must be solved to improve the clinical effects in patients with several types of cancer. Regarding that, CRAp53 vectors were developed to enhance E1-dependent virus replication in tumours.

CRAd, also known as oncolytic adenovirus, is a new class of anticancer agents with great therapeutic potential. They replicate selectively in cancer cells but rarely in normal cells, leading to cytolysis of tumour cells without adverse side effects. This tumour specificity can be achieved by placing the expression of adenoviral genes, such as the E1A and E1B genes, under the control of tumour- or tissue-specific promoters, or by completely or partially deleting the adenoviral genes that are necessary for viral replication in normal cells, but not in tumour cells. Furthermore, by carrying tumour suppressor genes, oncolytic adenoviruses could combine the advantages of gene therapy and virotherapy to enhance antitumor efficacy [71].

Therefore, several types of tumor-specific p53-expressing adenovirus vectors have been developed, such as ONYX-015, AdDelta24-p53, SCH-5800, SG600-p53, H101, among others. The application of these vectors varies between different kinds of cancers like cervical carcinoma, epithelial cancer, lung cancer, among others [9, 55].

The E1B-defective Ad was originally developed in the USA and was representatively called ONYX-015 (Onyx Pharmaceuticals, Emeryville, CA, USA). While ONYX-015 had been investigated for the clinical efficacy, a Chinese bioventure company, Shanghai Sunway Biotech (Shanghai, China), independently developed E1B-55 kDa-deleted Ad, completed their clinical studies and commercialized the medicine (Oncorine®) in 2006. Sunway Biotech has also acquired the exclusive license of ONYX-015 in the world. This virus contains a deletion between nucleotides 1496 and 3323 in the E1B region encoding the 55 kDa protein, enabling its selective replication and lysis of the tumour cells with deficient and/or dysfunctional p53 tumour suppressor gene activity [72]. These adenoviruses are responsible for

binding and blocking p53, preventing p53-induced cell death, and allowing viral replication and production of viral progeny. Moreover, replication-competent CRAdp53 vectors have been developed in which the promoters of cancer-related genes are used to enhance E1-dependent virus replication in a tumor-dependent manner [73]. Experiments with this drug demonstrated an efficiency to induce the cancer cells killing comparable to the one achieved with adenovirus for cervical carcinoma, colon carcinoma, glioblastoma and pancreatic adenocarcinoma. The treatment with this virus significantly reduced the tumour size of human deficient xenografts in nude mice and, there are also studies confirming that it could also sensitize p53-deficient cancer cells to radio and chemotherapy. Indeed, some clinical trials showed increased benefits when the radiotherapy was applied in conjugation with this virus [74, 75]. The application of this virus leaves some droughts regarding the mechanism of action and selectivity for p53 deficient cells since several studies have been demonstrated that wild-type adenovirus (wt-Ad5) and ONYX-015 replicate in various cells regardless of their p53 status [76]. However, the clinical trials have shown that the drug treatment induces objective responses in tumours with mutant or wt-p53 without substantial damage to normal tissue. In fact, Ries and co-workers (2000) studied this effect and observed that cell lysis mediated by ONYX-015 can occur in cells with wt-p53 if some mutations affect other genes in the p53 pathway, rendering the cell p53-deficient [31].

The full understanding of cell selectivity to the virus application is still to be accomplished, however, several studies have been performed in order to try it. In 2002, Vasey and collaborators, in a phase I trial, analyzed the replication of ONYX-015 in a primary human epithelial ovarian cancer, being the toxicities observed related to the virus administration, namely by inducing the flu-like symptoms, emesis and abdominal pain. However, the procedure failed probably due to the oncolytic effect of this virus on ovarian cancers that led to the expression of coxsackie/adenovirus receptors (CAR) and integrin co-receptors on the surface of ovarian tumour cells which could be the reason for the basis of resistance to adenovirus therapies [77].

SCH 58500 is a replication-defective recombinant adenoviral vector containing the human wild-type tumour suppressor gene p53. SCH 58500 is currently undergoing Phase I/II clinical trials for the treatment of tumour types that commonly express mutant p53 phenotypes. In a Phase II trial for NSCLC patients, three different cycles of regimen A, carboplatin plus paclitaxel, or regimen B, cisplatin plus vinorelbine in combination with intratumoral injection of SCH-58500 were applied. Through the results obtained, it was possible to observe that there was no significant difference between the response rate of lesions treated with p53 gene therapy combined with chemotherapy (52%) and lesions treated with chemotherapy alone (48%). There was no survival difference between the two groups, and the median survival of the cohort was 10.5 months. Conclusively, the authors suggested that intratumoral SCH-58500 therapy appeared to provide no additional benefit in patients receiving chemotherapy for advanced NSCLC [78]. This vector was applied alone or sequentially in combination with platinum-based chemotherapy in patients with recurrent ovarian, primary peritoneal, or fallopian tube cancer containing aberrant or mutant p53. As the main result, they observed extensive adenoviral-induced inflammatory changes concluding however that intraperitoneal SCH 58500 is safe and well tolerated and also when combined with platinum-based chemotherapy can be associated with a significant reduction of serum CA125 (a cancer antigen) in heavily pre-treated patients with recurrent ovarian, primary peritoneal, or fallopian tube cancer [79]. These studies established the safety and feasibility of the applicability of rAd-p53 SCH 58500 in the cancer treatment. In these trials, the drug is delivered by several routes, depending on the tumour type. Thus, the administration routes include intratumoral administration to breast, melanoma, head, neck, and non-small-cell lung tumours; intraperitoneal administration to tumours associated with the ovary; and hepatic artery (intra-arterial) administration to liver tumours. The administration can be performed as a single dose or a small number of daily doses over a short time span, or could also be cyclic in combination with chemotherapy [79-81]. The main conclusion taken by the studies performed with SCH-58500 was that no potential benefit on survival and tumour response rates of patients with various cancers is observed by the use of this vector.

H101 is a recombinant human type 5 adenovirus with a total deletion of E1B 55K gene and an additional deletion of 78.3-85.8 μm gene segment in the E3 region which may enhance the safety of the drug and also make it similar to ONYX-015. Xia and collaborators performed in 2004 a phase III clinical trial where chemotherapy and H101 were administrated in advanced head and neck cancers demonstrating a significant increase in the tumour response rate. Briefly, the intratumoral H101 injection was combined with cisplatin plus 5-fluorouracil (PF) regimen or adriamycin plus 5-fluorouracil (AF) regimen *versus* PF or AF regimen alone in treating patients with head and neck or esophagus squamous cell cancer. The combination of H101 with PF was the one that presented the higher response. Regarding the side effects, these were fever, injection site reaction and influenza-like symptoms [82].

4.3.1.4. p53MVA

The attenuated poxvirus modified vaccinia Ankara (MVA) is a viral-based vaccine and, in this case, p53MVA has the ability to express the wild-type p53 antigen. The main advantage for the use of this vaccine is its excellent safety profile and possibility to induce potent immune responses against recombinant antigens. Diamond and collaborators (2016) performed (based on this kind of therapy) the first clinical trial using p53MVA in combination with gemcitabine into over-expressed p53 ovarian tumor cells. The main results of this study were that the combinatorial therapy between p53MVA and gemcitabine was well tolerated, with the expected toxicities related to the use of gemcitabine, and no evidence of autoimmunity was verified [83].

Concerning these clinical trials, it is important to note that the majority does not present the final results. In fact, few clinical trials advance to the final stage, mainly due to safety concerns, cytotoxic effects or other issues that arise during the trial and restrain the continuity.

4.3.2. Non-viral Vectors

Although until now viral vectors have been the most used to deliver the p53 gene in the clinical trials, it is important to refer that some disadvantages are associated to these vectors, namely their complex preparation and their immunogenicity, which can be responsible for serious adverse effects and failure of repeated applications in the human body. Regarding that, this delivery system is far from being used as a safe gene delivery vector. To overcome these limitations and to efficiently and safely deliver p53, non-viral vectors, produced at a nanoscale with different biomaterials, are being evaluated. Usually, the size of these nanocomplexes should range between 10 and 100 nm, presenting a huge surface area to improve the ability for adsorption, concentration and protection of the nucleic acids [84].

The non-viral vectors present many advantages compared to the viral vectors as they (1) do not contain viral components, reducing immunogenicity; (2) are less expensive; (3) are easily produced and (4) have an easier manipulation. Thus, the non-viral vectors can overcome some problems like the safety, the tissue tropism and the immunogenic response. In fact, several biopolymers could be used without inducing immune response in contrast to what happens with the viral vectors. Despite all the benefits found for the non-viral vectors, few clinical trials have been conducted using these vectors to deliver p53. This could be due to the low transfection efficiency found and consequently the low expression rate of the protein.

The nanocomplex produced between the p53 and the biomaterial could efficiently insert the genetic information into a variety of cells. This occurs mainly due to the interaction between the positive charge of the nanocomplex with the negative charge of the cell membrane, enabling the nanocomplex to enter into the cell [85]. The main mechanism involved in the nanocomplexes entry inside the cells is endocytosis and, this process can be favoured by the coupling of specific molecules on the surface of the nanomaterials. These molecules can be antibodies, proteins, peptides, among others, with the ability to bind and to be recognized by specific receptors on the cell surface. Through this process the target gene can easily access the cancerous cells which makes the administration and transfection safer and more effective at inducing tumour cell death (Fig. **6**) [84].

There are several studies using polymeric nanoparticles (NPs) combined with p53 proving that the protection provided is crucial to achieve better results. For example, Sharma and collaborators (2011) have used poly (lactic-co-glycolic acid) (PLGA) NPs combined with the p53 gene to study the antiproliferative effect of this therapeutic product *in vivo*. Regarding that, mice with p53-null prostate cancer cells were treated with p53 NPs being the controls injected with saline solution, nude p53-DNA, and empty NPs. The results have shown that mice treated with local injections of p53 NPs demonstrated significant tumour growth inhibition, improving animal survival compared with controls. Tumour growth inhibition corresponded to sustained and greater p53 expression in tumours treated with p53 NPs than with nude p53-DNA [86].

Other approaches focused on the NPs conjugation with different drugs to enhance the therapeutic effects accomplished by gene or chemo/radiotherapy alone. Regarding that, several studies using different polymeric particles with the p53 vector have been combined with different drugs. For example, Wang and collaborators (2015) combined the chitosan-based NPs with doxorubicin and the p53 vector and, through the results was possible to observe a strong pDNA condensation, providing protection for pDNA against deoxy-

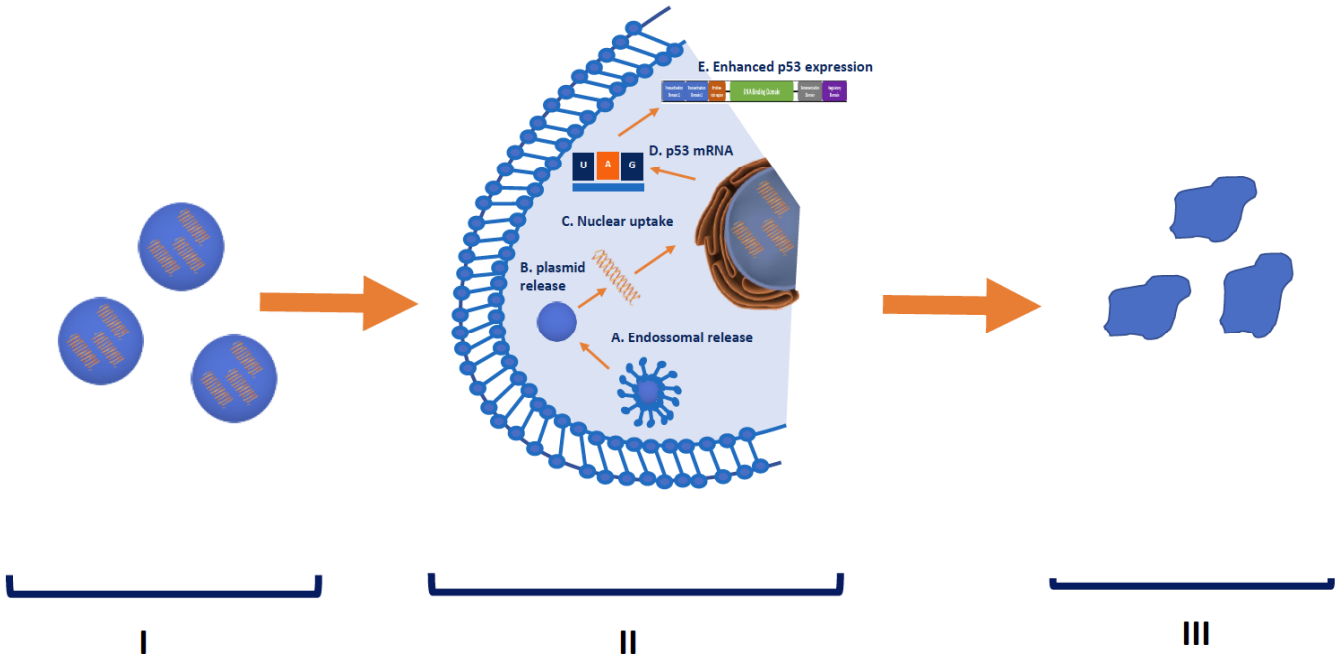

Fig. (6). Cellular uptake of a nanocomplex with pDNA: (I) pDNA encapsulated into a nanoparticle; (II) cellular uptake and release of the nanoparticle containing a p53 encoding plasmid; (III) tumour cells death.

ribonuclease I degradation. Good gene transfection efficiency *in vitro*, using HeLa cells, was accomplished. Finally, they proved *in vitro* that the complex of NP+Doxorubicin+p53 had a better inhibitory effect on HeLa tumour cells growth than doxorubicin or p53 alone [87].

To overcome problems such as the lack of suitable delivery agents presenting insignificant immunogenic response, serum compatibility, and early and easy detection of the transfected cell population, in 2014, Misra and collaborators successfully produced a nanoparticle combining the p53 gene with the green fluorescent protein (GFP)-encoding gene. With the p53-EGFP-C3, they were able to easily assess p53 mediated anti-tumor activity concluding that this nanocomplex was more efficient at inducing cell apoptosis when compared with a commercial reagent (Lipofectamine) [88].

Gaspar and collaborators (2011) already proved that the use of a chitosan-based system to transport and deliver the supercoiled p53 encoding plasmid enabled the reinstatement of the p53 protein expression. In this study, the authors also observed that the sc pDNA transfected cells exhibited the highest p53 expression levels when compared with other formulations [51].

The delivery of p53 has not been made only by polymers, and regarding that, other materials like silica have been successfully applied as NPs for the restoration of the p53 levels in different cancer cells. Li and collaborators (2017) have recently used a combination of bortezomib encapsulated in hollow mesoporous silica nanospheres as a biocompatible and effective drug-delivery system for NSCLC therapy. After this initial study, they decided to combine this delivery system with p53 and found that the restoration of the p53 expression by these NPs induced activation of early apoptosis which contributed to enhanced cell death later. Finally, they also found that several p53 downstream genes responded strongly and synergistically to bortezomib function and p53 restored expression. More specifically, the presence of p21 and Bax, the activation of caspase 3 and a down-regulation of Bcl-2 was observed [89].

In (Table **2**) it is possible to observe the different nanoparticulated systems used until now to deliver the p53 with or without association with other molecules.

Magnetic and gold NPs have also been used as effective vehicles applied in the p53 delivery. Actually, the magnetic NPs are interesting as they can be controlled in their concentration and distribution to a desired part of the body by using externally driven magnets and also because they are easy to track (Table **3**) [90].

Another example is that of PEI and iron oxide that have been combined with p53 and significantly higher p53 tumour suppressor gene expression and cellular viability were achieved, when compared to positive controls. Transgene expression of the p53 tumour suppressor gene was evaluated at the mRNA level and the results showed that this NPs could be used as efficient gene delivery carriers and also could be tracked by magnetic resonance imaging [91].

Xu and collaborators (2017) also used a nano-theranostic agent a gold nanoshell filled with PLGA that was conjugated with an anti p53 antibody which has high specificity for the p53 protein overexpressing in breast cancer. The results showed that this combination enables a suitable ultrasound contrast imaging acquisition [92].

4.4. Ligand Targeting

The NPs should have the ability to selectively choose the target, in this case, the tumour cells, in order to promote a more efficient delivery of the drug or gene. The strategies applied for this purpose should rely on the targeting agents or ligands ability to bind the tumour cell surface in an appropriate manner to trigger receptor endocytosis. Usually, these systems are dependent on the interaction established at the cell surface being the molecule responsible for the linkage activated only in the presence of the tumour cells [93].

Considering that p53 is regulated in normal cells, the delivery of this gene using functionalized nanocapsules only in cancer cells is crucial. To accomplish a good functionalization of the NPs, enabling the specific targeting of cancer cells, the most commonly used ligands are particular peptides and antibodies. The process used in the functionalization should be non-denaturing to maintain the structure and activity of the cargo [94]. There are several ligands used to enhance the recognition of the cancer cells by the NPs containing the p53 vector. Folic acid is widely used since cancer cells overexpress folate receptor at their surface while in normal tissues it has minimal expression [95, 96]. Chen and collaborators (2016), used ternary positively charged FK/p53/PEG-PLL(DA) complexes. The cellular uptake and electrostatic attraction were probably improved by the targeting property given by the folic acid moiety in the FK peptide. After internalization, the FK/p53 complexes were degraded in the cytoplasm under the stimulation of glutathione (GSH) and have further released the p53 plasmid to induce cell apoptosis by regulating both intrinsic and extrinsic apoptotic pathways. Both *in vitro* and *in vivo* studies demonstrated that the FK/p53/PEG-PLL(DA) complexes could enhance antitumor efficacy and reduce the side effects effectively [95].

Another receptor used is a transferrin antibody fragment since this receptor is also overexpressed in different cancer cells (*e.g.* pancreatic) when compared to normal tissue, suggesting that it may be a specific marker for malignancy. Another factor supporting the use of transferrin receptor as a target is the fact that it is recycled during internalization, in rapidly dividing cancer cells, thus improving the uptake of transferrin-vectors [97]. Rejeeth and co-workers observed this ability of NPs targeting tumour cells, by performing experiments with silica-NPs modified or unmodified with tranferrin. They found that transferrin-modified SiNPs inhibited cell growth 1.4 times more than the unmodified SiNPs. The expression of exogenous wild-type p53 was higher following transfection with transferrin–SiNPs–p53, in comparison with transfection with unmodified SiNPs–p53. Therefore, the increase in the efficacy of p53 gene transfer and the subsequent increase in p53 expression seemed to have an effect on MCF-7 growth inhibition [98].

Different targeting ligands such as EGFR, octa-arginine and others, have also been applied to drive different NPs containing p53, as presented in (Table **4**).

Table 2. Nanosystems used do deliver p53.

Based Material	Therapeutic	Cancer/Cell Type	Year	Ref.
DOTMA and dioleoyl phosphatidyletha-nolamine	-	Breast carcinoma	1995	[91]
DPEP and DOPE	-	Lung cancer	1998	[92]
1,2 dioleoyl-sn-glycero-3-ethylphosphocholine and DOPE	Antiangiogenic peptide of throm-bospondin I	Breast carcinoma	1998	[93]
PLGA	-	Breast cancer	2004	[94]
SLN	-	Lung	2008	[95]
Chitosan	-	HeLa	2011	[12]
PLGA	-	PC-3	2011	[87]
Sillica	-	Breast cancer	2012	[96]
Cholesterol gemini (Chol-5L) with DOPE	GFP	HeLa/ HEK293T	2013	[89]
Hydroxyapatite	GFP	HuH-7	2014	[97]
PEI and PEG with histidine and glutamic acid	-	NSCLC	2015	[98]
N-acetyl-l-leucine-modified PEI	-	HeLa/ PC-3	2015	[99]
N-isopropylacrylamide-modified PEI	-	HeLa PC-3	2015	[100]
POSS, PDMAEMA and PMPDSAH	Doxorubicin	MCF-7	2015	[101]
PEI	-	HeLa	2015	[102]
Poly-(N-ε-carbobenzyloxy-L-lysine) (PZLL) and chitosan (CS)	Doxorubicin	HeLa	2015	[88]
PEI-modified calcium carbonate	Green fluorescent	Hep3B, QSG-7701, H1299, 293a and HeLa	2016	[103]
Silica	Bortezomib	NSCLC	2017	[90]

Table 3. p53 magnetic nanoparticles.

Magnetic Material	Other Material	Cancer/Cell Type	Year	Ref.
Iron oxide	PEI	CT-26	2012	[105]
Gold	PLGA	Breast cancer	2016	[106]
Magnetite	Pullulan–spermine	U87	2016	[104]

Using active targeting strategies for NPs can enhance the intracellular concentration of drugs in cancer cells, while avoiding toxicity in normal cells, that represent a potentially powerful technology. Furthermore, when NPs bind to specific receptors to enter the cells, they are usually enveloped by endosomes *via* receptor-mediated endocytosis, thereby bypassing the recognition of P-glycoprotein, one of the main drug resistance mechanisms. However, the use of target receptors could also lead to some limitations such as the poor oral bioavailability, instability in circulation, inadequate tissue distribution, toxicity and decreased effectiveness of some drugs after being linked to targeting moieties [93].

4.5. Clinical Trials Using Nanoparticles

An efficient delivery of nucleic acids into target tissues is critical for the success of gene therapy and, as previously mentioned NPs have a huge potential as gene delivery systems however its use is recent and still need improvement. Regarding these, there is still few clinical trials using nanosystems delivering p53.

As is possible to observe in (Table **5**), there is only one nano delivery system applied for this purpose, which is the SGT-53 that was been developed by SynerGene Therapeutics, Inc. SGT-53. SGT-53 is a complex of cationic liposome that has on the surface an anti-transferrin receptor (TfR) single-chain antibody fragment for tumor-targeting and encapsulates a normal human wild-type p53 DNA sequence. The SGT-53 was designed to target tumour cells *via* the TfR, which is highly expressed on the surface of many tumour cells as previously mentioned. Regarding that, this nano complex is efficiently internalized *via* receptor-mediated endocytosis. It is also important to refer that this nano delivery system enhances the efficacy of chemo and radiotherapy in various pre-clinical models of human solid tumours [99]. The sensitization of tumours to chemotherapy by this tumor-targeted and efficient p53 gene delivery method could lower the effective dose of the drug, reducing the severe side effects, while decreasing the possibility of recurrence. Moreover, this approach is applicable to both primary and recurrent tumours, and more significantly, metastatic disease [100].

Pirollo and collaborators (2016) were responsible to conduct one phase I clinical trial in patients with advanced solid tumours, using the SGT-53. They had demonstrated that SGT-53 presented tumor-specific targeting as well as anti-tumor effect in several patients. Among the patients submitted to this treatment, three of these patients achieved partial responses with tumour reductions of 47%, 51% and 79%. Two other had stable disease with significant shrinkage (25%, and 16%) [101].

At the moment, Cho and collaborators are performing one of the two approved Phase II trials using this vector. This study will be applied to glioblastoma and will combine the tumour target SGT-53 with temozolomide which is the standard of care for glioblastoma multiforme brain tumours in order to test the efficacy, safety, tumour recurrence or progression [102].

Table 4. Functionalized nanoparticles to deliver p53.

Based Material	Therapeutic	Target Receptor	Cancer/Cell Type	Year	Ref.
Liposome	-	Transferrin	Squamous cell carcinoma of the head and neck	1999	[49]
DMRIE-cholesterol	-	Transferrin	Prostate carcinoma	2002	[113]
PEI	Avidin	Transferrin	COS7/ HepG2/ HeLa	2010	[114]
SGT	Gemcitabine	Transferrin	Hepatic metastatic	2013	[111]
tetra-peptide AVPI	Doxorubicin	Octaarginine	HeLa/B16/293T	2013	[115]
Thiolated gelatin	Gemcitabine	EGFR	Pancreatic adenocarcinoma	2014	[116]
PEG	-	Anti-Her2/ luteinizing hormone	HeLa	2014	[108]
DOTAP with DOPE	Temozolomide	Transferrin	Glioblastoma multiforme	2014	[117]
$CaCl_2$	-	Rhodamine 123	Normal human dermal fibroblast	2014	[118]
xPolyR8–KLA	Triphenylphosphonium	Octaarginine	HeLa	2015	[119]
PEI	Candesartan	rHDL	Bladder Cancer	2015	[120]
Sillica	-	Transferrin	Breast cancer	2016	[112]
PEG-PLL(DA)	-	Folic acid	HeLa	2016	[109]

Table 5. Clinical trials using SGT-53.

Therapeutic	Cancer/Cell Type	Phase of the Clinical Trial	Status	Year of the Last/Final Update	Clinicaltrials.gov
Topotecan Cyclophosphamide	Paediatric solid tumours	I	Recruiting	2016	NCT02354547
Nab-paclitaxel Paclitaxel	Metastatic Pancreatic	II	Recruiting	2016	NCT02340117
Temozolomide	Glioblastoma	II	Recruiting	2016	NCT02340156
Docetaxel	Neoplasm	I	Completed	2017	NCT00470613

5. CONCLUSION AND FUTURE TRENDS

P53 is widely reported as crucial in tumour progression/regression since it is directly and indirectly evolved in almost all of the reported cancers. Concerning that, several viral and non-viral systems have been used to promote p53 reestablishment. The cell delivery of the wtp53 by various viral vectors, in particular, retroviral and adenoviral vectors, has been reported to successfully suppress the growth of various types of malignant cell. This information has incited the use of vectors in clinical trials that until now have led to the commercialization of two therapeutics based on the combination of viral vectors and p53 delivery (Advexin and Gendicin).

Despite the results provided by the use of viral vectors, these systems still present several drawbacks. To suppress these last problems, the new technology based on the use of NPs has been successfully applied, being reported until now several studies for the delivery of p53. However, this kind of systems still has a long way to go since only SGT-53, a complex cationic liposome system, has been used in phase I and II clinical trials. Perhaps researchers should invest on the specific targeting of the nanocomplexes in order to minimize the costs of this procedures and maximize the effectiveness of these therapeutics, enabling a quick and direct delivery of the nucleic acids-based drugs to target sites.

Concerning the future, researchers worldwide have been continuously searching and developing new technologies in order to create a more effective and successful cancer gene therapy. Among these new systems, there are the exosomes (cell-derived nanovesicles) that are responsible for communication between tumour and "normal" cells in the tumour microenvironment. In addition, the interior of the exosomes could be manipulated in order to promote the delivery of specific nucleic acids or proteins, like p53, which increase is suitability to cancer gene therapy [103, 104]. However, this new technology present some drawbacks like the isolation, purification, characterization and delivery of treated exosomes as well as an associated immune response in patients [105, 106].

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). J.F.A. Valente also acknowledges PhD fellowship (Ref SFRH/BD/96809/2013). Authors would like to acknowledge Doctor Adriano Raposo for the help with the images.

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PMID: 29336259

Paper II

Dilemma on pDNA purification: binding capacity vs selectivity

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Abstract

Plasmid DNA chromatography is a powerful field in constant development and evolution. The use of this technique is considered mandatory in the production of an efficient and safe formulation to be applied for plasmid-mediated gene therapy. Concerning this, the search for an ideal chromatographic support/ligand combination motivated scientists to pursue a continuous improvement on the plasmid chromatography performance, looking for a progression on the ligands and supports used.

The present review explores the different approaches used over the time to purify plasmid DNA, ambitioning both high recovery and high purity levels. Overall, it is presented a critical discussion relying on the relevance of the binding capacity versus selectivity of the supports.

Keywords: Chromatographic supports, DNA chromatography, binding capacity, selectivity, specific ligands.

Highlights

-Overview of the different chromatographic approaches used over the time to purify plasmid DNA (pDNA), ambitioning both high recovery and high purity levels;

-Describes and characterizes the chromatographic supports and ligands used in the pDNA purification process;

-Explores the dichotomy between selectivity and capacity among the used pDNA chromatographic matrices;

-Presents future perspectives in the pDNA chromatographic field.

Introduction

Gene therapy has been attracting a great interest in recent years namely, by using nucleic acids as active biopharmaceuticals to be applied in this therapy, as a way to correct genetic abnormalities, or in genetic vaccination to induce immune responses [1]. Non-viral vectors, like plasmid DNA (pDNA), are being investigated and considered in various current clinical trials and supercoiled (sc) plasmids are of particular interest due to their higher safety, integrity and biological efficiency [2].

The production of pDNA is usually performed in a recombinant *Escherichia coli* (*E. coli*) host through fermentation, accounting with around 3% w/w [3, 4] of the global components of the *E. coli* extract (Figure 1). Considering all the impurities present in the pDNA-containing lysate, regulatory agencies such as Food and Drug Administration (FDA, USA) and European Medicines Agency (EMA) impose several criteria that must be accomplished in order to prepare sc pDNA for therapeutic applications [5, 6]. Thus, it is imperative to proceed to the total isolation of the pDNA from cellular debris and other impurities like RNA, proteins and gDNA. Presently, the processes used for the pDNA recovery and purification comprise several sequential operations, which are combined to achieve a maximum sc pDNA recovery and purity level, minimizing the pDNA degradation (Box 1). This review is focused on the purification of the pDNA, since this is one of the most important steps with great impact on the target biomolecule quality as well as on the efficiency, sustainability and robustness of the global biotechnological process. The application of different types of chromatography for pDNA purification has already been extensively reviewed. So, in a different perspective, this review intends to show how research efforts have been focused on the improvement of supports and on the design of ligands as a way to overcome the pDNA purification challenges, trying to answer to the general dilemma related with the pursue of higher binding capacities or enhanced selectivity.

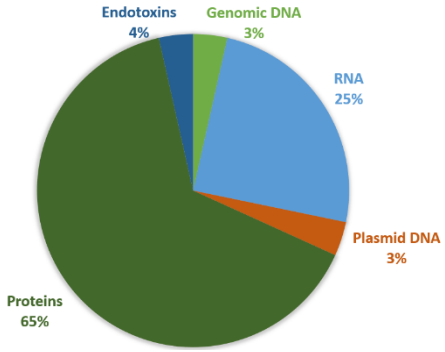
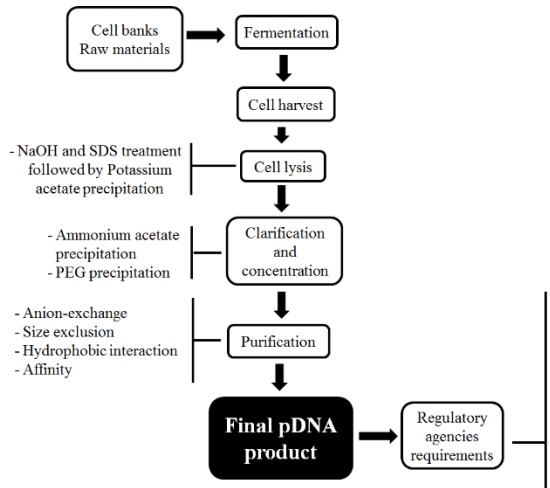


Figure 1 - Average composition of an *E. coli* lysate - adapted from [7].

Box 1- Generic upstream and downstream processing steps used for pDNA production- adapted from [2].



Specification	Approval criteria	Recommended assay
Plasmid		
Appearence	Clear, colourless solution	Visual inspection
Identity of plasmid	Restriction map as expected, sequence homology	Restriction mapping sequencing, PCR
Plasmid concentration	According to application	A260, HPLC, fluorescenece
Potency	According to application	Cell transfection
Impurities		
Proteins	Not detectabel, <0.01 µg/dose	Bicinchonic acid (BCA) assay, SDS-PAGE
RNA	Not detectable	0.8% agarose gel
gDNA	<0.05 µg/µg plasmid, <0.01 µg/dose	Hybridisation blots, PCR, fluorescence
Endotoxins	<0.1 EU/µg plasmid, <5 EU/ kg body weight	Limulus ameobocyte lysate (LAL) assay

pDNA purification by chromatography

Plasmid purification by chromatography deals with some concerns associated to the diversity of biomolecules present in the lysate extracts and also their characteristics, such as size, shape, conformation, and rheological properties. Moreover, most of the impurities like RNA, genomic DNA (gDNA) or endotoxins, share with pDNA some properties of molecular mass, charge or hydrophobicity [8]. The elimination of such impurities is so imperative and difficult that researchers try their elimination or reduction in the steps prior to the main purification. The reduction of gDNA levels is usually achieved by denaturation, during the alkaline lysis procedure and the RNA content can be reduced by the addition of RNA-digesting enzymes. This last approach however, cannot be applied for the purification of biotherapeutics, since the use of animal-derived enzymes (RNases) is not allowed [9]. Endotoxins removal is extremely important since this lipopolysaccharide component of *E. coli* can produce symptoms of toxic shock syndrome if present in sufficient quantities *in vivo* [10]. The elimination of endotoxins is commonly performed in the purification step, by chromatography.

Historically, the first methods used on pDNA purification were based on sucrose or cesium chloride–ethidium bromide density gradients ultracentrifugation. However, these methods are time consuming and difficult to scale up, and also use toxic and mutagenic reagents making them undesirable for plasmid preparation [5]. To overcome these hurdles, liquid chromatography has been established as a central technique to guarantee the quality of the

recovered pDNA products [4]. Chromatography can explore properties like size, charge, hydrophobicity, accessibility of the nucleotide bases, topological features and/or affinity, to promote the interaction between pDNA and the chromatographic matrices, expecting some selectivity and resolution, to finally recover the desirable supercoiled pDNA specie [11]. Initially, it was found a huge limitation related with the low capacity of the available matrices to bind large molecules, such as pDNA. Also, in the case of resins that presented higher capacities, they usually presented lack of selectivity, due to the similarity between pDNA and impurities. This was the start point to define a strategy to reinvent the chromatographic supports, taking into account the properties of this target biomolecule.

Reinventing the chromatographic supports

Chromatography is a well characterized and well-established method, largely used by the pharmaceutical industry for proteins purification [12]. Matrices that were initially produced for the purification of proteins, were constituted by particles with a mean size between 50 to 500 μm and a pore size surrounding the 30 nm [4]. These dimensions can cause some restrictions to pDNA purification since usually the average pore size of common chromatographic resin is smaller or approximately of the same size as the radius of gyration of a pDNA molecule which disables pDNA to access pores in standard chromatographic supports [8]. Over the years, the development of chromatographic supports for pDNA purification has tried to overcome the limitations associated to the diffusion of these molecules or with the binding capacities, culminating with the study of different supports, like the superporous matrices, the monoliths or adsorptive membranes [13-15].

The most frequently used method for primary capture of pDNA is anion-exchange chromatography (AEXC) [16] however, other chromatographic techniques also enable the isolation of pDNA from lysates, such as the size exclusion chromatography (SEC) [17], the hydrophobic interaction chromatography (HIC) [4, 18] and the affinity chromatography (AC) [6, 9, 19]. In Box 2 it is presented a summary of these chromatographic techniques, as well as the main advantages and disadvantages of each one.

Box 2- Different chromatographic methods applied in pDNA purification.

Anion-exchange chromatography

Uses the electrostatic interactions between negatively charged pDNA and positively charged stationary phases:

Advantages: Ability to selectively isolate the sc from the oc pDNA and also from other components of the lysate;

Disadvantages: Co-elution of biomolecules with charge and structure similar to pDNA like gDNA, endotoxins and some RNAs, and also, the low capacity to bind pDNA.

Size Exclusion Chromatography

Explores the different hydrodynamic sizes of the pDNA and their impurities in order to purify the target molecule:

Advantages: Good removal of RNA and proteins; Enables buffer exchange;

Disadvantages: Difficulty to remove gDNA (it elutes near the pDNA).

Hydrophobic Interaction Chromatography

Uses the hydrophobic nature of the single-stranded nucleic acid impurities (RNA, denatured gDNA, and denatured pDNA) and endotoxins to promote the binding and retard the flow of these impurities through a hydrophobic support:

Advantages: Ability to isolate pDNA from endotoxins and single stranded nucleic acids;

Disadvantages: Elution at high salt concentrations.

Affinity Chromatography

Exploits the natural biological processes based on the molecular recognition for the selective purification of the sc pDNA ensuring that this sample is within the standards of regulatory agencies:

Advantages: One-step purification of pDNA from the lysate; pure sc pDNA can be obtained;

Disadvantages: Use of biological ligands that can be unstable and are also sometimes associated to low binding capacity.

As mentioned above, AEXC is the most used chromatographic technique namely in commercially available kits for pDNA purification, such as the ones available from Promega, Qiagen or NZYTech. The Q-Sepharose anion-exchanger contains quaternary amine groups (Q) and has been largely used for globular proteins purification. At least in theory, this support has not the most suitable characteristics for plasmids purification, mainly due to the small pore size, which limits these molecules to access to the pores. In addition, this kind of problem also can lead to low binding capacity (Table 1) [20]. To suppress this capacity limitation, matrices like Fractogel EMD DEAE from MERCK have been developed. This matrix is constituted by beads with large surface area, containing coupled polymer tentacles and large pores, which enables an efficient capture of the pDNA culminating in higher binding capacities (Table 1) [21]. Moreover, there are also in the market matrices made of superporous agarose beads that can contain two sets of pores, the diffusion pores and so-called superpores or flow pores, in which the chromatographic flow can transport substances to the interior of each individual bead increasing the surface area of the bead and also increasing the binding capacity of these matrices [22]. The superporous agarose beads have large connecting flow pores with sizes that range from 1/4 to 1/20 of the overall bead diameter [22, 23]. Superporosity not only improves the access of pDNA to the internal voids but may also allow convective pore flow to take place and consequently improve internal mass transfer. As an example, Deshmukh and collaborators developed in 2005 the CELBEADS that have approximately 3 μm of pore size and are able to achieve pDNA dynamic binding capacities of 1.4 mg/mL of adsorbent with recovery yields of 77% and 52% in batch and column modes, respectively (Table 1). The final plasmid product was also found to be free from RNA, gDNA, proteins and endotoxins [24]. Another example is the case of Cytopore (GE Healthcare, Sweden), also a superporous support with much larger pores, ranging the 30 μm, for which it was described a higher binding capacity, of about 13 mg/mL, but no information concerning the purity of the pDNA was available [25].

Meanwhile, particles with an easy manipulated particle/pore diameter were also produced to better adapt them to their purpose (for example, depending on the usage of the support in analytics or large-scale purification of nucleic acid) [26]. Butyl-6PW and the octyl-6PW from Tosoh are examples of this versatility on the support production. This matrices were sequentially used, being the first support responsible for the retention of RNA and proteins and the second responsible for the adsorption of pDNA and gDNA [27]. In this approach it was described a capacity of 1.1 mg/mL and a pDNA recovery yield of 90%. The process was completed with a final polishing step with AEXC, to completely remove all impurities [26].

With the development of the new methodologies, membranes like Mustang from Pall Corporation or CIM monoliths from BIA Separations have been developed and applied in pDNA isolation and, with these systems it was possible to reach high pDNA binding capacities (above 10 mg/mL) [28]. Concerning the monoliths, they display a higher porosity with large flow-through pores which enable the mobile phase to easily travel through the support and also,

allow it to have an increased permeability and a lower back pressure in the chromatographic systems [29, 30]. These unique characteristics allow fast and efficient separation in short processing times, reducing product degradation and buffer consumption, without diminishing the resolution and separation efficiency. Tarmann and collaborators in 2008 used different chromatographic supports to compare their performance with the CIM-DEAE monolith finding that this support presented the fastest adsorption rate and highest binding capacity (13 mg pDNA/mL) (Table 1) [31]. In a different approach, a pyridine-modified monolith was also used, focusing a HIC strategy, with interesting results concerning nucleic acids purification. In that research work, a pDNA binding capacity of 3 mg/mL was obtained, being also described the recovery of sc pDNA with a homogeneity of 98% and recovery yield of 96% [18]. Nonetheless, some drawbacks are pointed to this kind of chromatography, namely because of the need of high salt concentration, and conjugation with other steps, thereby increasing the operation time and costs [11].

In the case of membranes application for the isolation of pDNA from lysates, Mateus and collaborators in 2010 were able to isolate the pVAX1-LacZ plasmid from impurities, particularly from RNA, using a membrane modified with a linear alkyl chain ligand known for its hydrophobic behaviour. However, it is important to refer that although this membrane could isolate the pDNA from the impurities, no attempt to specifically purify the sc pDNA specie was mentioned. The plasmid was recovered in the flowthrough, with a yield of 73% and 60% of purity. The characterization of the binding capacity of these membranes revealed a really high value of about 32.5 mg/mL (Table 1). The method was described as fast, simple and effective and enabling the reduction of the number of steps in purification processes of pDNA [32].

Another emerged alternative for large molecules purification, such pDNA, are the cryogels. These materials present many advantages including their large pores and short diffusion path [33]. The large pores of cryogels, similarly to what was previously reported for superporous matrices, will allow penetration of large pDNA molecules to the internal surface area. High accessibility to binding sites and negligible internal mass transfer limitations are also characteristics of cryogels, making them attractive for pDNA binding studies [34, 35]. However, as for some of the other supports, there are no reports of cryogels for the sc pDNA separation from the other pDNA isoforms.

Overall, to improve parameters such as selectivity, capacity, durability and cost effectiveness, researchers are not only dedicated to the improvement of the base material of the chromatographic supports, but they are also paying a lot of attention to the design of ligands. In fact, researchers ae mainly trying to conjugated the optimization of the supports with specific ligands, independently on the chromatography type, to achieve the best chromatographic performance on pDNA purification (Figure 2). Concerning the previously mentioned, the following topic will focus on the description of the specific ligands applied in pDNA chromatography.

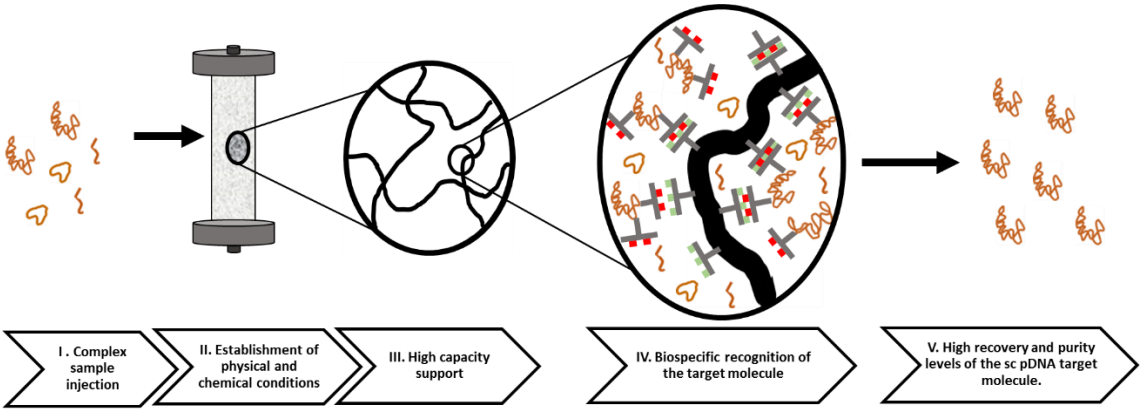


Figure 2 - Schematic representation of the ideal purification approach for supercoiled pDNA. (I) Complex sample injection; (II) Establishment of physical and chemical conditions; (III) High capacity support; (IV) Biospecific recognition of the target molecule; (V) High recovery and purity levels of the sc pDNA target molecule.

Table 1 *– Characterization of chromatographic supports used in pDNA purification.*

Stationary phase		Basic material	Functional group	Bead size (µm)	Pore size (µm)	pDNA (kb)	Adsorption Capacity (mg pDNA/mL)	Ref.
Beads								
	Q-Sepharose Fast Flow	Agarose	Q	90	<0.035	5.9	0.72	[20]
	Q- Ceramic Hyper D	Hydrogel/Ceramic	Q	50	0.3	5.9	5.3	[20]
	Fractogel EMD DEAE	Methacrylate	DEAE	40-90	0.8	5.9	2.45	[20]
	Sephadex G	Dextran crosslinked with epichlorohydrin		50	-	6.1	-	[36]
	Source 30Q	Polystyrene divinylbenzene	Q	30	0.002-0.1	6.9	0.707	[21]
Superporous beads								
	POROS 50 HQ	Polystyrene divinylbenzene	PEI Q	-	<0.8	5.9	2.12	[20]
	CELBEADS	Cellulose		150-500	≈ 3	9.8	1.4	[24]
	Cytopore		DEAE	230	30	4	13	[25]
	Superporous Agarose	Agarose	Q	45-75	4	7	1-2	[37]
	Superporous Agarose	Agarose	PEI	45-75	2	7	3-4	[37]
Membranes								
	Mustang	Polyethersulfone	Q	-	0.8	6.1	10	[38]
	Natrix	Hydrogel	Q	-	0.45	6.4	12.4	[39]
	Sartobinds Epoxy 75	Cellulose	Linear alkyl	-	0.45	6.05	32.5	[32]
Monoliths								

	CIM DEAE	Methacrylate	DEAE	1.5	0.7-0.95	4.9	13.42	[31]
	CIM C4	Methacrylate	Pyridine	-	-	4.7	3	[18]
	CIM C4 HLD	Methacrylate	Butyl	0.95-1.1	2	4.7	≥2.5	[40]
Cryogels								
		PHEMAH	Cibacron Blue F3GA	-	10-100	4.3	32.4 mg/g polymer	[41]
			N-methacryloyl-(l)-histidine methyl ester (MAH)	-	10-100	4.3	13.5 mg/g polymer	[33]

Design of specific ligands for sc pDNA isolation

Current processes for pDNA purification, aiming its therapeutic application, require several chromatographic steps, which make pDNA production not only time-consuming but also costly [11]. As referred in the previous topic, AC employs a specific immobilized ligand to interact with the target biomolecule loaded under particular conditions that favour the binding to the support and also intends to promote the largest pDNA recovery level as possible. Due to the improved selectivity, this technique is capable of achieving purification in a single process. However, the selectivity and specificity can only be achieved when ligands are able to recognize the molecule of interest which, in this case, is the sc pDNA [9, 42]. Previous affinity strategies used on pDNA purification include a DNA binding protein, triplex DNA formation, aromatic affinity chromatography and amino-acid affinity chromatography [9].

Considering this approach, nucleic acids isolation was already established using a DNA-binding protein as ligand. To the best of our knowledge, the first authors reporting the use of this chromatography was Woodgate and collaborators in 2002. They used a bifunctional zinc finger (ZNF) DNA-binding protein fused to Glutathione-S-transferase for the isolation of pDNA from a complex lysate, bearing the ZNF recognition. However, using this approach it was only observed an yield of 10% and no data about the elution of the pDNA was given [43]. This research group also used a different heterofunctional protein, the LacI-His6-GFP, and in this case they improved the recovery of pDNA to more than 80%, achieving a high purity in the final pDNA product that was free from detectable RNA and protein and with minimal genomic DNA contamination [44]. A different strategy is based on triple helix affinity, which involves the formation of Hoogsteen hydrogen bonds between thymine (T) and adenine (A) to form TAT triplexes, or between protonated cytosine (C+) that specifically recognizes guanine (G) to form CG-C+ triplexes [8]. To guarantee some stability during the use of these triplexes is imperative the use of acidic pH. The binding of pDNA occurs via an intermolecular triplex formation with a biotinylated oligonucleotide as ligand, and the recovery occurs by loading the column with a mild alkaline buffer which is responsible for the destabilization of the Hoogsteen H-bonds. Sherman and collaborators in 1997 used this kind of chromatography on the purification of the plasmid pXL2563 which contained a poly-GAA sequence targeting the CTT oligonucleotide covalently bound to the Sepharose column used. The maximum plasmid recovery was 42% and the impurities were totally eliminated (as it is the case of RNA) or significantly reduced (for proteins and gDNA) [45]. A less explored, but important strategy is related with the use of aromatic affinity ligands, being the most common the six-membered ring benzene [46]. These compounds have the ability to fully isolate the supercoiled isoform being described purity levels between 98.8% and 100% and recovery yields of about 100% as is possible to see in Table 2.

Amino acids have been also used in the last years for affinity chromatography, as a way to mimic the natural interaction phenomena occurring between nucleic acids and proteins in

biological organisms. Until now amino acids such as arginine, histidine, methionine among others have been successfully applied in the isolation of the sc pDNA isoform (Table 2) [6, 47]. These specific ligands revealed to be a promising approach for pDNA purification, enabling the selective biorecognition of the sc pDNA isoform and allowing the elimination of the remaining pDNA isoforms and other impurities [48]. The main advantage of these affinity ligands is the multitude of interactions (hydrophobic, electrostatic, cation-π, van der Waals forces and/or hydrogen bond) that can be established with the sc pDNA, promoting this kind of affinity towards the target biomolecule, which consequently results in a more effective and selective separation. Also, with some of these ligands, there is a possibility to mainly favour some particular interactions through the adjustment of the binding and elution conditions, such as the temperature, flow rate and buffer composition (pH, ionic strength or presence of competitive agents), giving more versatility to the method [3]. When amino acids were immobilized on beads the sc pDNA purity levels ranged between 97 to 100% being the recovery yields between 40 to 70% [47-50]. It is also important to refer that, although selectivity is of high importance, and ideal chromatographic support should also be able to promote the isolation of high amounts of pure pDNA. Regarding this, some intents have been made to combine the selectivity of these ligands with the capacity of modern supports, by using for example the monoliths. As is presented in Table 2, when the immobilization is performed on monoliths, the purity levels ranged between 98.3 to 100% and the recovery from 45.3 to 91.3% [19, 50-53]. Also, when the capacity of the different supports is evaluated is notorious that monoliths present a significantly higher capacity than conventional beads. For example, when histidine is immobilized on an agarose based matrix, the maximum DBC achieved was of about 0.5 mg/mL, while the immobilization of the same amino acid onto a monolithic support yielded a DBC of around 11 mg/mL [49, 53]. Also, arginine, a well-studied ligand for the sc pDNA isolation, was immobilized in both types of supports, and the higher DBC were found for the monoliths (5.18 mg/mL against 1.11 mg/mL in an agarose support) [50].

The search for the most suitable ligands lead researchers to use multimodal ligands able to promote multiple and different elementary interactions with the target molecule. This type of ligands presents more than one active site which could enable simultaneously ionic and hydrophobic interactions, and these can be differently explored during the chromatographic run, by adjusting the experimental conditions and combining gradients [54]. This versatility usually increases the selectivity and specificity of the chromatographic process [55, 56]. As an example, amino acids derivatives, such as histamine, were already exploited in this field, to accomplish the purification of sc pDNA. Černigoj and collaborators in 2013 exploited the hydrophobic (from the imidazole group) and also the ionic behaviour (from the amino group) of histamine to isolate pDNA from a sample made of oc and sc pDNA, combining a descending pH gradient with the use of $(NH_4)_2SO_4$ [46]. In another study, Silva-Santos and collaborators in 2016 used a commercially available multimodal matrix, the Capto adhere with a N-Benzyl-N-methyl ethanol amine ligand (from GE Healthcare Biosciences). In this work, hydrophobic interactions

from the benzyl group and ionic interactions from the amine and hydroxyl groups of this ligand were exploit. The results revealed that 91.8% of the pDNA collected in the first peak was in the oc form, whereas 92.2% of the pDNA collected in the second peak was supercoiled [57].

At the moment the pursuit of the ideal ligand motivates scientists to explore bioinformatic tools and combining them with molecular docking. All these technological tools move researchers to a new level of affinity ligands design ability, since they are able to use combinatorial methods for systematic generation and screening of large numbers of novel compounds [58]. Concerning the combinatorial approach, there are already some studies where different scaffolds with different linkage molecules (like triazine or Ugi) were used to quickly and easily search for the suitable affinity ligand to be applied for the recovery of the target biomolecule [59]. Nevertheless, from the best of our knowledge there are no research works concerning these techniques applied to pDNA chromatography. However, a diversity of studies simulating the DNA linkage to peptides or drugs for other kind of applications are easily found what brings the opportunity to use these techniques in the specific ligand design to improve pDNA chromatography [60-63].

Table 1 *– The influence of different ligands and supports in the pDNA adsorption, recovery and purity.*

		Ligand	pDNA (kb)	Main Conditions	pH	Capacity (mg/mL)	Recovery (%)	Purity (%)	Year	Ref.
BEADS	**Aromatic**	Phenyl		Hydrophobic	8		≈ 100	≈ 100	2005	[64]
		Phenyl boronate (3aPABA)	6.05	Hydrophobic	5.2	-	96	-	2011	[65]
		Mercaptopyrimidine	3.697	Hydrophobic	8	-	68.5	98.8	2013	[66]
		3,8-diamino-6-phenylphenanthridine (DAPP)	6.05	Ionic	5	0.337	74	100	2013	[67, 68]
		N-Benzyl-N-methyl ethanol amine	3.7	Ionic	8	-	-	92.2	2016	[57]
		1,3-bis(4-phenylamidinium) triazene (Berenil)	6.05	Hydrophobic	8	-	87	99	2014	[69]
	Amino acid	Histidine	6.05	Hydrophobic	8	0.530	40	100	2006/ 2007	[47, 49]
		Arginine	6.05	Ionic	8	-	79	97	2009	[48]
			8.702	Ionic	8	1.11	39.18	99	2013	[50]
		Lysine	6.05	Ionic	8	-	45.5	100	2011	[70]
		Methionine	6.06	Hydrophobic	8	-	39 µg/ mL	97	2014	[6]
	Triple helix	S-$(CTT)_7$	-	Hydrophobic	4.5	-	42	-	1997	[45]

		Polypyrimidine oligonucleotide	-	Ionic	5	0.028	38.8	-	1998	[71]
	Protein-DNA	LacI-His6-GFP	-	ionic	7.4	-	>80	-	2005	[44]
		Zinc Finger-Glutathione S-Transferase	-	-	8	-	10	-	2002	[43]
		LacI peptide	2.7	Ionic	7.4	0.022	81	92	2008	[72]
MONOLITHS	**Aromatic**	Carbonyldiimidazole (CDI)	6.05	Hydrophobic	8	-	74.4	100	2011	[73]
			14, 6.05 and 2.686	Hydrophobic	8	5.319, 5.478, 5.891	-	-	2014	[74]
	Amino acid	Histamine	5.1	-	variable	1.3	95	98	2013	[51]
				Hydrophobic	7.4	2.7	62	98.5		
				Ionic	5	4.2	-	-		
		Arginine	8.702	Ionic	7.9	5.18	83.5	100	2013/ 2015	[50, 52]
		Histidine	6.05	Hydrophobic	8	11.03	-	-	2015	[53]
		Agmatine	6.471	Ionic	9.6	-	45.3	99.6	2016	[19]

Concluding remarks and future perspectives

Selectivity and capacity in purification processes can present an opposite tendency, so researchers have been making a huge effort to counter this trend. In fact, important progresses on pDNA chromatography have been done improving drastically the suitability of the supports for large molecules. Concerning the described until now, it could be said that future will involve the use of supports with large surface contact areas and with large pores enabling not only a higher number of binding sites to the desired molecule, but also promoting a better flow of the molecules within the support. With this approach the support ability to bind and promote the recovery of large amounts of pDNA will increase, which will also enhance the economic feasibility of the chromatographic process applied on the recovery and purification of pDNA molecules. Therefore, the ligand development will be a crucial point if pure supercoiled pDNA is expected to be recovered. In fact, science and technology start to work synergistically on the development of specific ligands, which could enable a better recognition of the target molecule and, consequently could lead to high recovery and purity yields. Overall, the scientific community is more and more convinced on the relevance of focusing both on the improvement of binding capacity of the supports and enhancement of selectivity through the design of more specific ligands, to globally improve the pDNA purification performance.

Acknowledgements

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I – Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). J.F.A. Valente also acknowledges PhD fellowship (Ref SFRH/BD/96809/2013).

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Chapter III

Paper III

Selective purification of supercoiled p53-encoding pDNA with L-methionine–agarose matrix

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Analytical Biochemistry 459 (2014) 61–69

Contents lists available at ScienceDirect

Analytical Biochemistry

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

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ARTICLE INFO

Article history:
Received 3 March 2014
Received in revised form 5 May 2014
Accepted 15 May 2014
Available online 23 May 2014

Keywords:
Affinity chromatography
Biorecognition
L-Methionine
p53-encoding plasmid
Supercoiled isoform

ABSTRACT

The p53 tumor suppressor gene has been widely explored for gene therapy as an alternative to the common treatments. Recently, the supercoiled conformation of a p53-encoding plasmid proved to be more efficient in cell transfection and protein expression than the open circular conformation. To successfully isolate this isoform, several chromatographic techniques have been used, namely affinity chromatography with amino acids as ligands. However, the study of new matrices and ligands with higher specificity and robustness for supercoiled plasmid purification is still required. The present work explores for the first time a new matrix of L-methionine–agarose to efficiently purify the supercoiled p53-encoding plasmid. The binding/elution conditions, such as salt concentration and temperature, were manipulated and combined to attain the best strategy. Therefore, the supercoiled plasmid isoform was purified from a clarified lysate by using a decreasing stepwise gradient comprising 2.35 and 1.7 M ammonium sulfate in 10 mM Tris–HCl, pH 8.0, and finally 10 mM Tris–HCl, pH 8.0, at 5 °C. After accomplishing the purification process, we performed several tests to assess the quality of the supercoiled plasmid, revealing that the amounts of proteins, gDNA, RNA, and endotoxins were significantly reduced or undetectable in the final formulation.

Cancer is one of the most lethal diseases worldwide, which makes the research in this field imperative and very widespread. Researchers have found a relation between p53 loss or mutation and the appearance of cells with a carcinogenic behavior. The signaling pathway of this protein is activated in response to a variety of stress signals, allowing p53 to coordinate transcription programs that ultimately contribute to tumor suppression, thus being the unique gene capable of promoting a proficient selective induction of growth arrest and apoptosis in response to oncogenic or damage signaling [1,2]. In this regard, it becomes extremely necessary to maintain or reestablish the proper function of this protein in cells.

To solve this problem gene therapy and DNA vaccination have been used as new therapeutic approaches based on plasmid DNA (pDNA) delivery. These kinds of therapy rely on the possibility of rapidly evaluating and producing pDNA biopharmaceuticals aiming at the treatment of patients through the replacement of a copy of the defective gene, which has a huge impact compared with traditional clinical management [3,4].

To produce the p53-encoding plasmid, a recombinant *Escherichia coli* host was used and grown by fermentation. Through the biosynthesis of pDNA an extract highly enriched in sc (supercoiled) pDNA was obtained, which is advantageous since sc pDNA preparations have proved to be more efficient for gene transfection than other isoforms, such as linear or open circular (oc) pDNA [4]. The efficient access of pDNA to the cell nucleus improves gene expression in eukaryotic cells. Moreover, several studies evidenced that high supercoiling levels are required for eliciting an effective therapeutic gene expression and, ultimately, to correct a genetic defect or suppress an uncontrolled production of a specific protein [5]. Also, in accordance with the regulatory agencies, such as the Food and Drug Administration, pDNA should accomplish several specifications to be suitable for therapeutic applications. Among these specifications are the appearance (clear, colorless solution), the plasmid homogeneity (≈97% sc), the RNA and protein content (should not be detectable), the amount of genomic DNA (gDNA; needs to stay above 2 ng/μg of pDNA), and also the level of endotoxins (should not be more than 0.1 EU/μg of pDNA) [6,7].

Taking the above-mentioned conditions into account, it becomes extremely important to eliminate the cellular components of the *E. coli* host and also reduce the oc, linear, and denatured pDNA isoforms that appear because of conformational changes in sc pDNA [8].

To efficiently isolate and purify the sc pDNA vector, various chromatographic methodologies have been used, namely size exclusion, anion exchange, hydrophobic interaction, reversed phase, thiophilic adsorption, and affinity chromatography. Size-exclusion

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http://dx.doi.org/10.1016/j.ab.2014.05.011
0003-2697/

chromatography has limited capacity and selectivity for pDNA and hence is ideally used as a polishing step to remove residual contaminants from the sc pDNA sample. In the case of anion-exchange chromatography, selectivity toward pDNA or impurities (e.g., RNA, gDNA, and endotoxin) is poor because of their nonspecific binding to the anion-exchange resin. On the other hand, hydrophobic interaction allows an efficient separation of pDNA from endotoxins and single-stranded nucleic acids, owing to nonbinding of pDNA. Also, pDNA elution occurs at high salt concentration of ammonium sulfate, which induces a high environmental impact. Reverse-phase chromatography requires the use of organic solvents that are often toxic, which is a major disadvantage for this technique [6,9,10]. Thiophilic chromatography enables a high purity yield; however, to accomplish the sc isolation, high salt concentrations are needed [9,11,12]. In the case of affinity chromatography, other than other established techniques, one strategy based on amino acids as ligands has been successfully applied for sc plasmid recovery by our research group [2,7,10,13]. However, it is still essential to develop and study new matrices with new ligands with higher specificity and robustness and able to be integrated in processes with economic interest for the pharmaceutical industry [14,15]. In the case of p53-encoding pDNA an arginine-based matrix has been already used for the isolation of sc isoforms; however, the sc pDNA purification from a complex lysate and the characterization of the purified sc sample, with regard to the residual contaminants, was not performed until now. Moreover, Gaspar et al. [2] have reported that the purity degree achieved for the isolated sc pDNA was 95%, and in the present work, the sc plasmid was purified from the *E. coli* lysate with the L-methionine agarose support, presenting a reduced impurity level and a homogeneity above 97% for the sc isoform.

Accordingly, in this work a new chromatographic matrix of L-methionine–agarose was used to purify p53-based pDNA to obtain the sc isoform. Other plasmids, such as pUC19 and HPV-16 E6/E7, were also tested to better understand the range of applicability of this matrix. This amino acid is hydrophobic, being the mediator of a large number of contacts in protein–nucleic acid interactions [16]. The interaction between this matrix and various oligonucleotides was already studied by our research group, by manipulating the effects of temperature and salt concentration. Through this research it was possible to find that methionine can interact with nucleic acids, showing a higher affinity for thymine bases [16]. Overall, these findings strongly suggest the possibility of using the methionine ligand to specifically purify pDNA. To accomplish this, various salts were evaluated and the salt concentration, pH of the mobile phase, and also the temperature were changed and combined to specifically purify the sc pDNA from other isoforms and host contaminants.

Materials and methods

L-Methionine–agarose matrix was obtained from Sigma–Aldrich (St. Louis, MO, USA) and the NZY Maxi Prep Kit was purchased from NZYTech (Lisboa, Portugal). All the water used to prepare solutions was ultrapure grade, purified with a Milli-Q system from Millipore. Ammonium sulfate ($(NH_4)_2SO_4$) was purchased from VWR and tris(hydroxymethyl) aminomethane (Tris) was from Merck (Darmstadt, Germany). Elution buffers were filtered through a 0.20-μm pore size membrane (Schleicher & Schuell, Dasel, Germany) and degassed ultrasonically. The 6.07-kb pcDNA3-FLAG-p53 Addgene plasmid 10838 [17] and the 8.702-kp HPV-16 E6/E7 Addgene plasmid 8641 [18] were purchased from Addgene (Cambridge, MA, USA). The 2.7-kp plasmid pUC19 was purchased from Invitrogen (Carlsbad, CA, USA), and finally, all the reagents used in bacterial growth were obtained from Sigma–Aldrich. The DNA ladder was obtained from Bioline (London, UK).

Plasmid and bacterial growth conditions

In a cell culture of *E. coli* DH5α, the pcDNA3-FLAG-p53, pUC19, and also the HPV-16 E6/E7 plasmids were amplified. The growth was carried out at 37 °C, 250 rpm, in Erlenmeyer flasks with 500 ml of Terrific Broth medium (20 g L^{-1} of tryptone, 24 g L^{-1} of yeast extract, 4 ml L^{-1} of glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg ml^{-1} ampicillin for pcDNA3-FLAG-p53 and with 100 μg ml^{-1} for pUC19 and HPV-16 E6/E7 plasmids. The cells were grown until the log phase ($OD_{600\ nm}$ ± 9). Finally, the cells were collected by centrifugation and stored at −20 °C.

Lysis and plasmid isolation

In a first step the cells were disrupted by alkaline lysis and the p53-encoding plasmid was prepurified with the NZYTech kit according to the supplier protocol to obtain the native pDNA (sc and oc isoforms). Basically, after an alkaline lysis the pDNA was bound to the NZYTech anion-exchange resin under appropriate low-salt and pH conditions. Then, the impurities were removed by a medium salt wash and finally the plasmid was eluted through the increase in ionic strength.

To study a more complex lysate (of all plasmids), after the cells' recovery they were lysed through a modified alkaline method that was already described [7,19]. In this case, the extract was considerably more complex, containing pDNA and several impurities, namely RNA, gDNA, proteins, and endotoxins.

Isolation of nucleic acids

The isolation of gDNA and RNA was performed from a pDNA-free DH5α *E. coli*. For RNA isolation, the cells were lysed by a modification of the alkaline lysis method [20]. The resultant lysate was clarified by ammonium acetate precipitation and nucleic acids were concentrated by polyethylene glycol 6000 precipitation [21]. The isolation of gDNA was performed according to the manufacturer's instructions using the Wizard genomic purification kit from Promega (Madison, WI, USA).

Preparative chromatography

The chromatographic assays were performed in a fast protein liquid chromatography system (Pharmacia Biotech, USA). A 16 × 10-mm (approximately 8 ml) column was packed with a commercial L-methionine–agarose gel (Sigma–Aldrich). The manufacturer characterizes this support as a cross-linked 4% beaded agarose support with one-atom spacer and an extent of labeling between 2 and 10 μmol/ml. The experiments were performed using a circulating water bath to maintain the various temperatures under study. Then different samples (pDNA, gDNA, RNA, or complex lysates) were loaded onto the column using a 200-μl loop, at 1 ml/min. The absorbance of the eluate was continuously monitored at 280 nm. The column was equilibrated with various concentrations of ammonium sulfate depending on the experiment. The elution of bound species was carried out using a decreasing ammonium sulfate stepwise gradient. To elute all remaining species, 10 mM Tris–HCl, pH 8.0, was used. All the results were confirmed by performing at least three replicates.

Agarose gel electrophoresis

The horizontal electrophoresis was performed to analyze the peak fractions recovered from chromatographic experiments. A 0.8% agarose gel (Hoefer, San Francisco, CA, USA) was used, stained with GreenSafe Premium (0.01%; NZYTech). Electrophoresis was

carried out at 110 V with TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0). The gel was visualized under UV light in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal).

Plasmid quantification

The concentration, recovery yield, and purity of the pDNA were evaluated according to an adaptation of the analytical method previously described [22]. Briefly a phenyl–Sepharose column (Amersham Biosciences, USA) was connected to the ÄKTA Purifier system (GE Health-Biosciences, USA) to separate the double-stranded pDNA molecules that elute in the flowthrough from the more hydrophobic impurities that are delayed (gDNA and proteins) or retained (RNA and proteins) in the analytical column, as previously described [7].

The pDNA concentration was calculated taking into account a calibration curve designed with pDNA standards (1–100 μl) purified with a commercial NZYTech kit. The purity degree was defined as the percentage of the plasmid peak area related to the total area of all peaks present in the chromatogram.

Protein quantification

For protein quantification, the micro-BCA (bicinchoninic acid) protein assay kit from Pierce (Rockford, IL, USA) was used in accordance with the specifications of the supplier. A calibration curve with the standard protein bovine serum albumin (0.01–0.1 mg/ml) diluted in 10 mM Tris–HCl, pH 8.0, was determined. The samples were desalted with Tris–HCl, pH 8.0, and further analyzed.

Genomic DNA quantification

Real-time polymerase chain reaction (PCR) was used to evaluate the existence of gDNA in the purified samples. The analyses were performed in an iQ5 Multicolor real-time PCR detection system (Bio-Rad), as previously described [19]. Sense (5′-ACACGGTCCA GAACTCCTACG-3′) and antisense (5′-CCGGTGCTTCTTCTGCGGGT AACGTCA-3′) primers were used to amplify a 181-bp fragment of the 16S rRNA gene. For the quantification, the samples collected after lysis were diluted 100-fold beforehand. PCR amplicons were quantified by following the change in fluorescence of the DNA binding dye Sybr green (Bio-Rad). The concentration of gDNA was obtained through a calibration curve generated by serial dilutions of purified *E. coli* DH5α gDNA in the 5 pg/μl to 50 ng/μl range. Negative controls (no template) were run at the same time of each analysis.

Endotoxin quantification

The ToxiSensor Chromogenic *Limulus* amoebocyte lysate (LAL) endotoxin assay kit (GenScript, USA) was used for measuring the endotoxins, considering the manufacturer's instructions. The calibration curve (from 0.1 to 1 EU/ml) was performed using a provided stock solution of 8 EU/ml. To avoid the external endotoxin interference, all the samples were diluted or dissolved with non-pyrogenic water, which was also used as the blank. All the tubes and tips used to perform this analysis were endotoxin-free. The entire procedure was performed inside a laminar flow cabinet to ensure aseptic conditions.

Results and discussion

Chromatographic purification

Affinity chromatography has been used in sc plasmid purification since it simulates the phenomena occurring during the biological processes such as molecular recognition between a particular amino acid and nucleic acid bases [7]. This kind of chromatography takes advantage of the specificity and efficiency of immobilized ligands to promote the biorecognition between target biomolecules and particular ligands. This strategy also reduces the chromatographic steps into only one, increases recovery yield, and improves the economics of the purification process [19,23].

Although the *E. coli* lysate sample used in this work has been clarified by a partial elimination of impurities during the primary isolation, considerable amounts still remain, making its removal crucial for the clinical application of pDNA [13]. To separate the p53-based sc pDNA from impurities a new affinity strategy was explored using the L-methionine–agarose matrix.

Plasmid isoform separation

The initial experiments were performed using a prepurified p53-encoding plasmid DNA sample, composed of sc and oc plasmid isoforms. In this case, a screening of the type and salt concentration was performed to achieve the best binding/elution conditions. Through this analysis it was verified that the application of an increasing NaCl gradient was not able to promote an effective interaction between pDNA and the methionine ligand. Therefore, the selectivity to recover the sc pDNA was not achieved under these binding/elution conditions (data not shown). The separation of sc and oc isoforms was achieved by using a decreasing stepwise gradient of ammonium sulfate. First, the L-methionine–agarose column was equilibrated with 2.35 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl, pH 8.0. After injection of 200 μl of prepurified plasmid sample (oc + sc), a first peak was rapidly attained owing to the elution of unbound species (Fig. 1A). Thereafter, the elution step comprised a decrease in the ionic strength to 10 mM Tris–HCl, pH 8.0, and a second peak corresponding to the elution of the more retained species was obtained.

Temperature is a parameter that normally affects the purification of nucleic acids by affinity chromatography with amino acids as ligands. Thus, the temperature effect (5, 10, and 15 °C) on pDNA retention and selectivity was also evaluated, applying the same elution strategy (Fig. 1). In contrast to the effect of temperature in a hydrophobic-governed mechanism, these experiments evidenced that, while the temperature of the column increases, the sc isoform retention decreases, being partially eluted in the first peak together with the oc isoform. This tendency can be monitored by the different heights of the peaks and the electrophoretic profile achieved for both peaks in each temperature tested (Fig. 1). In fact, some previous studies describe that the increase in temperature can be responsible for conformational changes in the secondary structure of plasmid molecules, which consequently decreases the retention on the column [24]. In particular, the increase in temperature can change the torsion of the sc pDNA chain, which promotes relaxation [25] and in consequence enables a decrease in the interaction of the sc isoform with the methionine ligands.

The best results are shown in Fig. 1A and, in this case, a temperature of 5 °C was used and the oc isoform was eluted in the first step, while the sc pDNA remained strongly retained in the L-methionine column, being eluted only when the ionic strength was decreased. The retention behavior found in the methionine matrix was similar to what was described for the histidine ligand [24]. Similar to what is described for other amino acid-based matrices, the analysis of all the results suggest that several elementary interactions are responsible for the biorecognition of the ligands for a specific pDNA isoform. For example, in isoform separation with histidine as ligand the involvement of several interactions was demonstrated to be responsible for pDNA retention and selective purification of the sc pDNA isoform using an ammonium sulfate-based binding/elution strategy. In this case it was hypothesized that the pDNA backbone is not mainly involved in the retention

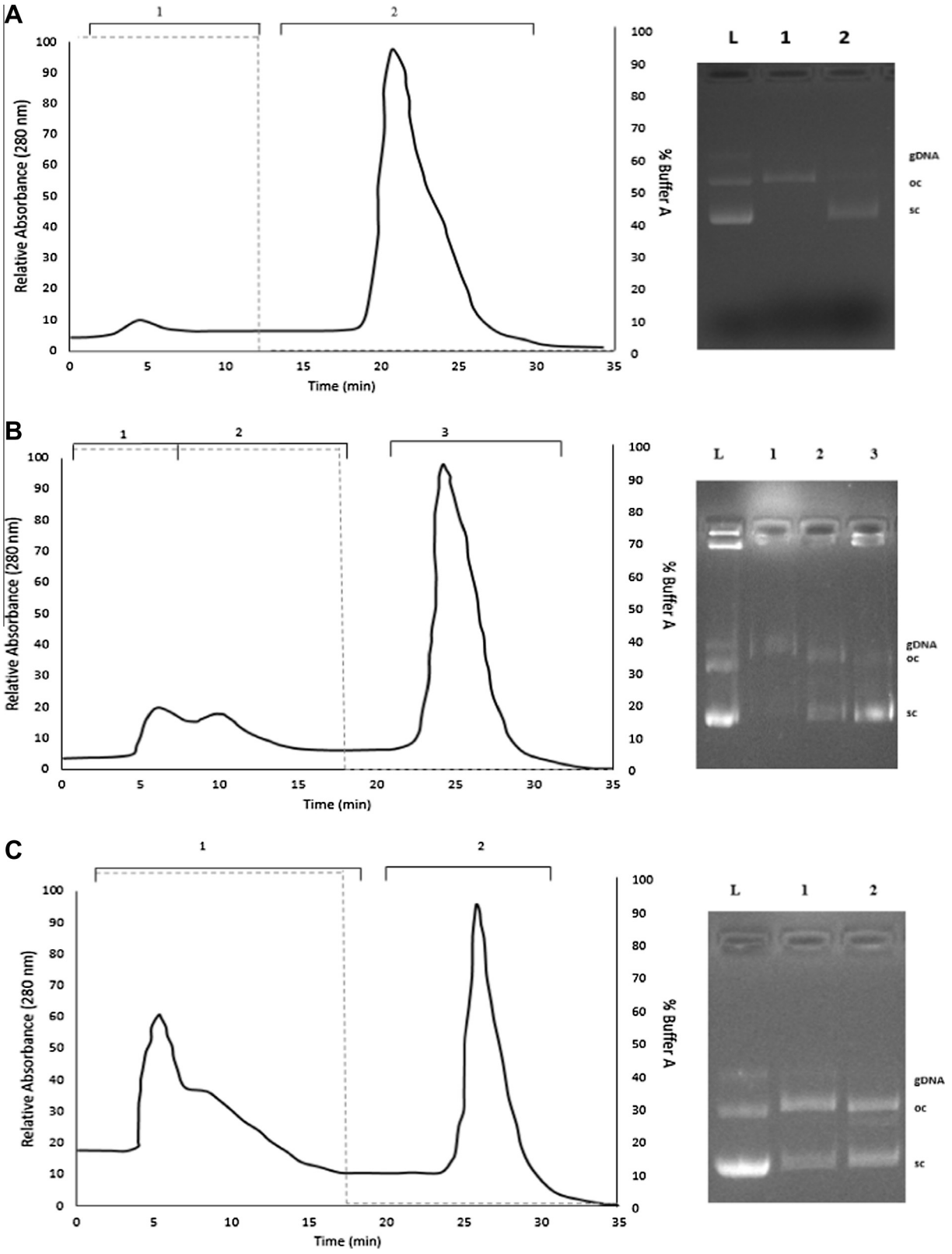

Fig.1. Chromatographic profiles and respective agarose gel electrophoresis of the plasmid isoform separation from a prepurified pDNA sample (sc + oc) in an L-methionine–agarose column using various temperatures: (A) 5, (B) 10, (C) 15 °C. The elution was performed by stepwise gradient of 2.35 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl, pH 8.0, to 10 mM Tris–HCl, pH 8.0, as represented by the dashed line. In the electrophoresis gel, lane L represents the prepurified plasmid sample and lanes 1, 2, and 3 represent the respective peaks of each chromatogram.

mechanism, since phosphate and sugar groups are equally exposed in both isoforms. The selectivity found for sc pDNA was associated with the higher nucleotide base exposure [13].

In the case of lysine and arginine affinity chromatography the elution strategy used was slightly different since these matrices showed to be effective in pDNA binding at low ionic strength. More recently, the possibility of using these amino acids as ligands to purify a specific pre-miRNA, using a decreasing ammonium sulfate gradient, was also described [26,27]. Lysine performs an interaction analogous to the end-on conformation by placing the N atom between more than one acceptor atom, which was explained as being the most likely cause of the different retentions of nucleic acids. In addition, atomic studies of the interaction of the arginine with pDNA show that the predominant interaction was with guanine base owing to bidentate and also complex interactions accomplished via hydrogen bonds [19].

With the methionine ligand the experimental conditions used suggest that the hydrophobic interactions play a critical role in the pDNA-specific retention, which could be mainly due to the thioether group present in the methionine amino acid [28]. Also thiophilic interaction with the polynucleotides could be considered; however, since the methionine does not have an aromatic group, this kind of interaction will be extremely weak and probably nonexistent [11,12]. Moreover, other important interactions between methionine and pDNA bases and backbone (sugar and phosphate groups) are also established, namely the hydrogen bonds, van der Waals contacts, and water-mediated interactions [29].

gDNA and RNA separation

After pDNA isoform separation, the behavior of gDNA and RNA impurities was analyzed through various binding/elution experiments, to recover useful information for further sc pDNA separation from the complex lysate. For this purpose, gDNA and RNA samples isolated from plasmid-free *E. coli* DH5α were loaded separately in the matrix and both experiments were performed at 5 °C.

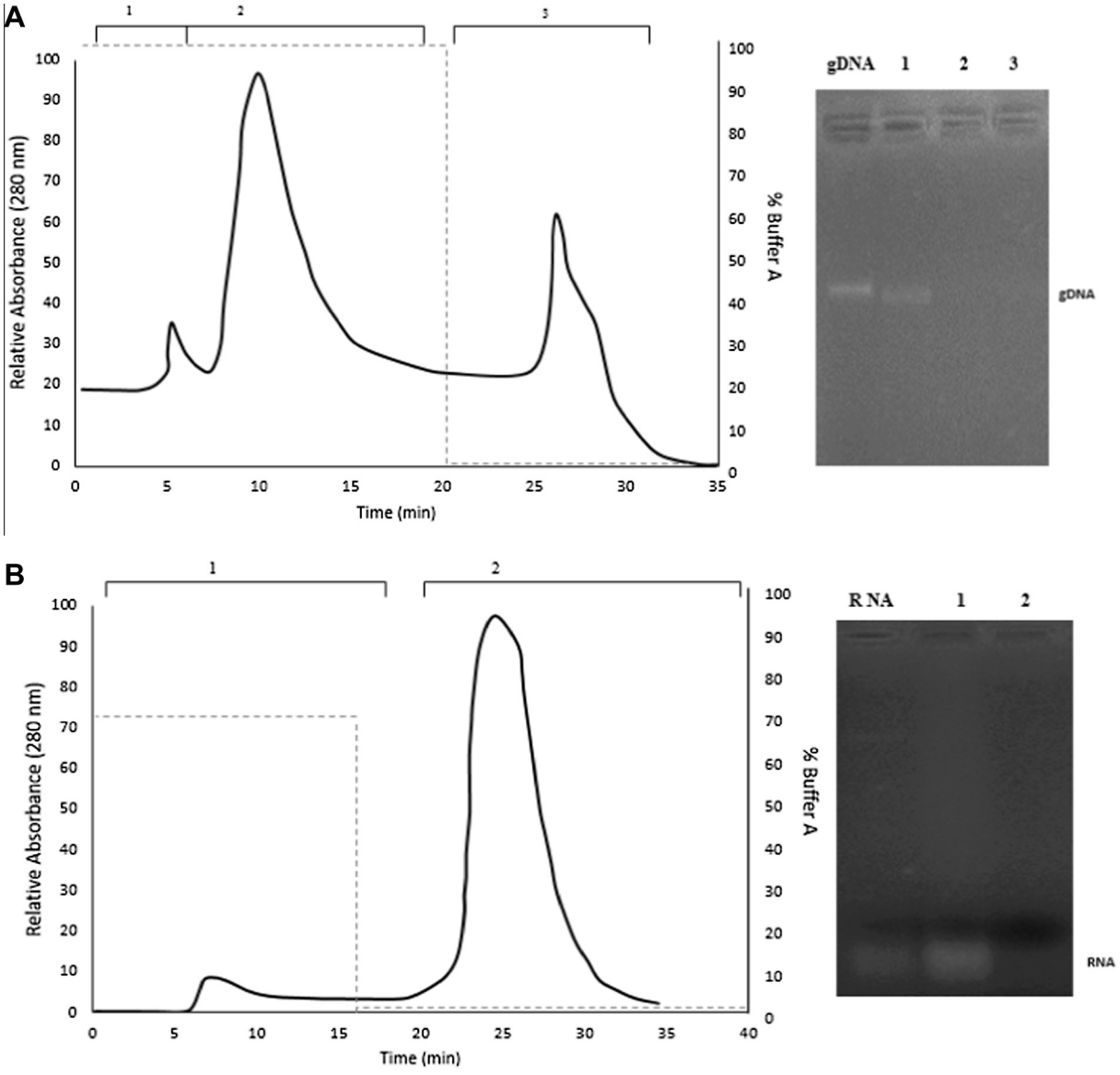

Fig.2. Chromatographic profile and respective agarose gel electrophoresis of (A) gDNA and (B) RNA injection in the L-methionine–agarose column. In (A) the elution was performed by stepwise gradient of 2.35 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl, pH 8.0, and 10 mM Tris–HCl, pH 8.0, and in (B) the elution was performed by stepwise gradient of 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl, pH 8.0, and 10 mM Tris–HCl, pH 8.0, as indicated by the dashed lines. In the electrophoresis the first lanes represent the loaded (A) gDNA or (B) RNA samples. Lanes 1, 2, and 3 represent the respective peaks of the chromatograms.

In Fig. 2A it is possible to observe the gDNA elution at 2.35 M ammonium sulfate, meaning that gDNA is not retained under these conditions. Owing to the polyanionic nature of gDNA the establishment of electrostatic interactions could be expected; however, this effect was not verified in a magnitude significant enough to promote binding. Once more, this could suggest that the specific recognition of biomolecules involves multiple interactions and is dependent on the degree of exposure of nucleic acid bases [30]. It is also important to note that owing to the alkaline lysis, gDNA suffers denaturation and its isolation can result in several double-stranded fragments. This heterogeneity is responsible for the appearance of multiple peaks on the chromatogram, which is also indicative of the different interaction of the biomolecules with the L-methionine matrix [13].

Generally, the nonrecognition of the gDNA by the matrix could be related to the inadequate exposure of nucleic acid bases, which is similar to what was verified with oc pDNA [19]. In this way, these results suggest that the gDNA could be eluted together with the oc isoform, not interfering with sc pDNA recovery.

As is possible to observe in Fig. 2B, host RNA is retained at high salt concentrations, being eluted only at 1.5 M $(NH_4)_2SO_4$ and with 10 mM Tris–HCl. Considering the pDNA retention pattern described above, the elution of sc pDNA is expected at higher salt concentrations. So, the use of 1.5 M ammonium sulfate or Tris–HCl to elute RNA will be advantageous since it allows the recovery of a pure sc pDNA fraction, free from contaminants. This strong interaction of RNA with the column can be due to the single-stranded nature of RNA, which makes its bases highly exposed and available for interaction with methionine [13]. Therefore its recovery is accomplished only by significantly decreasing the ammonium sulfate concentration in the elution step.

Selective isolation of sc pDNA from a complex lysate

Considering the possibility of isolating pDNA isoforms and the different retention pattern of impurities, the main goal was to evaluate the ability of the methionine matrix to purify sc pDNA from a complex extract. Fig. 3 shows a representative chromatogram of the injection of 200 µl of a clarified lysate onto the L-methionine–agarose column. First, the column was equilibrated with 2.35 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl (pH 8.0) and the experiments were performed at 5 °C since this was the best temperature found in previous assays. After the injection of the pDNA-containing lysate a first peak corresponding to the unbound material was obtained. Then, the ionic strength was decreased to 1.7 M $(NH_4)_2SO_4$ and to 10 mM Tris–HCl, pH 8.0, to elute the more retained species.

The chromatographic peaks were collected and then analyzed through agarose gel electrophoresis. Through this analysis it was possible to observe that peak 1 corresponded to the elution of species not interacting with the column, namely the oc isoform (Fig. 3, lane 1), while sc pDNA remained bound under the same conditions, being eluted only in peak 2 (lane 2), after decreasing the ionic strength of the buffer. Instead, and as expected, RNA showed to be the most retained species, being eluted when the salt concentration was drastically decreased, in peak 3 (lane 3). Therefore, using these conditions, it was possible to isolate sc pDNA in the intermediate elution step.

According to what was described above, the differential interaction between the L-methionine matrix and the nucleic acids is due to the different degrees of base exposure [7]. This justifies that species such as oc and gDNA are eluted first since they did not interact with the methionine column, and species such as RNA and sc pDNA are eluted only after a salt concentration decrease [13]. In all the different affinity matrices tested until now it is possible to establish a specific and stronger interaction of the sc isoform mainly because of the higher base exposure compared with the oc isoform [6]. Indeed, the sc pDNA conformation results from the supercoiling phenomenon, which is a consequence of deformations induced by the torsional strain, leading to a more compact molecule with higher base exposure than the oc isoform [8].

Also, plasmids with different molecular weights were studied to better understand the range of applicability of the L-methionine-agarose. Fig. 4A shows a representative chromatogram of the injection of 200 µl of a clarified lysate from the HPV-16 E6/E7 plasmid onto the column. A stepwise gradient of 2.3 and 1.7 M ammonium sulfate in 10 mM Tris–HCl (pH 8.0) buffer, followed by 10 mM Tris–HCl, pH 8.0, without salt was used.

Fig. 4B presents the sc isoform isolation from the clarified lysate (injection of 200 µl) of the small plasmid pUC19. In this case, the column was equilibrated with 2.35 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl (pH 8.0) at 5 °C, at which a first peak was eluted. Then, a second peak was achieved with 1.7 M $(NH_4)_2SO_4$ in 10 mM

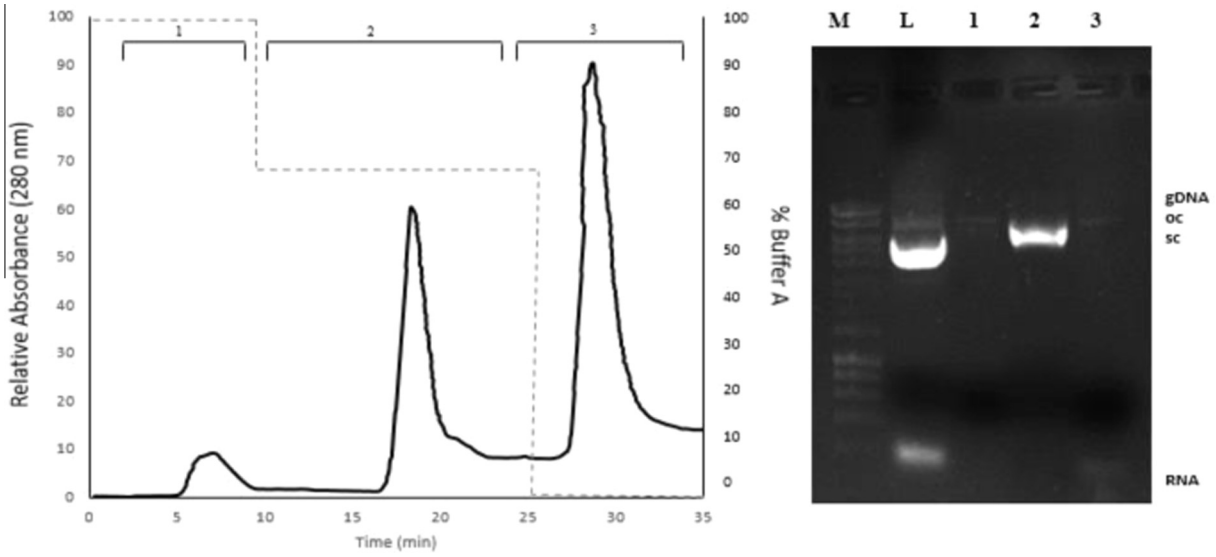

Fig.3. Chromatographic profile of the purification of sc pDNA from an *E. coli* lysate in an L-methionine–agarose column, at 5 °C. The various species were eluted from the column by a decreasing stepwise gradient of ammonium sulfate, as presented by the dashed line in the chromatogram. In the agarose-gel electrophoresis the lane M represents the molecular weight marker, lane L represents the lysate, and lanes 1, 2, and 3 represent the first, second, and third peaks of the chromatogram, respectively.

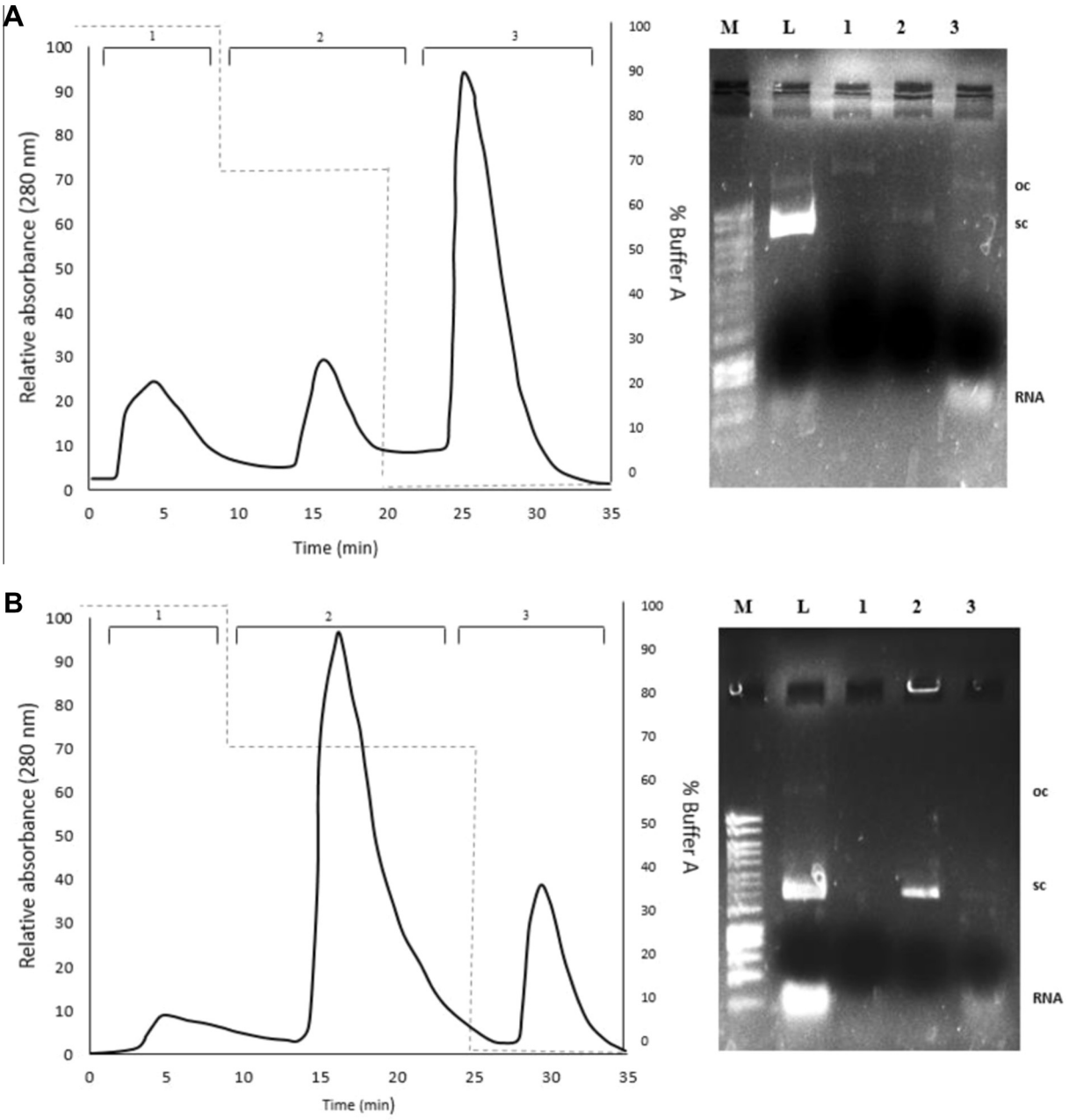

Fig.4. Chromatographic profile of the purification of sc pDNA from an *E. coli* lysate using the L-methionine agarose column, at 5 °C, of different plasmids: (A) HPV-16 E6/E7 and (B) pUC19. The different species were eluted from the column by a decreasing stepwise gradient of ammonium sulfate, as is represented by the dashed line in each chromatogram. In the agarose-gel electrophoresis the lane M represents the molecular weight marker, lane L represents the lysate, and lanes 1, 2, and 3 represent the first, second, and third peaks of the respective chromatogram.

Tris–HCl, pH 8.0, and finally a Tris–HCl (pH 8.0) elution step was applied to elute the retained species.

Similar to the purification results of the p53-encoding pDNA, also in HPV-16 E6/E7 and pUC19 plasmid assays, the sc pDNA isoform was eluted in the second peak obtained with 1.7 M ammonium sulfate, as is visible from the electrophoretic analysis of Fig. 4A and B.

From these results it was possible to observe that for the high-molecular-weight plasmid (HPV-16 E6/E7) a slightly lower concentration of ammonium sulfate was used to achieve the separation of oc and sc plasmid isoforms, compared with the salt concentration used for pUC19 and p53-encoding pDNA vectors. These results demonstrate that L-methionine–agarose can be an option for HPV-16 E6/E7 plasmid purification, since previous studies performed by our research group showed its purification from the other species of the lysate using an arginine-modified monolithic support and applying a stepwise gradient of increased NaCl concentration [31]. Finally, considering the efficient isolation of the sc pUC19 isoform with this strategy, the applicability of the L-methionine-based support for the purification of plasmids with lower molecular weight was also proven. The column was equilibrated with 2.3 M (NH4)2SO4 in 10 mM Tris–HCl (pH 8.0) at 5 °C and a first peak of unbound material was obtained under these conditions.

Plasmid purity assessment

The agarose gel electrophoresis of fractions pooled from the chromatogram representative of the purification of sc pDNA indicates that the sc pDNA eluted in peak 2 appears to be 100%

pure, RNA not being detectable. However, to ensure this result and also to accomplish the quality parameters established by the regulatory agencies, further characterization of the recovered sc pDNA is required.

The purity and quantification of pDNA in the sc pDNA peak resulting from the purification assay were performed in accordance with an analytical method previously described [22]. The percentage of purity assessed was over 97% of the sc pDNA isoform, which is in agreement with the previous results obtained with agarose gel electrophoresis. Through these analytical experiments and using a calibration curve made with a prepurified pDNA sample it was also possible to calculate the amount of sc pDNA recovered from the L-methionine experiment. The sc pDNA concentration was 39 μg/ml, with a standard deviation of 8% with $n = 3$. By comparing this result with the pDNA recovery achieved with other amino acid-based affinity strategies, namely with lysine matrix (45.5%) or arginine matrix (79%), it was verified that the application of the methionine matrix resulted in a lower pDNA recovery. However, when this result is compared with a monolith functionalized with arginine ligands, the recovery yield is very similar, since the monolith allowed a plasmid recovery of about 39%. This recovery yield of the sc p53-encoding plasmid achieved with the L-methionine–agarose could be due to plasmid characteristics and also some manipulation during the recuperation, concentration, and clarification steps. With regard to purity, the complete purification of sc pDNA was verified, using either the methionine matrix or other affinity chromatographic processes using amino acids as ligands [7,19,31].

Also, the oc peak was tested to calculate the recovery yield, and through these results it was possible to observe that the percentage of oc recovery yield was almost 2%, with a standard deviation of 1% at $n = 3$. Considering the potential therapeutic application of pDNA, the most important result is the recovery of the sc plasmid isoform, since it is already known that this is the biologically active conformation.

It is also important to note that the sc recovery yield of pUC-19 and HPV-16 E6/E7 plasmids was also evaluated and the results revealed a recovery yield of 20 and 30%, respectively. These recovery yields of the sc p53-encoding plasmid, pUC-19, and HPV-16 E6/E7 achieved with the L-methionine–agarose could be due to plasmid characteristics and also to some manipulation during the recuperation, concentration, and clarification steps.

The residual amounts of proteins, gDNA, and endotoxins also need to be quantified to ensure that these values are in accordance with specifications of the regulatory agencies. To accomplish that, the sc pDNA pool was first analyzed in terms of the presence of contaminating proteins through micro-BCA assay. The results (Table 1) indicated that this new chromatographic method enables the recovery of sc pDNA free of proteins. Moreover, real-time PCR was performed to quantify the gDNA present in the sc pDNA peak. This quantification is extremely important since some studies demonstrated that the existence of this species in a purified plasmid sample for clinical application was directly related with necrosis of the muscle cells [32]. Taking this into account, the gDNA quantification demonstrated that the amount of gDNA in the sc peak was undetectable, revealing that this impurity was eliminated during the purification process (Table 1). Finally, the amount of endotoxins in the clarified lysate and in the sc pDNA recovered peak was assessed through a LAL endotoxin kit. These results demonstrated that the amount of endotoxins in the sc peak was 10 times lower than in the clarified lysate sample. In fact, it was possible to confirm the previous analysis reporting the high purity achieved in the sc-pDNA fraction recovered from the methionine–affinity chromatographic matrix.

Table 1
Quantitative analysis of proteins, endotoxins, and gDNA in the purified sc fraction.

Sample	Protein (μg/ml)	Endotoxin (EU/μg pDNA)	gDNA (μg/ml)
Lysate	5.75 ± 1.6	31 ± 2	700 ± 43
sc	Undetectable	3 ± 0.3	Undetectable

Data are shown ±SD, $n = 3$.

Conclusions

The L-methionine–agarose matrix was successfully used for the first time to efficiently purify the sc p53-encoding plasmid from a clarified lysate. Moreover, it was also proven that a methionine-based matrix can be successfully applied for the purification of the sc isoform of various plasmids, with different sizes and base compositions. Through this matrix it was possible to observe the selectivity achieved with the sc pDNA isoform present in a prepurified pDNA sample (oc and sc), by combining a decreasing stepwise gradient of ammonium sulfate with low temperature. Afterward, specific interactions were explored with several isolated nucleic acids (RNA, gDNA, and pDNA) by using the same elution strategy, and different retention patterns were observed. Further studies using the sc pDNA sample recovered from the purification step demonstrated that the sc plasmid was obtained with a purity above 97%. And finally, quality control tests performed with the methodologies recommended by the regulatory agencies showed that the levels of impurities (gDNA, RNA, proteins, and endotoxins) were reduced or in some cases undetectable compared with the initial clarified lysate sample.

Through this study it was possible to increase the knowledge in the pDNA purification field by establishing a new method able to promote a selective purification of the sc p53-encoding plasmid from a lysate using an L-methionine–agarose matrix.

Acknowledgments

The authors thank Dr. Thomas Roberts for providing the pcDNA3–FLAG–p53 construct through Addgene, Reference 10838; Dr. Munger for the construction of HPV-16 E6/E7 through Addgene, Reference 8641; and Patricia Pereira for her help in the real-time PCR study. This work was supported by FCT, the Portuguese Foundation for Science and Technology, through Project PTDC/EBB-BIO/114320/2009, and PEst-C/SAU/UI0709/2011 COMPETE. J.F.A. Valente and A. Sousa also acknowledge Ph.D. and postdoctoral fellowships (SFRH/BD/96809/2013 and SFRH/BPD/79106/2011, respectively).

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Paper IV

DoE to improve supercoiled p53-pDNA purification by O-phospho-L-tyrosine affinity chromatography

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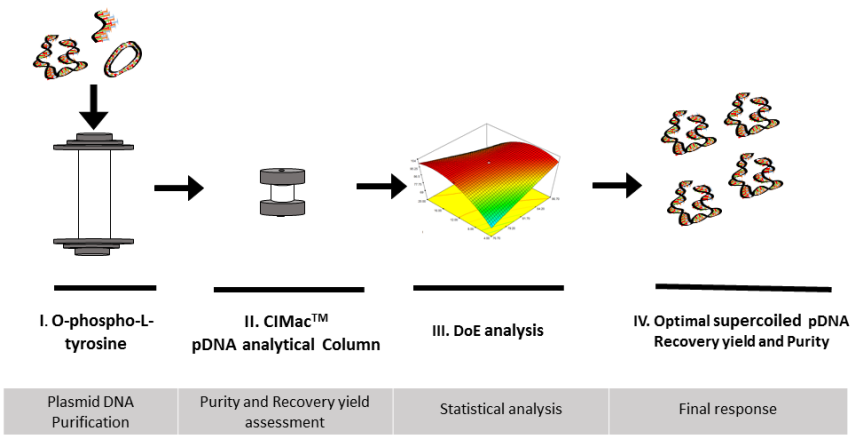
Abstract

P53 is implicated in various cellular functions and several studies have shown that transfection of cancer cells with wild-type p53-expressing plasmids could directly drive cells into growth arrest and/or apoptosis. In the present work, the 6.07 kbp pcDNA3-FLAG-p53 plasmid, which encodes the p53 tumor suppressor, was produced and recovered from a recombinant cell culture of *Escherichia coli* DH5α. Following plasmid biosynthesis, the O-Phospho-L-tyrosine chromatographic matrix was explored to purify the supercoiled p53-encoding plasmid. In order to quickly determine the optimal chromatographic performance and to obtain the required purity degree, maximizing the recovery yield of the supercoiled plasmid DNA, the Composite Central Face design was applied. The model revealed to be statistically significant (p-value < 0.05), with coefficient of determination of 0.9344 for the recovery yield and 0.9581 for purity and the central point was successful validated. After the chromatographic process optimization by using the design of experiments tool, 49.7% of the supercoiled p53-encoding plasmid was recovered with 98.2% of purity, when a decreasing ammonium sulphate gradient was applied. Finally, the purified sample was analyzed to assess the con-tent of endotoxins, proteins and genomic DNA, showing that all these impurity levels were below the recommendations of the regulatory agencies.

Graphical abstract



Keywords: Affinity chromatography; Composite Central Face design; Design of Experiments; O-Phospho-L-tyrosine support; Supercoiled p53-encoding plasmid.

Highlights

-TP53 gene is one of the most frequently mutated genes in human cancers;

-O-Phospho-L-tyrosine resin was used to purify the supercoiled p53-encoding plasmid;

-CCF design was successfully applied to optimize the chromatographic performance.

Introduction

The TP53 gene, which encodes p53, is one of the most frequently mutated genes in human cancers. It is reported that approximately half of all cancers have the p53 inactivated. This tumor suppressor is implicated in a number of known cellular functions, including the DNA damage repair, the cell cycle arrest in G1/S and apoptosis [1]. Several studies have shown that cells transfection with wild-type p53-expressing plasmids could directly drive cells into apoptosis and/or growth arrest when p53 is overexpressed, suggesting that the gene therapy approach for cancer treatment can re-establish the normal p53 function [2].

Recent advances have led to many different strategies to p53-targeted cancer therapy, including TP53 gene therapy, p53 vaccines, and rescue of mutant p53 function by small-molecule inhibitors. The cases of TP53 gene therapy and p53 vaccines have been studied extensively in patients with solid cancers [3-5]. In fact, several preclinical studies have shown that adenoviral vectors engineered with a complementary DNA copy of the p53 gene trigger a dramatic tumor regression response in cancers, such as head and neck cancer [6], lung cancer [7], colorectal cancer [8], among others [9].

For gene therapy purposes, the use of plasmids to replace or correct a defective gene has been evaluated. Plasmid DNA (pDNA) is the most used non-biologic vector in DNA-based therapies, due to its simplicity, low-cost manufacture, acceptable expression levels and lack of immunogenicity [10, 11]. Amongst the different conformations that pDNA can acquire, it has been proved that the supercoiled isoform (sc) is more advantageous as it is more effective for gene transfection [12, 13]. Also, in accordance with regulatory agencies, such as Food and Drug Administration (FDA) or the European Medicines Agency (EMA), pDNA should accomplish several specifications to guarantee its purity and global quality, in order to be suitable for therapeutic applications [14, 15].

To isolate and purify the sc pDNA vector, several chromatographic methodologies have been used, namely size exclusion, anion exchange, hydrophobic interaction, reversed phase, thiophilic adsorption and affinity chromatography [14]. In particular, the affinity chromatography has already been explored to successfully isolate the sc p53 encoding plasmid by using matrices modified with arginine and methionine ligands. In these studies, the arginine support allowed the recovery yield of sc pDNA with 95% of purity, while 97% was obtained with the methionine matrix [12, 16]. However, the recovery yield is usually low-to-moderate and the methodology commonly used to optimize the ideal elution conditions is based on trial and error approaches, which implies a huge number of experiments until the suitable conditions for the sc pDNA isolation are reached. Regarding this, it is still essential to develop and study new matrices and methods to accomplish the sc pDNA purification with higher specificity, recovery

yield, robustness, in a more cost-effective manner to be considered in biopharmaceutical industry.

In the present work, it was explored the O-Phospho-L-tyrosine chromatographic matrix to purify the sc p53-encoding pDNA. This affinity matrix was already studied in our research group for RNA isolation, showing an interesting potential for the purification of nucleic acids [17] To accomplish this aim, the Composite Central Face design (CCF) was applied to optimize the chromatographic process regarding the maximization of pDNA purity and recovery yield. The design of experiments (DoE) tool enables to systematically and simultaneously vary several parameters to obtain more information about the purification process with few experiments. Thus, we are able to faster reach the best purification conditions, to accomplish the final result, which allows a reduction of the costs associated to the random experiment approach in the search for the suitable isolation conditions.

Experimental section

Materials

O-phospho-L-tyrosine agarose matrix was obtained from Sigma Aldrich (St. Louis, M.O., USA) and the NZY MaxiPrep Kit was purchased from NZYTech (Lisbon, Portugal). All the water used to prepare solutions was ultra-pure grade, purified with a Milli-Q system from Millipore. Ammonium sulphate $(NH_4)_2SO_4$ was purchased to VWR and tris(hydroxymethyl) aminomethane (Tris) was from Merck (Darmstadt, Germany). Binding and elution buffers were filtered through a 0.20 µm pore size membrane (Schleicher Schuell, Dassel Germany) and degassed ultrasonically. The 6.07 kbp pcDNA3-FLAG-p53 Addgene plasmid 10838 [18] was purchased to Addgene, and all the reagents used in bacterial growth were obtained from Sigma-Aldrich. The DNA ladder was obtained from Bioline (London, UK).

Methods

Plasmid and bacterial growth conditions

The pcDNA3-FLAG-p53 plasmid was amplified in a cell culture of *Escherichia coli* (*E. coli* DH5α) harbouring this plasmid. The growth was carried out at 37 ºC, 250 rpm, in an Erlenmeyer with 500 mL of Terrific Broth medium (20 g L^{-1} of tryptone, 24 g L^{-1} of yeast extract, 4 mL L^{-1} of glycerol, 0.017 M KH_2HPO_4, 0.072 M K_2HPO_4) supplemented with 30 µg mL^{-1} of ampicillin. The

cells were grown until the log phase (O.D. 600 nm ± 9) and then were collected by centrifugation and stored at -20 ºC.

Lysis and plasmid isolation

In order to study a complex lysate sample, after cells recovery, they were lysed through a modified alkaline method previously described. Completed the lysis procedure, and expecting the elimination of some impurities, the pDNA was first precipitated with isopropanol and then, an ammonium sulphate precipitation step was performed to eliminate proteins and RNA [15, 19].

Preparative chromatography

Chromatographic assays were performed in an ÄKTA purifier system with UNICORN 5.11 software (GE Healthcare, Uppsala, Sweden). A 16×40 mm (approximately 8 mL) column was packed with a commercial O-Phospho-L-tyrosine agarose gel (Sigma Aldrich). This matrix is characterized by the manufacturer as a crosslinked 4% beaded agarose support with a one-atom spacer and an extent of labelling between 5-15 μmol/mL. The experiments were performed by using a circulating water bath to control the temperature. After the matrix equilibration, the lysate was loaded onto the column using a 100 μL loop, at 1mL/min. The absorbance of the eluate was continuously monitored at 260 nm. The column was equilibrated with different concentrations of ammonium sulphate depending on the experiment. The elution of bound species was carried out by using a decreasing ammonium sulphate stepwise gradient.

Design of experiments

To optimize the sc pDNA purity and also maximize its recovery yield, a CCF design was applied. Concerning that, three factors (inputs) were taken into account, namely i) the sc binding step: to efficiently bind the sc pDNA to the column and at the same time eliminate some of the impurities; ii) the sc elution step: to elute most of sc pDNA with the required purity degree; iii) the Temperature. The inputs were studied at three levels (-1; 0; +1) and the range was defined according to preliminary results obtained from the random experiments approach. The responses (outputs) evaluated were the % purity, that should be higher than 97% of sc pDNA and the % recovery yield, that should be maximized maintaining the required purity.

To perform the chromatographic experiments, the column was equilibrated within the binding concentration range of 2.3 M to 2.6 M ($(NH_4)_2SO_4$ in 10 mM Tris–HCl buffer pH 8, the elution step was performed with lower ionic strength within the range of 1.8 M to 2.1 M ($(NH_4)_2SO_4$ in 10 mM Tris–HCl buffer pH 8. A last step consisting on the application of 10 mM of Tris–HCl buffer pH 8 was always performed to guarantee the total elution of the remaining bound species. The Temperature of the different chromatographic experiments was changed in the range between

4 and 20 ºC. Each peak was pooled and concentrated to a final volume of 500 µL. Statistical analysis was performed through the use of UNICORN™ 6.1 software and Design Expert version 9 trial software. The generalized second-order polynomial model equation used in the response surface analysis is presented below (Eq. (1)):

$$\mathbf{Y} = \beta_1 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + X_{23} X_2 X_3 \quad (1)$$

Plasmid Quantification

A modified quantification method using a CIMAc™ pDNA analytical column was applied to determine the sc pDNA purity and recovery yield [20, 21]. The calibration curve was constructed in the range of 1–25 µg/mL, by considering the peak area corresponding to sc pDNA standards. The recovery yield was calculated by the ratio between the obtained sc pDNA concentration and the sc pDNA concentration present in the lysate sample, while purity was calculated by the ratio between sc pDNA peak area and total peak area present in the analytic chromatogram.

Protein quantification

For protein quantification, the micro-BCA (Bicinchoninic acid) protein assay kit from Pierce (Rockford, USA) was used in accordance to the supplier's specifications. A calibration curve with the standard protein Bovine Serum Albumin (BSA) (0.01-0.1 mg/mL) diluted in 10 mM of Tris-HCl pH 8.0 was determined. Before analysis, samples were desalted with Tris-HCl pH 8.0 to avoid salt interference.

Genomic DNA quantification

Real-time polymerase chain reaction (PCR) was used to evaluate the level of genomic DNA (gDNA) in the pDNA purified samples. The analysis was performed in an iQ5 Multicolor real-time PCR Detection System (Bio-Rad), as previously described [19]. Sense (5'-ACACGGTCCAGAACTCCTACG-3') and antisense (5'-CCGGTGCTTCTTCTGCGGGTAACGTCA-3') primers were used to amplify a 181-bp fragment of the 16S rRNA gene. For the gDNA quantification in lysate samples, a 100-fold dilution was prepared. PCR amplicons were quantified by following the change in fluorescence of the DNA binding dye Syber Green (Bio-Rad). The concentration of gDNA was obtained through a calibration curve generated by serial dilutions of purified *E. coli* DH5α gDNA in the 5 pg to 50 ng/µL range. Negative controls (no template) were run at the same time of each analysis.

Endotoxin quantification

The ToxiSensor™ Chromogenic Limulus Amoebocyte Lysate (LAL) endotoxin assay kit (GenScript, USA, Inc.) was used for endotoxins measurement, considering the manufacturer's instructions. The calibration curve (from 0.005 to 0.1 EU/mL) was performed using a provided stock solution of 8 EU/mL. In order to avoid the external endotoxin interference, all the samples were diluted or dissolved in non-pyrogenic water, which was also used as the blank. All the tubes and tips used to perform this analysis were endotoxins-free. All the procedure was performed inside of a laminar flow cabinet to assure aseptic conditions.

Results and Discussion

Affinity chromatography has been widely used in the supercoiled pDNA recovery and purification, since this technique allows the reduction of the number of chromatographic steps, increasing yields and improving process economics mainly due to the specificity and selectivity achieved by the ligands [22]. Basically, affinity chromatography mimetizes biologically occurring phenomena, such as the interactions established between nucleic acids and protein domains, involving particular amino acids, that regulate several cellular mechanisms [23]. In this regard, and as it was previously described in different studies, the use of amino acids as chromatographic ligands results in the establishment of several interactions with nucleic acids, increasing the biorecognition of the target and promoting the sc pDNA isolation, thus eliminating other pDNA isoforms and major contaminants [24].

In this work, tyrosine amino acid was selected as specific ligand to isolate the sc p53-encoding pDNA isoform from a complex lysate. To accomplish this, different conditions, such as buffer composition, pH and Temperature were tested in a random experiment approach to define the first binding/elution settings. Thus, pDNA retention was first evaluated in low ionic strength conditions, but the loaded sample immediately eluted during the flowthrough (data not shown). Conversely, total pDNA retention was achieved by using ammonium sulphate in the binding buffer and its elution was verified by decreasing the salt concentration. This behaviour suggests that hydrophobic interactions between pDNA and O-Phospho-L-tyrosine are favoured, mainly due to the existence of a benzyl aromatic ring in the ligand [25].

Temperature was also a parameter under evaluation, being verified that it definitely plays an important role in sc pDNA isolation in the O-Phospho-L-tyrosine agarose matrix (Figure 1). Concerning this, a lysate sample made of oc and sc pDNA and also RNA was injected in the matrix and, the results have shown a decrease on pDNA binding with the temperature increase, also indicating that the pDNA binding is not exclusively due to hydrophobic interactions, which

typically are favoured by increasing temperature. In fact, the biorecognition results from the establishment and conjugation of multiple non-covalent elementary interactions, such as hydrogen bonds, Van der Waals contacts and hydrophobic interactions [16]. The last peak of the chromatogram is supposed to be mostly made of RNA since this specie strongly binds to the matrix.

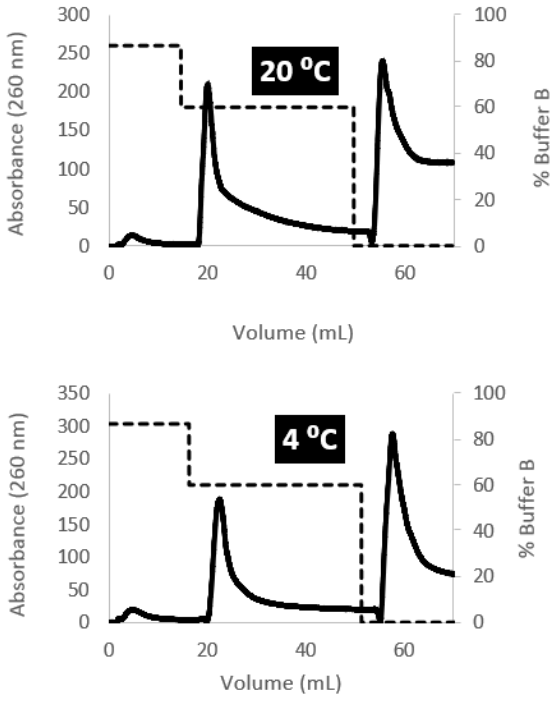


Figure 1 - Chromatographic profiles of the sc p53 encoding plasmid loaded onto the O-Phospho-L-tyrosine agarose column at 4 and 20 ºC. The elution was performed by a stepwise gradient of 2.6 M of $(NH_4)_2SO_4$ in 10 mM Tris-HCl pH 8.0, then 1.8 M of $(NH_4)_2SO_4$ in in the same buffer and finally 10 mM Tris-HCl pH 8.0, as represented by the dashed line.

Design of experiments

The DoE model used in this work was CCF, because it is more inclusive and does not consider points outside of the ranges established for the inputs (star points) [17]. Considering the aim of this work and expecting a pDNA quality in accordance with the regulatory agencies criteria, the responses (outputs) chosen were the % of pDNA purity, that should be higher than 97%, and simultaneously, the maximization of the % of recovery yield.

According to the preliminary studies, a chromatographic method was outlined by considering three steps. First, it was established a sc pDNA binding step, ranging between 2.3 M and 2.6 M

of $(NH_4)_2SO_4$, that intends to efficiently bind this target molecule to the column and at the same time to eliminate some of the impurities in the flowthrough. Secondly, the sc elution step, ranged between 1.8 M and 2.1 M of $(NH_4)_2SO_4$, was performed to elute most of sc pDNA with the required purity degree. Finally, the last step at 10 mM of Tris buffer pH 8 was used as a washing step to elute the remaining bound impurities. Given that, the outputs are the sc pDNA purity and recovery yield, the binding step and sc elution step were chosen as inputs. In addition, it was also included the Temperature as an input (ranging between 4 and 20 ºC), as the initial results indicated a significant influence of this parameter on pDNA retention and selectivity. The different experiments designed by UNICORNTM 6.1 software and the respective responses are available in supporting information (SI). The three central points are presented in grey (Table in SI), which were tested in the same conditions in order to access the model reproducibility. The regression equations (2) and (3) are resultant from the Design of Expert 9.0 and provide the level of responses for recovery yield and purity as a function of the different variables chosen ("sc binding step", "sc elution step" and "Temperature"). The signal of each factor designates a positive or negative effect on the response.

$$\textbf{Recovery yield=} + 40.53 + 13.63 \times A - 4.33 \times B - 3.4 \times C + 0.89 \times AB + 0.49 \times AC - 4.04 \times BC + 4.07 \times A^2 - 11.83 \times B^2 + 0.52 \times C^2 \quad (2)$$

$$\textbf{Purity =} + 98.58 + 1.96 \times A - 1.56 \times B + 5.68 \times C - 0.69 \times AB - 3.56 \times AC + 1.59 \times BC + 1.56 \times A^2 + 2.06 \times B^2 - 9.24 \times C^2 \quad (3)$$

In Table 1 it is possible to observe the information extracted from the multiple regression equation, corresponding to the main effects of the chosen parameters.

Table 1. Summary of the multiple regression equation (main effects of the factors).

Parameter	Recovery effect	Purity effect
sc binding **(A)**	Positive	Positive
sc Elution **(B)**	Negative	Negative
Temperature **(C)**	Negative	Positive
sc binding × sc elution	Positive	Negative
sc binding × Temperature	Positive	Negative
sc elution × Temperature	Negative	Positive
sc binding^2	Positive	Positive
sc elution^2	Negative	Positive
Temperature^2	Positive	Negative

From these results, it is possible to verify that the "sc binding step" condition is positive for both responses, which can indicate that higher ammonium sulphate concentrations intensify interactions established with the sc pDNA, strengthening its retention on the matrix. Thus, the sc pDNA recovery yield and purity could be increased in the subsequent elution step. Concerning the "sc elution step", it is possible to verify negative responses either for recovery yield as well as for purity. This mean that if low ammonium sulphate concentrations were applied the matrix will retain not only the sc pDNA but also impurities and the opposite could also happen (when high concentrations of ammonium sulphate were applied the elution of the sc and impurities will be promoted). Which means that high recovery and purity levels are only obtained when a very specific salt concentration is applied, being not allowed large variations.

On the other hand, the input "Temperature" present a different contribution for each response (negative for recovery yield and positive for purity). Actually, in accordance with what was previously observed in preliminary experiments, the decrease of Temperature seems to intensify the interactions between the ligand and the nucleic acids, which are only eluted in the washing step, favouring the purity but also decreasing the recovery yield of the sc pDNA in the elution step. These results show the influence of the salt concentration and Temperature in the purity and the recovery yield of sc pDNA in the elution step, being fundamental to determine the optimal point by a combination of these factors through experimental design tool, aiming to maximize the intended responses.

To evaluate the presence of potential outliers the Cooks Distance analysis was performed concluding their inexistence [26]. Moreover, the evaluation of the significance of the model was based on the analysis of the variance (ANOVA), as it is presented in Table 2. Looking closely to this analysis, it is confirmed that the model data are statistically significant for both responses, since the probability value (p-value) was always lower than 0.05 for purity and recovery yield [27, 28]. Also, for recovery yield, the "Model F-value" was 12.95, with only 0.14

% of chance of it occur due to the noise and, in case of purity, the “Model F-value” was 17.78 having 0.05 % of chance to occur due to the noise.

Table 2. ANOVA table for CCF design model.

Response	Degrees of freedom	Sum of squares	Mean of squares	*F*-value	*P*-value	R^2	Adj R^2	Adeq Precision	Q^2	P- value (prob >F)	Lack of Fit
Recovery	9	2724.25	302.69	12.95	0.0014	0.9344	0.871	13.949	0.784	0.8721	0.31
Purity	9	761.76	84.64	17.78	0.0005	0.9581	0.904	13.684	0.7	0.0594	16.12

The Lack of Fit is responsible to describe if the model is appropriate to correctly express data or if a more complex model should be applied. To achieve this parameter, a comparison between variability of the current model residuals and the variability between observations and replicative settings of the factors is used [29]. The "Lack of Fit F-value" to the output Recovery yield was 0.31, implying that the Lack of Fit is not significant relatively to the pure error, and there is 87.21 % of chance that a "Lack of Fit F-value" this large could occur due to noise. This Lack of fit showed to be non-significant which is a positive result since we want that the model fits. In case of the output Purity, the "Lack of Fit F-value" was 16.12, which implies 5.94 % of chance for this large "Lack of Fit F-Value" occur due to the noise. Also, in this case, this parameter showed to be non-significant.

The coefficient of determination (R^2) shows if data points fit the statistical model, indicating the high significance of the model. Although, being acceptable a value ranging between 0 and 1, ideally it should be closer to 1 [28]. In this case, the R^2 were 0.9344 and 0.9581 for recovery yield and purity respectively, suggesting that both responses fit the models. Adjusted R^2 (Adj R^2) comprises the variation of the ordinary R^2 and is adjusted for the "size" of the model (the number of the factors) [17]. The results obtained from the ANOVA analysis present good adjusted R^2 for both responses.

Moreover, the usefulness of the model to predict new data is associated with positive predicted values variation (Q^2), while negative Q^2 is related to models that do not have a predicted power [30]. The Q^2 data for purity (0.7) and for recovery yield (0.7837) responses presented positive values, suggesting that the model can predict new data. However, the value of Q^2 for purity was lower than the value of Q^2 for recovery yield, indicating that the prediction will be better for the recovery yield response. This result does not demerit the present work, since it is intended to maximize the sc pDNA recovery yield, maintaining the pDNA purity higher than 97%. Actually, this affinity chromatographic methodology based on amino acids as specific ligands already proved to be efficient on sc pDNA purification, while the major drawback is usually the recovery yield. For example, in a previous purification strategy of sc p53-encoding pDNA by using the methionine matrix, it was only possible to achieve a recovery yield of about 8% [16]. So, the present results indicate that the use of DoE tool can help in the definition of the best experimental conditions, to improve the recovery yield and purification of pDNA. Finally, the "Adequate Precision" is the parameter responsible by the measure of the signal to noise ratio, being desirable a value greater than 4. The results obtained in this field were 13.949 and 13.684 for the recovery yield and purity respectively, suggesting that both models can be used to navigate the design space.

Three-dimensional response surface plots

The surface and contour plots show the three-dimensional representation of the multiple regression equations [29]. By the analysis of plots illustrated in Figure 2, it is easily observed the relation between the chosen parameters (sc binding step, sc elution step and Temperature) and the outputs (recovery yield and purity). The different colours intensity represents the range for optimal points. Thus, blue represents the lowest response followed by green, yellow and finally red, which represents the largest interaction. At the same time, the ellipticity obtained in the plots is indicative of the interaction order that occurs between the chosen variables. In general, by analysing the colour and ellipticity, it is visible that Temperature has more influence in the purity response (Figure 3) than in the recovery yield response (Figure 2). Also, through the ellipticity and colour of the recovery yield contour plots, it is possible to verify that the interaction between sc elution step versus sc binding step (Figure 2A) or Temperature versus sc binding step (Figure 2B) is more pronounced than in the case of Temperature versus sc elution step (Figure 2 C). These results indicate that Temperature has a less pronounced effect on the sc elution and may influence in a more effective way its binding to the matrix. In purity plots (Figure 3), it is depicted that all the variables chosen for the design have a crucial role, being red the most observed colour, indicating a very expressive interaction between sc binding step versus sc elution step (Figure 3 A). In fact, to maximize the sc pDNA purity it should be found a compromise between the salt concentration of the sc binding step and the sc elution step.

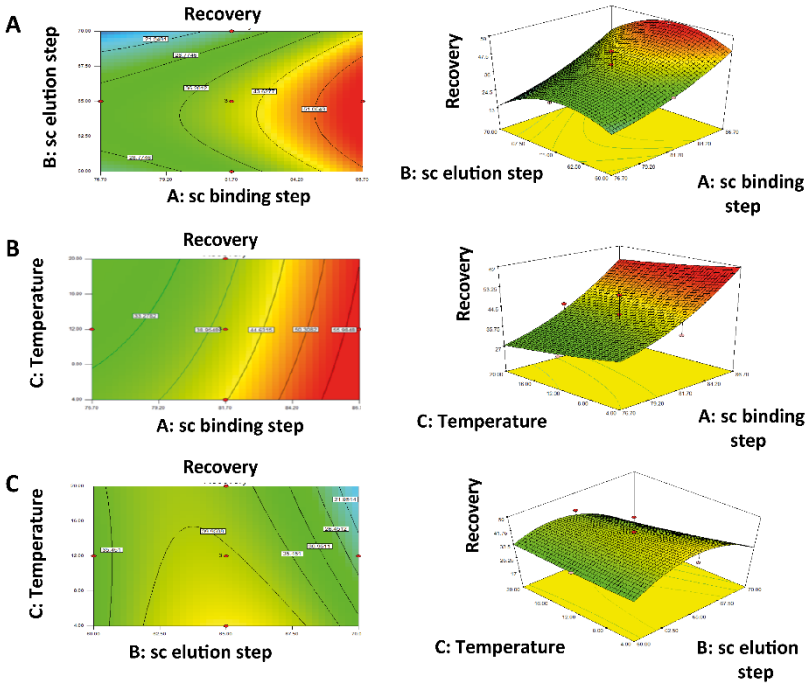


Figure 2 - Contour and three-dimensional response surface plot of the interaction of the different variables (binding sc; elution sc; Temperature) and its effect on % of recovery yield.

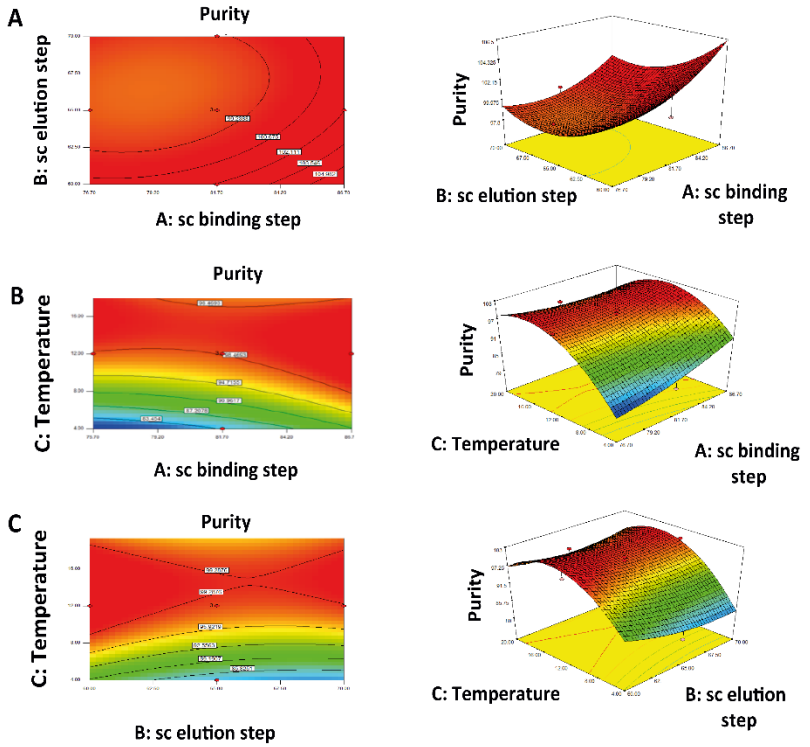


Figure 3 - Contour and three-dimensional response surface plot of the interaction of the different variables (binding sc; elution sc; Temperature) and its effect on % of purity.

Model validation

After the analysis of the responses given by the experiments provided by DoE, it was predicted the optimal point, consisting in the best conditions to achieve the global aim of this study, which is the sc p53-encoding pDNA preparation with a purity degree higher than 97% and maximizing the recovery yield. So, the best conditions, as well as the obtained responses, are presented in Table 3. Therefore, the optimal conditions suggested by the software (2.58 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer pH 8, for the sc binding step, 1.92 M of $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer pH 8 for the sc elution step and 9.5 ºC for the Temperature) were used to perform the experiment in triplicate. All chromatograms revealed the same profile, suggesting that the method was suitable and reproducible. Figure 4 shows one representative chromatogram from the triplicate assays that illustrate the total retention of the target molecule in the binding step, the selective isolation of sc p53-encoding plasmid in the second step (peak 2, lane 2) and finally the elution of impurities such as RNA (peak 3, lane 3) in the washing step at 10 mM Tris-HCl buffer pH 8. It is important to notice that the purification of sc pDNA was achieved from a complex lysate containing gDNA, oc isoform, sc isoform, RNA and also proteins and endotoxins. From the analysis of the electrophoretic profile of Figure 4 it is also visible that the sc pDNA is recovered in peak 2 with high purity level, but a small amount is also eluting in the final step, justifying the obtained recovery yield. The samples of peak 2 from triplicates were analysed by the CIMac pDNA analytic column and the mean value for purity was 98.2% with a standard deviation of 1.3 % and for recover was 49.7% with a standard deviation of 1.9 %.

Table 3. Optimal conditions selected by DoE to recover and purify sc p53-pDNA.

Predicted inputs	Value
Sc binding ([$(NH_4)_2SO_4$] in 10 mM Tris-HCl buffer, pH 8)	2.58 M
Sc elution ([$(NH_4)_2SO_4$] in 10 mM Tris-HCl buffer, pH 8)	1.92 M
Temperature	9.5 ºC
Predicted output	**Value**
Recovery	56.5%
Purity	100%

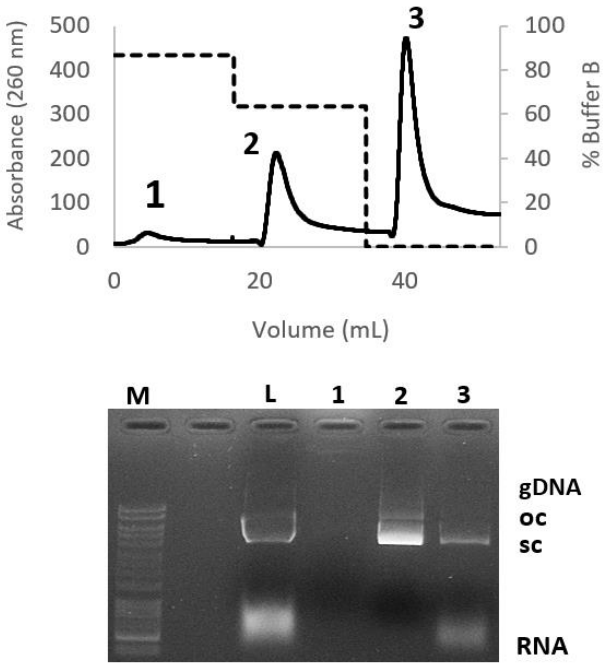


Figure 4 - Chromatographic profile of the sc pDNA purification from an *E. coli* lysate by using the O-Phospho-L-tyrosine agarose column, at 9.5 ºC. The different species were eluted from the column by a decreasing stepwise gradient of ammonium sulphate, as it is presented by the dashed line in the chromatogram. In the agarose gel electrophoresis, the lane M represents the molecular weight marker; lane L represents the lysate sample; and lanes 1, 2 and 3 represent the first, second and third peaks of the chromatogram, respectively.

In Table 4 is represented the confidence intervals (CI) for both responses analysed. Comparing these intervals with the obtained values for the recovery yield and also purity it is possible to conclude that they are between the predicted CI. The purity values obtained are in accordance with the recommendations of regulatory agencies and, comparing the recovery yield of sc p53-encoding pDNA with the values obtained with other matrices, like L-methionine with 8% of sc recovery yield, it is possible to conclude that the O-Phospho-L-tyrosine matrix was significantly more efficient [16]. These results suggest a huge potential of this matrix to be integrated into a downstream platform in order to obtain this biopharmaceutical product for further application in gene therapy studies.

Table 4. Confidence intervals for the two responses.

Response	95% CI low	95% CI high
Recovery	49.6	63.3
Purity	97.4	103.6

Based on the fact that sc pDNA preparations have proved to be more efficient for cells transfection than other isoforms and, considering that the efficient access of pDNA to the cell nucleus improves gene expression in eukaryotic cells, it is extremely important to guarantee that the final sample is enriched in sc pDNA [13].

However, besides the requirement of 97% of sc homogeneity, the regulatory agencies also indicate other specifications that must be accomplished by pDNA to be suitable for therapeutic applications. In particular, pDNA solution must be clear and colourless, the RNA and proteins content should not be detectable, the amount of gDNA needs to stay below 2 μg gDNA/mg of pDNA and also the level of endotoxins should not exceed the 10 EU/mg of pDNA [31]. In this way, the second peak of the replicate chromatograms containing sc pDNA was analysed regarding these impurities. The results (Table 5) indicated that this new chromatographic method enables the recovery of sc pDNA free from proteins. Moreover, real-rime PCR was performed in order to quantify the gDNA present in these samples. This assessment is extremely important because some studies demonstrated that the existence of gDNA in a purified plasmid sample for clinical application was directly related with necrosis of the muscle cells [32]. The gDNA quantification demonstrated that the amount of gDNA in the sc peak was below the limit given by the regulations (Table 5). Finally, the endotoxins content in the sc pDNA preparations was assessed through a LAL endotoxin kit, and once again the values were below the recommended limit given by the regulatory agencies

Table 5. Quantitative analysis of proteins, endotoxins, and gDNA in the purified sc fractions.

	Proteins (μg/mL)	Endotoxins (EU/ mg pDNA)	gDNA (μg gDNA/mg DNA)
sc elution peak	Undetectable	8.5	1.1

Conclusions

This research work used O-Phospho-L-tyrosine combined with CCF design in order to achieve the optimal conditions for the isolation of the sc p53-encoding plasmid. To perform that, three parameters were used and combined ("binding sc", "elution sc" and "Temperature") and finally the percentage of recovery yield and also purity was evaluated as responses. It was possible to see during the experiments the interactions of all inputs and their influence on the final outputs. The model revealed to be statistically significant (p-value < 0.05), with coefficient of determination of 0.9344 for recovery yield and 0.9581 for purity and the central point was successful validated. The central point was repeated three times being achieved 49.7% of recovery yield and 98.2% of purity. The quality of the resulting sc pDNA was assessed, being confirmed that the levels of impurities are in agreement with all the parameters required by the regulatory agencies such as EMA and FDA.

DoE demonstrated to be a very powerful and quick tool for the establishment of the optimal conditions for the sc p53-encoding plasmid purification. In addition, it was established a purification methodology based on the O-Phospho-L-tyrosine matrix that allows an improvement of the recovery yield of this plasmid, being a promising tool for the downstream process of this biopharmaceutical product.

Acknowledgments

The authors would like to thank Dr. Thomas Roberts for providing the pcDNA3–FLAG–p53 construct through Addgene, ref: 10838 and to Patricia Pereira for her help in the real-time PCR study. This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). J.F.A. Valente and A. Sousa also acknowledge PhD and Postdoctoral fellowships (Ref SFRH/BD/96809/2013 and Ref SFRH/BPD/102716/2014, respectively).

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Supporting information

Table S1. CCF design matrix and responses of the sc p53-pDNA in terms of recovery yield and purity.

Experiment	sc binding step (M)	sc elution step (M)	Temperature (ºC)	% Recovery	% Purity
1	2.3	1.8	4	26	85
2	2.6	1.8	4	50	98
3	2.6	2.1	4	52.6	90
4	2.3	1.8	20	23.6	98.9
5	2.6	1.8	20	51	98.4
6	2.3	2.1	20	6.5	100
7	2.6	2.1	20	36	96
8	2.3	1.95	12	29.9	99.3
9	2.6	1.95	12	56.3	99.4
10	2.45	1.8	12	32.9	100
11	2.45	2.1	12	21.5	99.7
12	2.45	1.95	12	41.4	100
13	2.45	1.95	12	49.8	100
14	2.3	2.1	4	23.6	79
15	2.45	1.95	4	39	80.8
16	2.45	1.95	12	36.4	98.9
17	2.45	1.95	20	40.1	96.3

*The three central points are presented in grey, which were tested in the same conditions in order to access the model reproducibility.

Paper V

Macroporosity in affinity chromatography: supercoiled p53 encoding plasmid isolation

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Abstract

p53 is a tumor suppressor gene that has been explored for cancer gene therapy as a possible alternative to the common treatments. The use of plasmid DNA (pDNA) to carry the therapeutic gene has been considered, but it is a requisite to preserve its supercoiled (sc) structure, for eliciting a more effective gene expression and therapeutic action. The purification of the sc pDNA using amino acids-based affinity chromatography has been successfully applied, exploring different amino acids and supports. From these studies, it stood out the selectivity of arginine for the recognition of sc pDNA. However, some limitation on the binding capacity was found in the arginine-agarose support, and in the case of monoliths, fouling and clogging could induce major problems on sequential runs. By using a macroporous support modified with arginine it was expected to take advantage of the selectivity of the ligand combined with the flow properties and binding capacity offered by the support. The arginine-macroporous support was characterized by SEM, EDX and FTIR also to verify the correct immobilization of arginine, and then used for pDNA purification. The support showed to be effective on the sc p53-pDNA isolation, and the robustness was also achieved by accomplishing the purification of plasmids with different sizes, only by slightly adjusting the experimental conditions. Regarding the dynamic binding capacity of the arginine-macroporous support, it was achieved an improvement of more than 50% in the pDNA binding capacity when compared with their homologous arginine-agarose commercial resin, suggesting a potential economic feasibility in case of scale-up.

Keywords: Affinity chromatography, arginine, biorecognition, p53-encoding plasmid, supercoiled isoform, macroporous beads.

Introduction

Tumorigenesis is a multistep process that results from the sequential accumulation of genetic and epigenetic modifications leading human normal cells to a progressive transformation into highly malignant derivatives. Usually, these changes make the tumour self-sufficient in relation to growth signals, promote insensitivity to antigrowth signals, facilitate evasion from programmed cell death, induce an unlimited replicative potential, sustained angiogenesis, and finally stimulate metastasis. Amongst the massive amount of genes that can be mutated and involved in this process, there is one that stands out, which is the p53 tumour suppressor gene (also known as TP53) [1, 2]. The p53 expression and activity regulation in cells is extremely important since this protein is responsible for the control of a broad range of downstream effector pathways including the cell cycle arrest, cellular senescence, apoptotic cell death, coordination of DNA repair and coordination of metabolism [3, 4]. Regarding that, the p53 gene has been extensively studied in the last decades as a potential role player in gene therapy. Actually, different p53 gene-based products, including viral- or non-viral systems, have been developed to be applied in gene therapy of cancer diseases [5, 6]. In the case of non-viral vectors, the use of the supercoiled (sc) p53 encoding pDNA conducted to promising results for potential cancer therapy application, since this isoform proved to be more efficient for gene delivery, cells transfection and protein expression than other plasmid isoforms (linear or open circular (oc) pDNA), leading to higher rates of apoptosis [7, 8].

To obtain the sc pDNA isoform with the required purity degree for therapeutic applications, amino acids-based affinity chromatography has been applied [9-12]. This method has been widely used by our research group, focusing on a mimetic approach of the biological processes occurring in cells, namely the molecular recognition and specific interaction between particular amino acids and nucleic acid bases. Regarding that, one of the first amino acids used as a chromatographic ligand for the sc pDNA isolation was arginine, which led to the establishment of an interesting purification methodology allowing the recovery of the sc isoform of different plasmids, with good recovery yields and purity degree, only requiring minor adjustments on the experimental conditions. [7, 9, 13]. In addition, this amino acid was already immobilized in different supports, such monoliths and agarose beads matrices demonstrating ability for the sc recovery [9, 13]. However, problems concerning the binding capacity of the commercial arginine agarose matrix or fouling and clogging problems in case of monoliths, are real handicaps for the industrialization of the arginine chromatographic supports developed until now. Therefore, the need to develop a more efficient and effective chromatographic support still remains creating the opportunity for the study of new supports such as the macroporous matrix with arginine ligands. The macroporous matrices present large flow pores, allowing the increase of contact surface area as well as the increase of the intraparticle flow [14]. Due to these properties, the macroporous matrices have been already used across the years for the purification of pDNA and other biomolecules, even after the linkage of different chemical and

biological species [15, 16]. For example, Per-Olof Larsson had been using macroporous matrices since the 90's for affinity chromatography using NAD^+ as ligands [17].

In this work a macroporous matrix was modified with arginine ligands and was then completely characterized to be used in the isolation of the sc p53-encoding pDNA. Thus, we take advantage of the selectivity of the arginine ligand combined with the flow properties and binding capacity offered by the macroporous support to promote the sc p53-encoding pDNA isolation. Finally, the robustness of this arginine macroporous resin was also tested, trying the purification of different plasmids.

Materials and Methods

Toyopearl® AF-Epoxy-650M was kindly provided by Tosoh Bioscience (Stuttgart, Germany), arginine was purchased to Sigma-Aldrich (St. Louis, MO, USA) and the NZYMaxiprep Kit was from NZYTech (Lisbon, Portugal). All the water used to prepare solutions was of ultrapure grade, purified with a Milli-Q system from Millipore. Sodium chloride (NaCl) was purchased to Sigma-Aldrich, tris(hydroxymethyl) aminomethane (Tris) was from Merck (Darmstadt, Germany) and EDTA was purchased to VWR (Alfragide, Portugal). Binding and elution buffers used in chromatography were filtered through a 0.20 μm pore size membrane (Schleicher & Schuell, Dassel, Germany) and degassed ultrasonically. The 6.07-kb pcDNA3-FLAG-p53 Addgene plasmid 10838 [17] and the 8.702-kp p1321 HPV-16 E6/E7 Addgene plasmid 8641 [18] were purchased to Addgene (Cambridge, MA, USA). The 2.7-kpb plasmid pUC19 was from Invitrogen (Carlsbad, CA, USA), and finally, all the reagents used in bacterial growth were obtained from Sigma-Aldrich. The DNA ladder was obtained from Bioline (London, UK).

Plasmid and bacterial growth conditions

The pcDNA3-FLAG-p53, pUC19, and the p1321 HPV-16 E6/E7 plasmids were independently amplified in cell cultures of *E. coli* DH5α. The different bacteria cultures were carried out at 37 °C, 250 rpm, in Erlenmeyer flasks with 500 mL of Terrific Broth medium (20 g/L of tryptone, 24 g/L of yeast extract, 4 mL/L of glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg/L ampicillin for pcDNA3- FLAG-p53 and with 100 μg/L for pUC19 and p1321 HPV-16 E6/E7 plasmids. The cells were grown until the late log phase (OD 600nm ± 9), and then collected by centrifugation at 4000 rpm and stored at -20 °C, until further use.

Lysis and plasmid isolation

To recover the produced pDNA, *E. coli* cells were first disrupted by alkaline lysis and the p53-encoding pDNA (sc and oc isoforms) was then pre-purified with the NZYTech kit according to

the manufacturer's instructions. Briefly, after the alkaline lysis, pDNA molecules were bound to the NZYTech anion-exchange resin under appropriate low-salt and pH conditions. Then, the impurities were removed by a medium salt wash and finally, the pDNA was eluted through the increase in ionic strength.

Immobilization of Arginine in the 650M epoxy support

The macroporous resin (1 g) was washed with 1 L of milli-Q water and then suspended for 72 h in water, to perform the swelling of the material. Then, the water was discharged, and an arginine-saturated solution prepared in 2 M of sodium carbonate at pH 9.8, was added. To accomplish the immobilization reaction, this preparation was keep under stirring for 72 h at 200 rpm, at 65 ˚C. Finally, the matrix was washed with 1L of milli-Q water.

Characterization of the arginine-support

In order to verify if the beads suffered from morphological modifications and to confirm the arginine immobilization, different techniques were applied to characterize the support.

SEM

To analyse the effect of the immobilization process in the beads morphology, the samples were evaluated, before and after the immobilization process, using SEM (Hitachi S-3400N, Tokyo, Japan), operating at an accelerating voltage of 20 kV, at variable magnifications. The samples were fixed on a brass stub using double-sided tape and then made electrically conductive by coating with gold using a Quorum Q150R ES sputter coater.

Fourier transform infrared spectroscopy

To search for evidence of successful immobilization with arginine, Fourier transform infrared spectroscopy (FTIR) analysis was performed. Briefly, the beads were mounted on a diamond window and compressed to improve spectrum signal to noise ratio. For each sample, 128 scans were acquired with a spectral width ranging from 4000 to 500 cm^{-1} and a spectral resolution of 4 cm^{-1} [18]. The non-immobilized matrix was used as the blank. The spectra were recorded on an FTIR spectrophotometer Nicolet iS10 (Thermo Scientific, Waltham, USA).

Energy dispersive X-ray analysis

To confirm if the arginine was immobilized, an energy dispersive X-ray analysis (EDX) (Rontec) was carried out. For that, samples were placed on aluminium stub supports, air-dried at room temperature, and sputter-coated with gold [18]. Then, elemental analysis was performed to search for evidence of the N atom in the immobilized and non-immobilized sample.

Chromatographic analysis

The chromatographic experiments were performed in an Äkta Pure System (GE Healthcare Biosciences, Uppsala, Sweden). A 16 x 40 mm (approximately 8 mL) column was packed with the macroporous arginine matrix. The experiments were performed using a circulating water bath to maintain and control the temperature under study. Then, the samples were loaded onto the column using a 100 µL loop, at 1 mL/min. The absorbance of the eluate was continuously monitored at 260 nm. To find the best binding/elution conditions, different increasing sodium chloride gradients were evaluated. Thus, the column was equilibrated with various concentrations of sodium chloride depending on the attempts. The best conditions achieved for the sc p53 encoding pDNA isolation using the arginine-macroporous matrix included an initial equilibration with 10 mM Tris-HCl and 10 mM EDTA, pH 8.0 at 4 ºC. After sample injection, the ionic strength was stepwise increased to 310 mM NaCl using the same buffer (10 mM Tris-HCl and 10 mM EDTA, pH 8.0) and finally to 1M NaCl to sequentially elute the bound species. All the results were confirmed by performing at least three replicates.

Dynamic binding capacity of plasmid DNA

A 10 × 7 mm column (GE Healthcare Biosciences, Uppsala, Sweden) was packed with the macroporous arginine matrix giving a total bed volume of 500 µL. The column was connected to an ÄKTA Pure system (GE Healthcare Biosciences, Uppsala, Sweden), and used for determination of the DBC of the support using a pDNA feed stock solution. These experiments were conducted at 1 mL/min and 0.5 mL/min and using a pDNA concentration of 50 µg/mL. The column was equilibrated with 10 mM Tris–HCl with 10 mM EDTA, testing two different pH conditions, namely pH 6.0 and pH 8.0. In each case, the pDNA solutions were prepared in the same buffer and pH value. The determination of DBC was carried out by recording breakthrough curves and calculating the amount of bound pDNA per mL support at 10 and 50% breakthrough. The DBC values were obtained by subtracting the value achieved under non-binding conditions, according to the following equation (Eq. 1):

$$\mathrm{DBC} = \frac{(\mathrm{VL}-\mathrm{V0})\times \mathrm{Cp}}{Vc} \qquad (1)$$

where DBC is the dynamic binding capacity (mg/mL), VL corresponds to the volume loaded up to the breakthrough point (mL), V0 is the void volume of the column (mL), Cp corresponds to the concentration of pDNA (mg/mL) and Vc is the volume of the column (mL). The elution of the bound pDNA was achieved by increasing the NaCl concentration in the mobile phase to 2 M in a stepwise manner.

Results and Discussion

Macroporous matrices have already been used for biomolecules separation, with particular application on large biomolecules purification [14, 19, 20]. In this research work, the advantages associated to these supports, namely the flow properties and binding capacity, were combined with the ability of the arginine amino acid for the biospecific recognition of the sc p53 encoding pDNA. This kind of affinity chromatography enables, through a slight adjustment of the adsorption and elution conditions, to achieve improved recovery yields and purity degrees of the target biomolecule when compared with other kinds of biological ligands, such as antibodies [21]. The mild conditions that can be used and the naturally-occurring interactions between the amino acids and the nucleic acids are also important factors to ensure the structural integrity of the purified molecules [22].

Characterization of the macroporous-arginine support

After the immobilization process of arginine in the macroporous resin, the matrix was characterized in order to verify if there are modifications in the shape or morphology of the support, in comparison with the initial beads. The first analysis was performed by SEM (Figure 1) and the results indicate that there is no modification on the shape or morphology of the beads during the immobilization process. Actually, even the analysis of the SEM images obtained with higher magnitudes (Figure 1 C and F) show that there are no differences in the integrity of the beads surface, being this result indicative of the maintenance of the physical properties inherent to the support used. In fact, this kind of result was already predicted since the immobilization process does not involve harsh conditions.

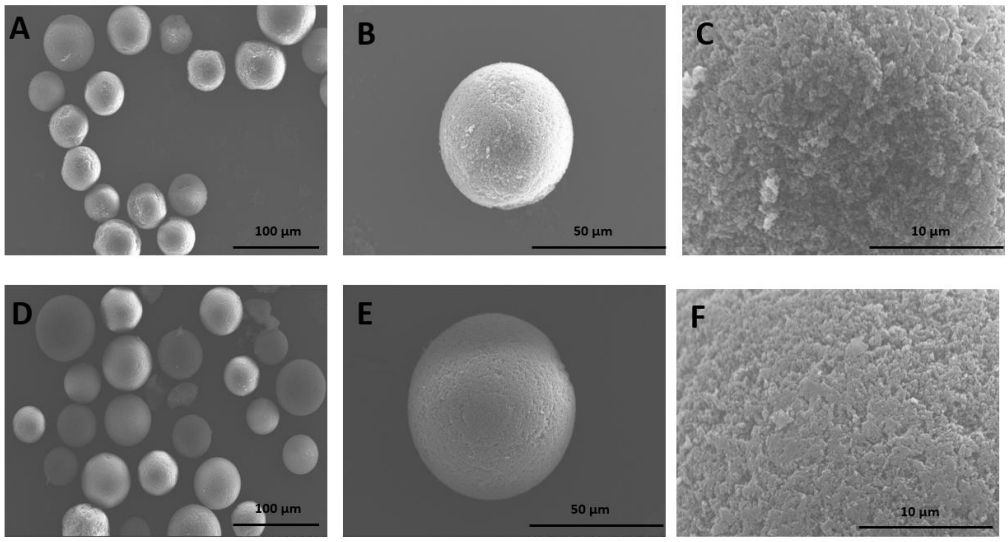

Figure 1- SEM images of the non-modified macroporous beads: A- 300 X, B- 1000 X, C- 5000 X, and macroporous beads modified with L-arginine: D- 300 X, E- 1000X, F- 5000 X.

FTIR was then performed to search for evidence of successful modification of the support with the arginine. In the acquisition of the spectrum (Figure 2) and comparing with the raw resin that was used as blank, it was possible to identify peaks that correspond to the CN linkage, which guarantee the success of the immobilization process. Moreover, the spectrum shows an N-H deformation of the primary amines near the 1671 cm^{-1} (Figure 2 A). The presence of such a strong band could indicate the presence of a guanidine group. Lastly, secondary amines could be shifted towards lower wavenumbers (1574 cm^{-1}) (Figure 2 B) [23, 24]. Taking these results into consideration, it was possible to confirm that arginine was bound to the raw support.

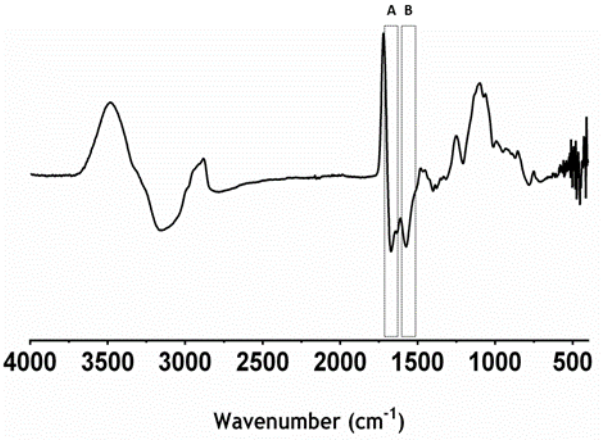

Figure 2- FTIR spectra of the beads immobilized with arginine: (A) N-H deformation at the primary amines; (B) secondary amines.

To obtain an additional confirmation of the arginine immobilization on the support, EDX was also performed. Comparing the results with the blank (beads without arginine), it was possible to identify the elements that were present in the sample and also to have a relative measurement of the percentage of each element. Thereby, it was verified the presence of N atoms in the modified matrix (Figure 3), proving the arginine ligand immobilization. With this measurement, it was observed that N atoms represent 4% of the total percentage of elements composing the sample. The remaining 96% of the sample composition mainly corresponded to C and O, which was expected due to the raw sample composition.

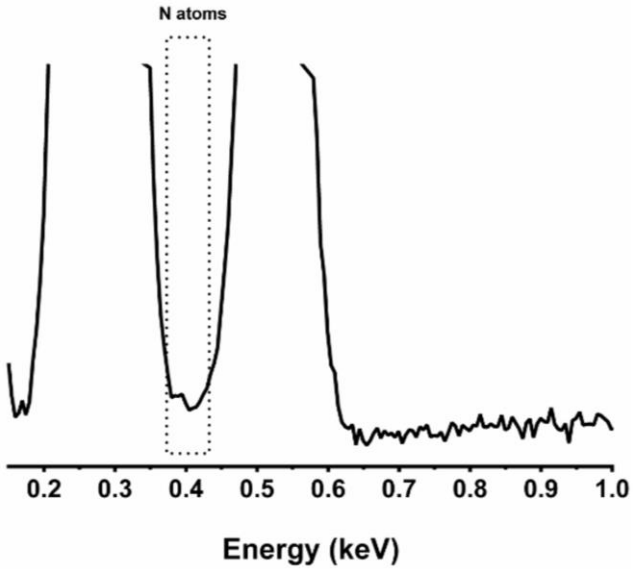

Figure 3- EDX analysis of the beads modified with arginine.

p53 encoding plasmid purification

Considering the versatility of the arginine ligand and the satisfactory results of recovery yields and purity degrees achieved in previous works [7, 9, 13], the arginine was immobilized in a macroporous matrix. After the immobilization process, the matrix was used to promote the sc pDNA isolation from a native sample (containing the sc and oc isoforms) of p53-encoding pDNA, previously prepared with a commercial purification kit.

Some initial experiments were performed to understand the behavior of the matrix and the nature of the interactions established with the pDNA. Therefore, different binding/elution strategies were evaluated by using ammonium sulphate and NaCl gradients. Arginine ligand was already described as an affinity ligand able to promote multiple non-covalent interactions with nucleic acids, which is in the basis of the biorecognition achieved for particular species. The establishment of these different interactions can be modulated by manipulating the

chromatographic conditions, namely the type of gradients. Previously, arginine supports have been used for pre-miRNAs purification with ammonium sulphate gradients, that favored hydrophobic interactions [25]. In this work, it was also tested the possibility to explore this type of interactions with the p53-pDNA, and a decreasing ammonium sulphate gradient was tested. Under these conditions, the pDNA was not retained on the column, and the sample was immediately eluted (data not shown). When a different strategy was applied, namely by using the NaCl gradient in order to exploit mainly ionic interactions, all the pDNA sample was bind to the matrix, and when a stepwise gradient of NaCl was applied the sample was selectively eluted. With these results, it was possible to conclude that the arginine support is more effective on the binding of the pDNA under ionic conditions, which indicates that the favored interactions involved are the electrostatic, which is also in agreement with previous works [13, 26].

After these preliminary studies, the isolation of the sc pDNA was evaluated, by adjusting the gradient conditions, regarding the salt concentration and pH value. The best binding conditions were characterized by the arginine-macroporous matrix equilibration with 10 mM Tris-HCl and 10 mM EDTA, pH 8.0 at 4 ºC. After the injection of 100 µL of p53-encoding pDNA (sc and oc isoforms), under these conditions, it was allowed the elution of unbound species. Afterwards, the ionic strength was stepwise increased to 310 mM NaCl using the same buffer (10 mM Tris-HCl and 10 mM EDTA, pH 8.0) and finally to 1M NaCl to sequentially elute the bound species. Figure 4 shows a representative chromatographic profile and the resulting agarose gel electrophoresis, used to identify and characterize the biomolecules eluting in each chromatographic step.

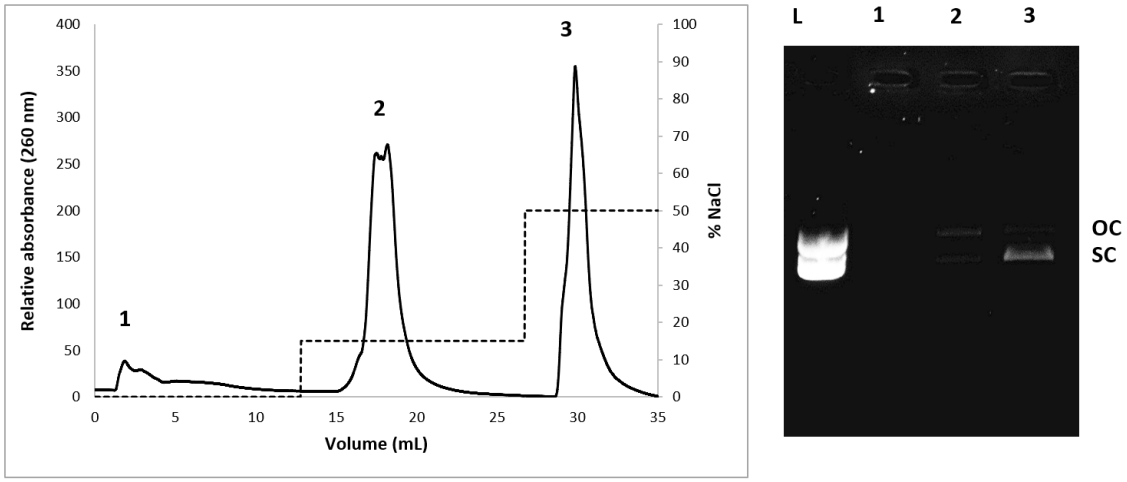

Figure 4- Chromatographic profile and agarose gel electrophoresis of the pDNA isoforms separation, from a pre-purified native pDNA sample (sc+oc), in the L-arginine macroporous matrix. The elution was performed by stepwise gradient comprising a first step with 10 mM Tris-HCl with 10 mM EDTA, a second step with 310 mM of NaCl and then a final step of 1 M of NaCl in the same buffer (10 mM Tris-HCl with 10 mM EDTA, pH 8), at 4 ˚C. In the electrophoresis gel, lane L represents the pre-purified pDNA sample and lanes 1, 2 and 3 correspond to the respective peaks of the chromatogram.

The analysis of the agarose gel electrophoresis reveals that there is no detection of species eluting in the first peak, however, the application of this binding step demonstrated to be crucial to the selectivity and ability to isolate the sc pDNA from the contaminant oc isoform, which is not accomplished if the binding step is omitted. The oc isoform (lane 2) and the sc isoform (lane 3) eluted in the second and third peaks, respectively. Through this result, it can be suggested that the biorecognition of the sc isoform by the arginine ligand is related with supercoiling phenomenon that is a consequence of deformations induced by torsional strain, leading to a higher bases exposition of the sc isoform when compared with the oc isoform [27]. Also, the higher compaction gained with this supercoiled structure, increases the negative density charge per surface area of the pDNA molecule, which also favors the establishment and strengthening of the electrostatic interactions [28].

Performing the chromatographic experiments some evidences were also noticed when changing the temperature or the pH value of the buffer. Actually, an increase in the temperature to 20 ˚C (Figure 5A), maintaining all the other experimental conditions, resulted in a significant increase on pDNA binding. In fact, this result is in agreement with other studies performed with arginine as ligand [26, 29]. Moreover, previous experiments with anion exchangers showed that the elution of double-stranded DNA fragments are shifted to higher salt concentrations when chromatographic experiments are performed at higher temperatures [30]. In a similar way, the pH modification to 6 (Figure 5 B) also promoted an increase in the pDNA binding and, once again, these results are in agreement with the ones previously obtained with arginine immobilized in a monolith. From these results we can conclude that higher pH values lead to a higher competition from the negatively charged ions. Besides, since the pKa of arginine is 12, a lower working pH will promote a more effective binding of negatively charged molecules to

the positively charged ligands [31]. This can improve the pDNA recovery, while higher pH can lead to a weaker ionic interaction, not disregarding the involvement of other non-covalent multiple interactions.

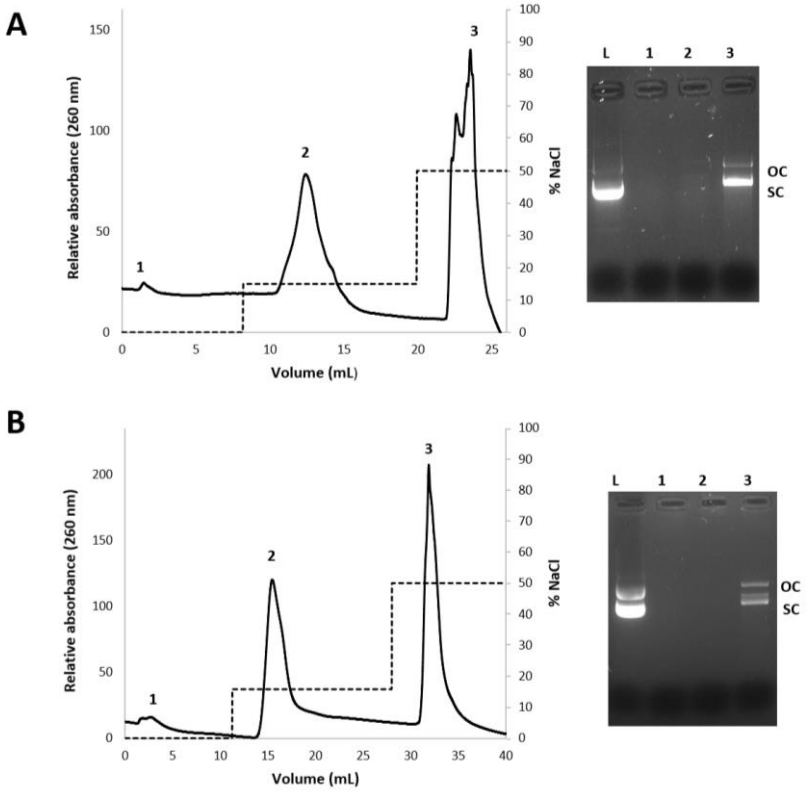

Figure 5- Chromatographic profile and respective agarose gel electrophoresis of the pDNA isoforms separation from a pre-purified pDNA sample (sc+oc) in an L-arginine macroporous matrix using (A) 20 ºC or (B) pH 6. The elution was performed using a stepwise gradient comprising a first step with 10 mM Tris-HCl with 10 mM EDTA, a second step with 310 mM of NaCl and then a final step of 1 M of NaCl in the same buffer (10 mM Tris-HCl with 10 mM EDTA). In the electrophoresis gel, lane L represents the pre-purified pDNA sample and lanes 1, 2 and 3 represent the respective peak of the chromatogram.

Since plasmids are large biomolecules, their binding to the chromatographic supports is likely to occur only at the surface, which is usually considered an important capacity limitation [32]. Therefore, in order to improve the characterization of this arginine macroporous support, the dynamic binding capacity was also determined. Breakthrough experiments were performed using variable temperature and pH conditions at 1 mL/min, with 0.05 mg/mL of a pDNA solution containing 90% of sc conformation (Figure 6 and Table 1). It is important to note that it was not possible to perform the experiments with the original, non-modified matrix, since this support had not the ability to bind pDNA under ionic binding conditions. Actually, this different retention pattern is also a proof of the successfully modification of the support with arginine.

Table 1. Comparison of the dynamic binding capacity of the L-arginine macroporous matrix to the p53 encoding pDNA, using different flow rates.

pH	Temperature (°C)	Flow rate (mL/min)	DBC (mg pDNA/ mL support)		
			10%	50%	Total
8	4	0.5	0.256	0.431	1.113
		1	0.257	0.438	1.736
	20	1	0.277	0.457	1.495
6	4	1	0.211	0.406	1.622

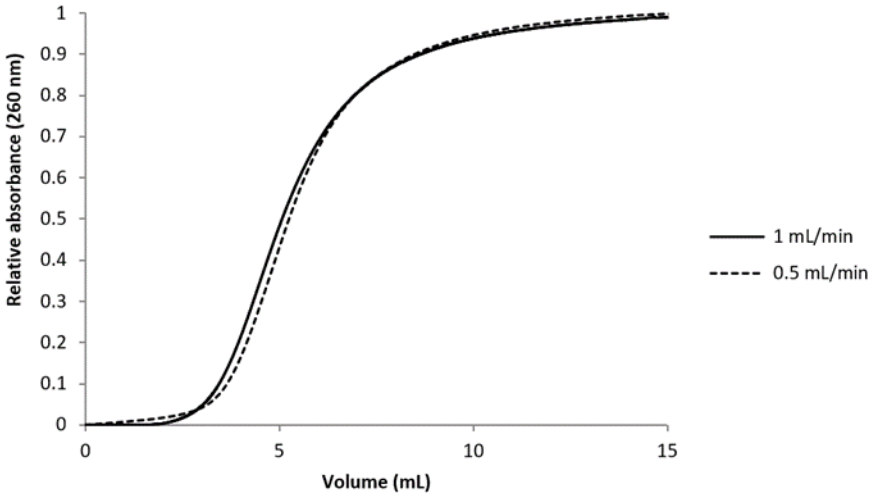

Figure 6- Breakthrough experiments with arginine macroporous. Flow rate: 1 mL/min and 0.5 mL/min; feedstock: native p53-encoding pDNA solution (0.05 mg/mL) in 10 mM Tris–HCl buffer at pH 8 and 4 ºC.

Through the analysis of these results and in comparison with the ones achieved for the arginine-agarose support [13] at the same flow rate (1mL/min), it is verified that the macroporous matrix presents a higher binding capacity, of 1.7 mg/mL comparing with 1.1 mg/mL in the case of the commercial arginine-agarose matrix, which is an advantage for the purification of large biomolecules. In fact, this represents more than 50% of increase on the DBC offered by the modified macroporous matrix, when compared with the value achieved with the homologous agarose-based commercial resin. If a scale-up of the pDNA purification process is intended, this capacity increase could be extremely valuable, since it could reduce the production runs and costs, increasing the amount of pDNA recovered per experiment and the feasibility of the

downstream process. The lower DBC achieved for the commercial arginine-agarose support can be related to the restricted number of available ligands on the particles surface, limiting the binding capacity to large molecules, such as pDNA [33]. In the case of the arginine-monolith, it occurs exactly the opposite, and better DBC values are associated to monolith structural characteristics [13]. Therefore, monoliths have already proved in the past to present a high ability to bind large molecules such as pDNA due to a high quantity of available binding sites on the three-dimensional interconnected channels, also showing excellent mass transfer properties [34, 35]. However, the use of monoliths has some drawbacks, like the fouling and clogging that can occur in most of the monolithic columns. This phenomena increase the pressure drop across the column, which decreases significantly their life span [36]. Another major concern related to the use of monoliths is the need to prepare and characterize each affinity column separately, as opposed to slurry-based methods (like the one used to prepare the arginine macroporous matrix used in this research work) which can be used with particulate supports for the simultaneous production and packing of multiple columns [37].

Regarding the effect of experimental conditions on the DBC, it can be referred that the slightly higher DBC found for the lowest temperature in study, can be related with the three-dimensional behaviour of the DNA, since a decrease on temperature promotes a compaction of the DNA. Then, during the binding, it is expected that a more compact molecule will occupy a lower number of ligands or binding sites, leaving more contact points available for the binding of additional molecules. Concerning the pH influence, it was not possible to verify any significant difference on the DBC, in the pH range studied.

Expecting to prove the versatility and robustness of the new support, another study was carried out, using plasmids with different sizes. Therefore, the selected pDNA were the pUC19 with 2.9 kpb and also the p1321 HPV-16 E6/E7 with 8.7 kpb. In Figure 7, it is depicted the representative chromatogram of the injection of 100 µL of p1321 HPV-16 E6/E7 pDNA (8.7 Kbp) and pUC19 pDNA (2.9 Kbp) pre-purified (sc and oc isoforms) samples onto the column. To perform this experiment, an adaptation of the binding and elution conditions was used. For p1321 HPV-16 E6/E7 pDNA, an increasing stepwise gradient of sodium chloride from 635 mM to 1.5 M in 10 mM Tris-HCl with 10 mM EDTA, pH 6.0 was applied, while, in the case of pUC19, the sc pDNA was isolated from the column by using a stepwise gradient of sodium chloride from 490 mM to 1.5 M in 10 mM Tris-HCl with 10 mM EDTA, pH 6.0.

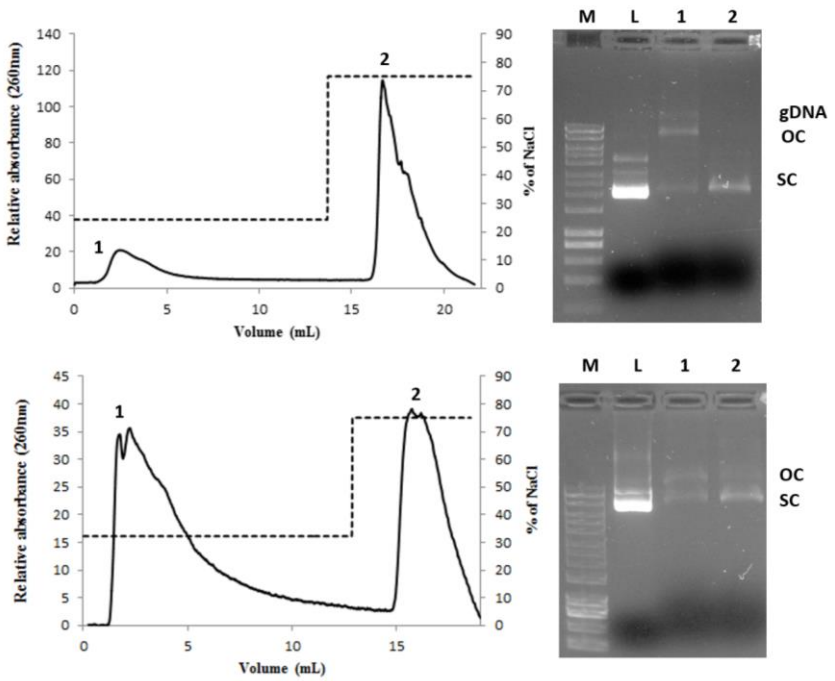

Figure 7- Chromatographic profile of the sc pDNA purification from pre-purified pDNA samples (sc and oc isoforms) with different sizes in an arginine macroporous matrix, (A) p1321 HPV-16 E6/E7 pDNA and (B) pUC19 pDNA. The different species in p1321 HPV-16 E6/E7 and pUC19 were eluted from the column by using increasing stepwise gradients, as presented by the dashed lines in the chromatograms. In the agarose gel electrophoresis the lane M represents the molecular weight marker; lane L represents the loaded pDNA sample (sc+oc); and lanes 1, 2 represent the first and second peaks of the respective chromatograms.

From the analysis of the retention profile of these plasmids, it is notorious the trend for using increased salt concentrations to achieve the required selectivity, allowing the sc pDNA isolation. This behaviour of the larger pDNA can be explained by the higher negative charge associated, which enables the establishment of more contact points, increasing the number of the electrostatic interaction between the larger pDNA and the matrix. On the other hand, it can be hypothesized that the behaviour found for the smaller pDNA, can be associated to the higher compaction of the molecules, which increases the surface negative charge and promotes higher retention.

Overall, with these results it was possible to confirm that the separation of the sc p1321 HPV-16 E6/E7 pDNA and pUC19 pDNA was accomplished using this new macroporous arginine matrix, only by adjusting the experimental conditions. Thus, the ability of this new matrix to promote the isolation of the sc isoform from plasmids with different molecular weights was proved, without compromising the required resolution and with no indication of clogging problems.

Conclusion

L-arginine was successfully immobilized on the macroporous matrix and this immobilization process was confirmed through the use of different techniques like FTIR and EDX analysis. The sc p53-encoding pDNA was isolated by this new matrix through an increase stepwise gradient of NaCl, from a native sample (sc and oc isoforms). This purification process was very influenced not only by the temperature but also by pH, which is in accordance with other studies that used arginine as ligand. The DBC of this new support was also characterized and the results indicated an improvement of more than 50% when compared with the commercial arginine-agarose matrix. This result could be considered a major advantage for the application of this matrix for industrial purposes since the high binding capacity could consequently enable an increase in the amount of pDNA recovered per experiment which will also increase the feasibility of the downstream process. Another important aspect filled in this research work was the range of applicability of this new macroporous matrix to isolate the sc isoform of plasmids with different molecular weights being only needed to perform some adjustments on the sodium chloride gradient. Regarding all these results, it was verified that a new and promising chromatographic support was developed for the sc pDNA purification field, that could be applied as a powerful and useful tool for the purification of several therapeutic plasmids.

Acknowledgements

The authors would like to thank to Dr Thomas Roberts and Dr Karl Münger for providing the pcDNA3–FLAG–p53 and p1321 HPV-16 E6/E7 plasmids through Addgene, ref: 10838 and 8641, respectively, to Tosoh Bioscience for kindly provide the 650M epoxy matrix and to Eng. Ana Paula for her help in SEM and EDX analysis. This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). J.F.A. Valente and A. Sousa also acknowledge PhD and Postdoctoral fellowships (Ref SFRH/BD/96809/2013 and Ref SFRH/BPD/102716/2014, respectively).

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Paper VI

The biological performance of purified supercoiled p53 plasmid DNA in different cancer cell lines

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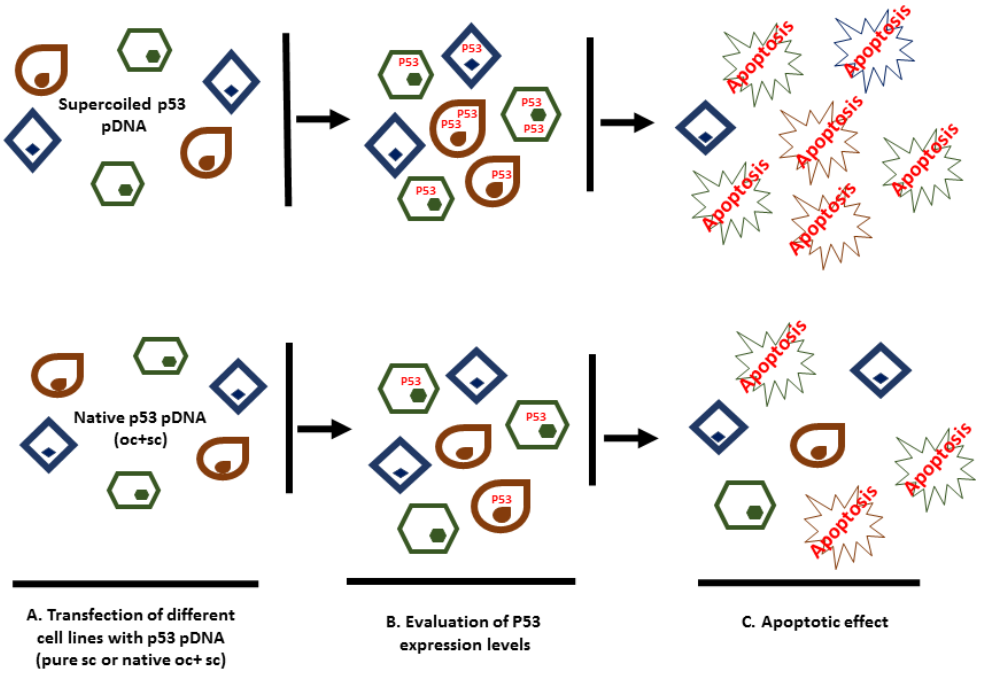
Abstract

Tumor suppressor p53 remains one of the most interesting therapeutic targets in cancer gene therapy due to its consistent mutation in numerous cancers. Thus, the reinstatement of the p53 expression and function can be seen as an effective alternative for cancer treatment, motivating research in this field. In this study, L-methionine matrix was used to purify the supercoiled topoisoform of a plasmid DNA encoding the p53 protein. This pure biopharmaceutical was conjugated with liposomes to comprehensively analyse its *in vitro* performance and therapeutic potential in different cancer cell lines, including the lung and cervix models. A different profile of cellular responses was attained after the transfection of these cancer cell lines with the p53-pDNA. Actually, the *in vitro* transfection with pure sc p53-pDNA resulted in a higher expression of the tumor suppressor protein in cancer cells when compared with the native pDNA samples (oc+sc topoisoforms). Also, wild-type p53 expression following transfection was significantly higher in HeLa cervix cancer cells in comparison to that obtained in A549 lung cancer cells. Overall, our findings emphasize the potential of sc pDNA gene-based therapy, also raising awareness of the need to adjust the therapeutics, considering the feature of high heterogeneity of cancer cells.

Graphical abstract



Keywords: Gene Therapy; L-methionine affinity chromatography; Supercoiled plasmid DNA; Tumour suppressor p53.

Highlights

- p53 pathway inactivation could be responsible for tumour development and progression;

- L-methionine affinity matrix successfully purified the sc p53-pDNA;

- Apoptosis induced by sc p53-pDNA was assessed in different cancer cell lines;

- The biological performance of pure sc p53 pDNA was higher than the native samples.

Introduction

Tumorigenesis is a process that combines the sequential accumulation of genetic and epigenetic modifications in key oncogenes and tumor suppressor pathways. Usually, these transformations are the responsible for the cancer cells growth with distinct characteristics that include: (i) self-sufficiency to growth signals, (ii) insensitivity to anti-growth signals, (iii) evasion from programmed cell death, (iv) unlimited replicative potential, (v) sustained angiogenesis, and (vi) the ability to invade and metastasize – the so-termed Cancer Hallmarks [1]. Regarding the plethora of genes involved in tumorigenesis, the p53 transcription factor (encoded by the TP53 human gene) is one of the most important [1, 2]. Most of the cancers known until now present mutations in TP53 with rates that vary between 10 % (e.g., in hematopoietic malignancies) and close to 100 % (e.g., in high-grade serous carcinoma of the ovary) [3].

The tumor suppressor p53, also termed the guardian of genome, is involved in key physiological processes including DNA damage response, upon which it triggers cells senescence and apoptosis [4]. The ablation of TP53 could be due to the single base substitution and loss of alleles, mediated by viral and cellular proteins, which are reported to play a major role in specific cancers. Some cancers including breast carcinomas, sarcomas, brain tumors, and adrenal cortical carcinomas, Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) syndromes have already demonstrate an early genetic predisposition to TP53 mutations. Moreover, since TP53 is extremely polymorphic in coding and non-coding regions, some of these polymorphisms have been related to an increase in cancer susceptibility [5]. Another important element is that other tumor suppressors are usually inactivated by frameshift or nonsense mutations, with the TP53 missense mutations being caused by single amino acid changes at many different positions [2]. Due to its biological relevance p53 is, therefore, a very valuable therapeutic target and different strategies have been explored so far to mimic or reinstate its activity.

The use of pDNA (pDNA) transgene expression vectors in non-viral cancer gene therapy has been emerging as a valuable methodology to reinstate the expression of wild-type p53 into malignant cells and restore its tumor suppressive function. Regarding the use of p53 as a therapeutic target, our research group has recently demonstrated that a purified supercoiled (sc) isoform of p53-encoding pDNA is the most efficient isoform for promoting non-viral pDNA-based transgene expression [6]. To obtain highly pure biopharmaceutical formulations, different strategies have been reported so far (like size exclusion, anion exchange, hydrophobic interaction, reversed phase, thiophilic adsorption, and affinity chromatography) [7, 8]. In this focus, our group has been exploring amino acids as ligands for DNA/RNA affinity chromatography as a successful method for the isolation of the sc isoform of different plasmids namely the pcDNA3-FLAG-p53 vector. For this specific transgene expression cassette, two different commercial matrices with L-arginine and L-methionine ligands have been investigated [6, 9].

Herein, an agarose matrix functionalized with L-methionine ligands was employed in order to promote the isolation of a sample composed only by sc pDNA. The final sample quality should be analyzed and the results should be in agreement with the parameters established by the regulatory agencies such as Food and Drug Administration (FDA), USA and European Medicines Agency (EMA) [9]. By using this purification technology, we successfully recovered pure sc p53-pDNA and investigated its biological performance following liposome-mediated delivery in different cancer cells. The use of different cell lines can help to a better understanding of the behavior of this suggested therapy *in vivo*, mainly because tumors are very different and even the same cancer is not homogeneous, being composed by different cells highly permutable, which makes mandatory the use of strategies able to efficiently target all the modified spots. Regarding this approach and, to the best of our knowledge, this is the first time that a comprehensive analysis of pure sc pDNA transfection by using commercial cationic delivery systems is performed in different cancer cell lines. To investigate such potential, several experiments were designed in order to initially verify the biocompatibility of the pDNA after the chromatographic procedure. Then, the amount of p53 expressed in the different cell lines was analysed and correlated with the induced apoptosis (Figure 1), considering the specific characteristics and the p53 metabolic pathways of the cells under study. Overall, sc pDNA transgene expression efficacy has higher than native pDNA samples and the therapeutic effect of the p53 encoded transgene was dependent on cancer type. Such pre-clinical findings may influence future applications of this approach.

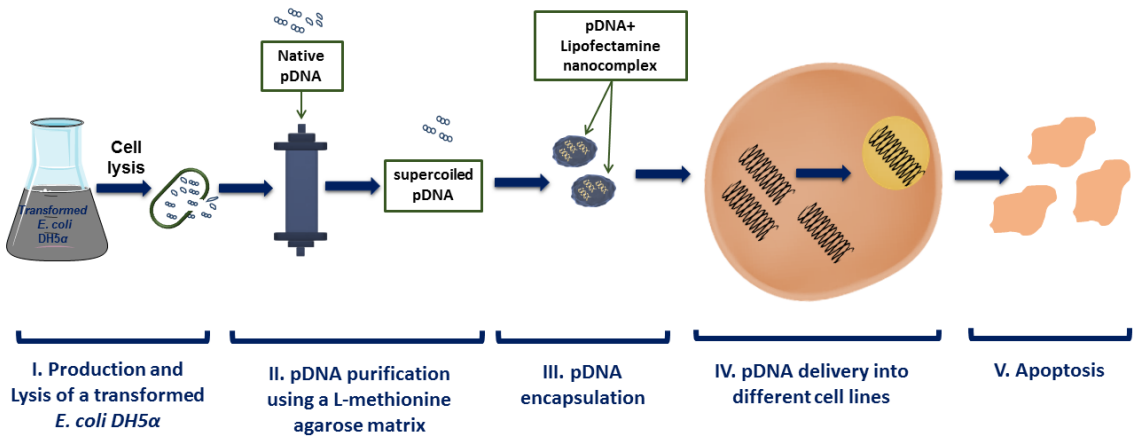


Figure 1 - Schematic representation of the biotechnological approach used in the present work. (I) Production and lysis of an *E. coli* DH5α strain transformed with the pcDNA3-FLAG-p53 vector; (II) pDNA purification and recovery of sc topoisoform by using an L-methionine affinity chromatography agarose-based matrix; (III) DNA complexation with Liposomes using commercially available cationic liposomal formulation Lipofectamine 2000; (IV) pDNA delivery to A549, HeLa cancer cells and normal human dermal fibroblasts (hFIB); (V) Evaluation of sc p53-pDNA mediated apoptosis of cancer cells.

Materials and methods

Materials

The NZYtech Maxi Prep Kit was purchased from NZYTech (Lisbon, Portugal). Ammonium sulphate ($(NH_4)_2SO_4$) was purchased to VWR and tris(hydroxymethyl) aminomethane (Tris) was obtained from Merck (Darmstadt, Germany). The 6.07 kbp pcDNA3-FLAG-p53 Addgene plasmid 10838 [10] was purchased from Addgene (Cambridge, MA, USA), Resazurin sodium salt, L-methionine agarose matrix and all the reagents used in bacterial amplification were obtained from Sigma-Aldrich (St. Louis, M.O., USA). The DNA ladder was obtained from Bioline (London, UK). The Annexin V-FITC/PI apoptosis kit was purchased from Calbiochem (La Jolla, CA, USA). The Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich and the Lipofectamine 2000 was purchased from Thermo Fisher Scientific (Inc., Lisbon, Portugal). All reagents were of research grade and used without further purification.

Methods

Plasmid production and pre-purification

pcDNA3-FLAG-p53, a plasmid with 6.07 kbp from Addgene, Cambridge, MA, USA (plasmid 10838) was amplified in a cell culture of *E. coli* DH5α. This *E. coli* was grown at 37 ºC in an Erlenmeyer flask containing 250 mL of Terrific Broth medium (20 g L^{-1} of tryptone, 24 g L^{-1} of yeast extract, 4 mL^{-1} of glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) and 30 µg mL^{-1} of Ampicillin. In advanced log phase (OD600 ≈ 9) the bacteria growth was suspended and cells were recovered by centrifugation. The plasmid DNA was recovered and pre-purified with the NZYtech Maxi Prep Kit, according to the supplier's instructions.

Preparative chromatography

For the chromatographic experiments, an ÄKTA purifier system with UNICORN 5.11 software (GE Healthcare, Uppsala, Sweden) was used. L-methionine-agarose gel (Sigma Aldrich) was then packed in a 16×40 mm (approximately 8 mL) column. The datasheet supplied characterizes this resin as containing an one-atom spacer and an extent of labelling between 2-10 µmol/mL. A circulating water-bath was used to fix the temperature (5 ºC) along the chromatographic runs. A 200 µL loop was used to load the pDNA onto the column, at 1 mL/min. The elution was monitored using an UV detection at 260 nm. To perform the complete isolation of the sc plasmid isoform it was used a decreasing stepwise gradient comprising initially 2.35 M of $(NH_4)_2SO_4$ in

10 mM Tris-HCl pH 8.0 and then 10 mM of Tris-HCl, pH 8.0, at 5 ºC. Finally, in order to check the eluted species, an agarose gel electrophoretic analysis was performed as previously described, using a Uvitec Cambridge Fire-reader UV transilluminator equipped with a CCD camera (Uvitec Cambridge) [9].

Cell culture and transfection

Cell culture experiments were performed with two cancer cell lines of different origins, the A549 non-small lung carcinoma cell line and HeLa cervix cancer cell line, and a non-malignant cell line, the human dermal Fibroblasts (hFib). DMEM-F12 medium supplemented with 10 % v/v heat activated FBS and with streptomycin (100 µg/mL)) was used for cell culture at 37 ˚C, under a 5 % CO_2 humidified atmosphere. Initially, cells were seeded in 25 cm^3 T-flasks until confluence was attained. Afterwards, the cells were sub-cultivated by incubation on 0.18 % trypsin (1:250) with 5 mM EDTA.

The *in vitro* transfection experiments were carried out by seeding 2×10^4 cells in a 96 well plate with 200 µL of DMEM-F12 complete medium and incubated for 24 h. Then, medium without FBS and antibiotic was used to promote transfection. The transfection of pDNA was then performed with a commercially available transfection reagent (Lipofectamine 2000 (LP2000)). Briefly, in each well of a 96 plate, 0.28 µL of LP2000 and 0.14 µg of DNA were diluted in 5.15 µL of Opti-MEM® I medium, according to the supplier protocol. Before the complexation reaction, LP2000 was incubated for 5 min at room temperature (RT). The LP2000-pDNA complexes were added to cells and then, were incubated for a period of 6 h after which the medium was exchanged to DMEM-F12 complete medium.

Plasmid DNA Fluorescent Labelling

The labelling of pDNA biopharmaceuticals with the FITC dye was performed to allow the follow-up of its cellular uptake and intracellular localization. Briefly, 5 µg of pDNA was added to 71 µL of labelling buffer (0.020 g of sodium (di)tetraborate in 1 mL of H_2O) and 2 µL of FITC (100 mg of FITC in 200 µL of sterile DMSO). After this, the solution was stirred for 4h at RT, protected from light. Finally, 85 µL of 3 M NaCl and 212.5 µL of absolute ethanol was added to precipitate FITC labelled pDNA by overnight incubation at -20 ºC.

Cellular Uptake Analysis by Confocal Laser Scanning Microscopy (CLSM)

To evaluate pDNA-lipoplexes cellular uptake kinetics, 1×104 cells were seeded in complete DMEM-F12 in Ibidi µ-Slide 8-well cell culture treated chambers (Ibidi GmbH, Germany) and cultured overnight. Transfection was performed when 70 % of cells confluence was achieved. Then, cells were incubated for 20 min with Hoechst 33342® (1:1000) (Invitrogen™ Molecular Probes™) and subsequently rinsed 3 times with PBS (pH = 7.4). Transfection was performed

during 0, 2, 4 and 6 h, with LP2000 nanosized lipoplexes loaded with native p53-pDNA (sc+oc) or sc p53-pDNA biopharmaceuticals. Following the incubation period, the DMEM-F12 medium was exchanged and 4 % paraformaldehyde in PBS was used for transfected cells fixation (for 20 min, at RT). To enable a better visualization, transfected cells were washed three times with PBS. Visualization was finally performed using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., USA) equipped with a plane-apochromat 63×/DIC objective.

Cytotoxicity

The cytotoxicity of liposomal-pDNA formulations was evaluated by using the resazurin assay. For this evaluation A549, HeLa and hFIB cells were seeded in 96-well plates as described above. Resazurin (10 µL, 2.5 mM) was added to each well two days after transfection. Plate was then incubated in the dark for 4 h, at 37 °C, in a humidified atmosphere of 5 % CO_2. After incubation, resofurin was measured using a plate reader spectrofluorometer (Spectramax Gemini XS, Molecular Devices LLC, US), at an excitation/emission wavelength of λex = 560 nm and λem = 590 nm. Data represents the mean of three independent experiments.

Western Blot Analysis

The expression of p53 protein mediated by cells transfection with p53-pDNA vectors was evaluated by western blot. Briefly, following transfection with LP2000-pDNA lipoplexes, cells were rinsed with ice-cold PBS and homogenized in cell lysis buffer: 25 mM Tris-HCl buffer, pH 7.4; 2.5 mM EDTA; 1 % Triton X-100; 2.5 mM EGTA; 25 mM phenylmethylsulfonyl fluoride and complete, EDTA-free protease inhibitor cocktail (Roche). Cell extracts were then centrifuged at 11500 rpm for 7 min at 4 °C and the supernatant was analyzed using Bradford Protein Assay (BioRad) accordingly with the supplier's instructions and then fractionated by electrophoresis on 10% SDS-PAGE. Proteins were denatured (95 ºC for 10 min) and transferred to polyvinylidene difluoride filter (PVDF) membranes (100 V for 40 min). Then, TBS-T supplemented with 5 % BSA was used for the blocking. The anti-p53 primary antibody (1:100 in 5 % BSA in TBS-T) (Santa Cruz Biotechnology) was used to incubate the membranes at 4 °C overnight. Membranes were then washed three times with TBS-T, and then incubated with the p53 anti-rabbit secondary antibody diluted 1:25000 in TBS-T. The membrane was then washed and incubated in ß-actin primary antibody (1:20000 in TBS-T) (Santa Cruz Biotechnology) for 2 h and finally incubated in the ß-actin secondary antibody (Santa Cruz Biotechnology). ECL substrate (BioRad) was used to signal detection according to manufacturer's instructions and images were acquired by using a ChemiDoc™ XRS system (BioRad) and analyzed with the Image Lab software (BioRad).

Flow cytometry Analysis

Apoptosis in malignant and non-malignant cells transfected with p53 expressing pDNA was evaluated by flow cytometry through Annexin V-FITC/PI staining (Calbiochem, USA). For this purpose, $5x10^5$ cells were initially seeded in sterile 6-well culture plates containing DMEM-F12 culture medium supplemented with 10 % FBS. Cell growth was then promoted at 37 ºC, 5 % CO_2, in a humidified atmosphere, for 24 h. In the following day, the culture medium was removed and the cells were transfected with LP2000 cationic liposomes loaded with different pDNA formulations (sc p53-pDNA and native p53-pDNA (oc + sc)), according to the manufacturer's instructions. After 48 h of transfection, cells were detached by using trypsin/EDTA and pelleted by centrifugation (1500 rpm, 5 min, RT). Binding buffer was used to ressuspend cells that were then labelled with Annexin V-FITC and PI according to the manufacturer's instructions. Flow cytometry experiments were performed on a BD FACS Calibur flow cytometer (Becton Dickinson Inc., USA) equipped with a 15 mW, 488 nm laser and a 635 nm red-laser. Different region of interest (ROI) were selected to data acquisition for the different cell lines used. A total of $1x10^4$ events were collected in the FL-1 (530/30 nm) and FL-2 channel (585/42 nm). As controls for ROI delimitation and detectors gain/voltage adjustment non-treated cells were used. Hydrogen peroxide (H_2O_2) was used as cell-death-inducing agent in order to have, positive controls for apoptosis and necrosis. In brief, positive control cells were seeded in 24-well culture plates and treated with 160 mM of H_2O_2 for 12 h, at 37 ºC, 5 % CO_2, in culture medium supplemented with 10 % FBS and antibiotics/antimycotics (complete medium). The cells were then recovered as before mentioned and analyzed by Annexin V-FITC/PI staining. Data processing was performed in FCS Express version 5 Research Edition (De Novo Software™, LA, USA).

Statistical analysis

Each experience was performed at least three times using independent cell cultures. Data are expressed as a mean ± standard error (S.D.) or standard error means (S.E.M). The statistical analysis performed was one-way analysis of variance (ANOVA), followed by multiple comparison test Turkey. A p-value below 0.05 was considered statistically significant. Data analysis and statistical tests were performed in GraphPad Prism 6 software.

Results and Discussion

Methionine-based purification of p53 supercoiled pDNA

The p53 tumor suppressor gene is a valuable biological target for gene-based cancer therapies since common small therapeutic molecules are unable to reinstate its activity, rendering p53 as one of the so-termed "undruggable targets". Recently, phase II clinical trials (NCT02340156) using p53 encoding pDNA have been initiated to explore the potential of this therapeutic strategy. Nevertheless, in these trials, the purity and supercoiled content of the pDNA used in the injected liposomal particles is not disclosed, what could be an important information, as it is known that the pDNA conformation can have an impact on biological activity of pDNA. Actually, several reports demonstrate that sc topology outdoes the biological performance of native pDNA samples that contain simultaneously both oc and sc topoisoforms [6, 11]. Also, to ensure the purity of the DNA is extremely relevant since the presence of impurities like endotoxins, genomic DNA or RNA could lead to undesirable reactions on application [12, 13]. To the best of our knowledge, the formulation of liposomal-sc pDNA and the evaluation of its biological performance in different cancer cell lines has not yet been explored. Such performance data can support the optimization of liposomal-sc pDNA formulations and contribute to their translation into other newer clinical trials. Exploring this aspect, is paramount from a regulatory context, since pDNA biopharmaceutical preparations intended for human use should have a content in supercoiled topoisoform of approximately 97 % [14]. Moreover, these preparations should have undetectable amounts of RNA and proteins, should present a clear and colorless appearance, the endotoxins levels should be below 0.1 EU/µg of pDNA and, the amount of genomic DNA (gDNA) should not exceed the 2 ng/µg of pDNA [15]. Taking these guidelines into consideration our research group has been developing affinity chromatography-based pDNA purification platforms, which allow to obtain purified plasmid preparations that conform to these guidelines [16]. The developed purification methodology takes advantage of affinity-based biomolecular interactions between pDNA vectors and different amino acids such as L-arginine or L-histidine [17, 18].

In particular, the L-methionine agarose matrix was previously explored and characterized by Valente and co-workers (2014), in order to recover the supercoiled topoisoform of different plasmids, including the p53-encoding plasmid vector (pcDNA3-FLAG-p53), from *E. coli* lysates, accomplishing the quality parameters required by the regulatory agencies [9]. We have reported that the sc p53-encoding plasmid, purified by this matrix, does not present in its composition proteins or gDNA, the endotoxin level was below the recommended by the regulatory agencies and also the content of the sc isoform was above 97 % [9]. The L-methionine amino acid regulates different protein-nucleic acids biomolecular interactions and could therefore be a valid ligand for amino acid-nucleic acid affinity chromatography [9].

Interestingly, the behavior of this amino acid is highly influenced by temperature and ionic strength conditions. Regarding its interaction with the different nucleic acids, the higher affinity was presented for the thymine bases [19].

In the present work, we have taken advantage of the L-methionine agarose matrix to adjust and optimize the purification strategy in order to separate the sc and oc pDNA isoforms from the pre-purified pDNA sample. A decreasing stepwise gradient comprising 2.35 M of $(NH_4)_2SO_4$ in 10 mM Tris-HCl pH 8.0 and 10 mM of Tris-HCl, pH 8.0, at 5 ºC was established, as demonstrated in Figure 2.

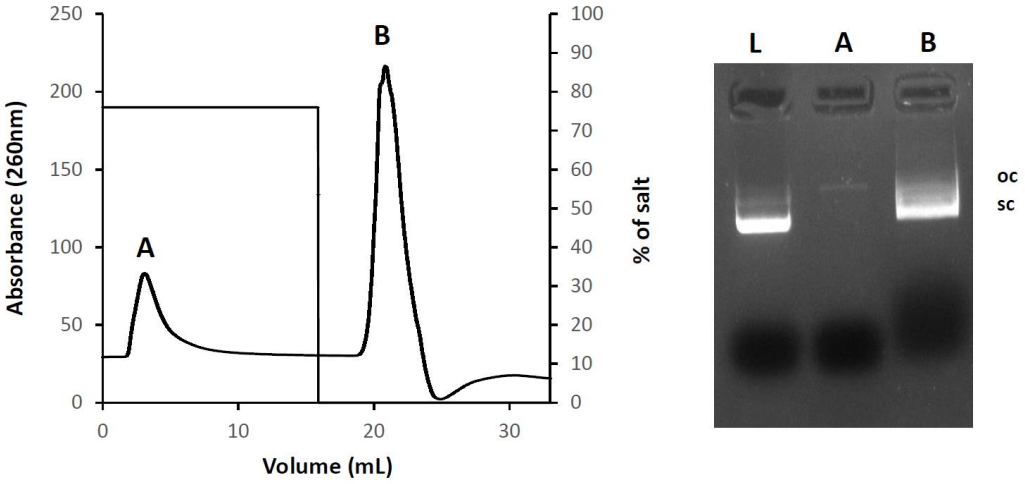


Figure 2 - Chromatographic profile and respective agarose gel electrophoresis of the plasmid isoforms separation from a pre-purified pDNA sample (sc+oc) (lane L) in an L-methionine agarose column at 5 ºC. The elution was performed by a stepwise gradient of 2.35 M of $(NH_4)_2SO_4$ in 10 mM Tris-HCl pH 8.0 (A) to 10 mM Tris-HCl pH 8.0 (B), as represented by the dashed line. In the electrophoresis gel, lane L represents the pre-purified plasmid sample (sc+oc) and lanes A and B represent the respective peaks of each chromatogram.

Evaluation of the transfection behavior

After the purification process, pure sc pDNA was successfully conjugated with commercial cationic liposomes (LP2000). This product is commonly used in pDNA delivery into different cells [20-24], and, as in this study the main objective is to compare the biologic activity of pure sc pDNA in different cell types, this standard transfection reagent is adequate.

Concerning the above mentioned, the cellular uptake kinetics (at 0, 2, 4 and 6 h of transfection) of different pDNA lipoplexes was evaluated in different cell lines to study possible differences in cellular entry and delivery among formulations. The obtained profiles after 6 h of transfection with sc pDNA lipoplexes are presented in Figure 3. In this image, it is possible to

observe the FITC-labelled pDNA presence in the nucleus of different cell lines after 6 h of transfection with LP2000. Therefore, it was also inferred that these transfection conditions are suitable for further experiments with the cell lines under study.

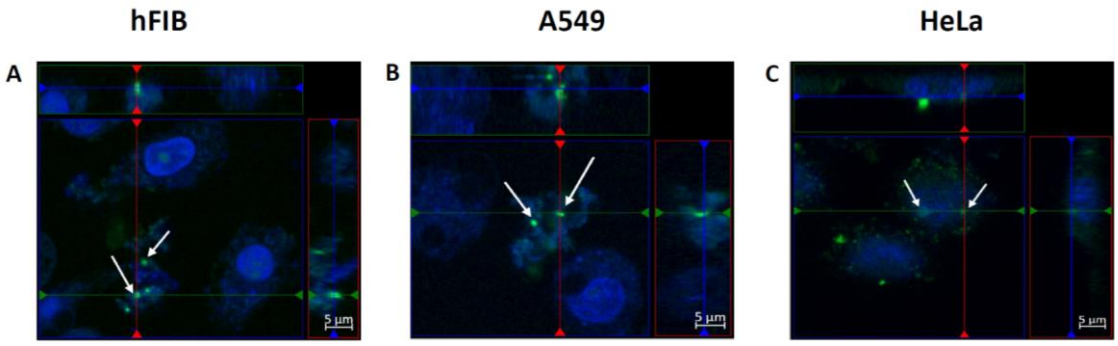


Figure 3 - Orthogonal view of live cell imaging six hours after pDNA complexes addition to the different cell lines (A- hFIB; B- A549; C- HeLa). The blue staining represents the cell's nucleus and the green staining represents FITC-labelled pDNA.

In addition, a time course transfection study was performed at different time-frames, 0, 2, 4 and 6 h, respectively, in order to evaluate the internalization profile of pDNA /lipoplexes in each cell line (Figure 4).

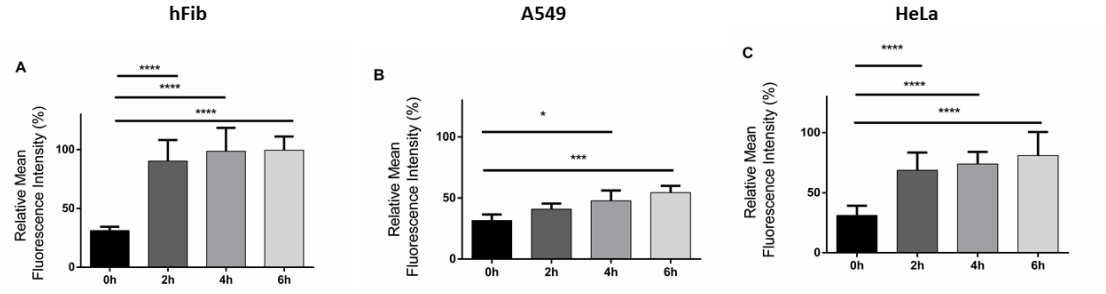


Figure 4 - Relative Mean fluorescence intensity (M.F.I.) values of p53 labelled FITC in the different cell lines (A- hFIB; B- A549; C- HeLa). Data is represented as mean ± S.D., n=10.

As it is possible to see in Figure 4, after 2 h of transfection, all cell lines presented some cellular uptake of FITC/sc pDNA-lipoplexes, being more evident in hFIB. However, it is also visible that among the cancer cells, pDNA-lipoplexes seems to be faster and more internalized in HeLa cancer cells than in A549 cells. Regarding A549, it was demonstrated through the relative mean fluorescence intensity (M.F.I.) graphs (Figure 4) that the amount of fluorescence in these cells is lower when compared with HeLa or hFIB cells. Previous research works had also demonstrated low transfection results for A549 for both viral and non-viral vectors showing that these cells are harder to transfect even using commercial liposomal formulations [6, 25, 26]. Concerning the therapeutic application perhaps it should be necessary to adjust and optimize the transfection to reach a more effective result.

Plasmid DNA-loaded Lipoplexes cytotoxicity

To evaluate the cytotoxic profile of pDNA liposomal formulations, Resazurin assays were performed at 48 h following particles administration. As demonstrated by Figure 5 A, the administration of sc p53-pDNA and native p53-pDNA (sc+oc) in normal hFib does not elicit any cytotoxic effect. In Figure 5 B there is a negligible difference between the cytotoxic effect of native and sc-pDNA transfected A549 cells. Contrariwise, the administration of sc p53-pDNA/LP2000 lipoplexes in HeLa cancer cells elicits a slight cytotoxicity (Figure 5 C). These results could indicate a possible contribution of p53 expression to the decrease in cell viability that was obtained, since as demonstrated in the previous section, this kind of cells presented the highest internalization of lipoplexes. Actually, the verified cell death can be a signal of apoptosis induced by the p53 expression, instead of the cytotoxic effect of the pDNA formulation. The validation of this hypothesis can be a good result as it may indicate the success of the delivery and expression of p53-pDNA, as well as the importance to obtain and use the sc topoisoform of pDNA to achieve a more significant biological effect. A similar effect was also previously described by Gaspar and collaborators were at 24h after transfection no cytotoxicity was observed, while at 72h a decrease in HeLa cell viability was reached. Also, in that research work no changes have been verified for fibroblasts viability [6].

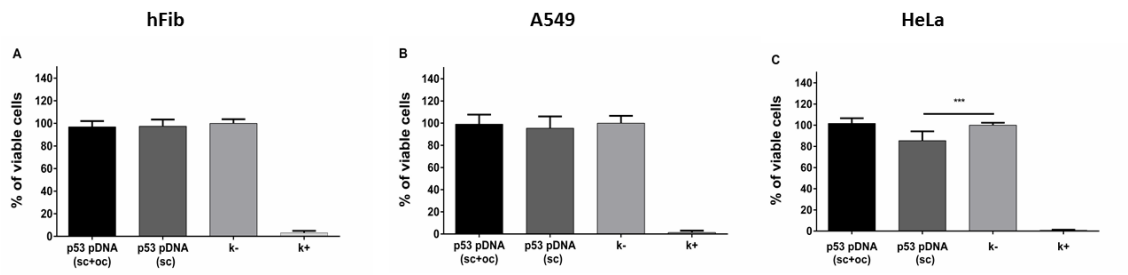


Figure 5 - Evaluation of cell viability following transfection with LP2000 lipoplexes loaded with p53-pDNA in different cell lines, at 48 h after administration. (A) human dermal fibroblasts; (B) A549 non-small lung cancer; (C) HeLa cervix carcinoma. Data is represented as mean ± S.D., n=3.

To further evaluate if the observed cytotoxicity in cancer cell lines is correlated with the p53 expression, a western blot analysis of the expressed wild-type protein was performed.

Analysis of p53 transgene expression

As demonstrated in Figure 6, the expression of the p53 tumor suppressor protein is significantly higher when the purified sc pDNA vector is used. It should also be noted that, for all the malignant cell lines tested, the purified sc pDNA isoform yielded a higher amount of p53, when compared with that obtained with native pDNA (oc+sc) formulations. These results are in agreement with previous ones, regarding to the improved biological performance of pDNA vectors administered in the supercoiled topoisoform [6, 27].

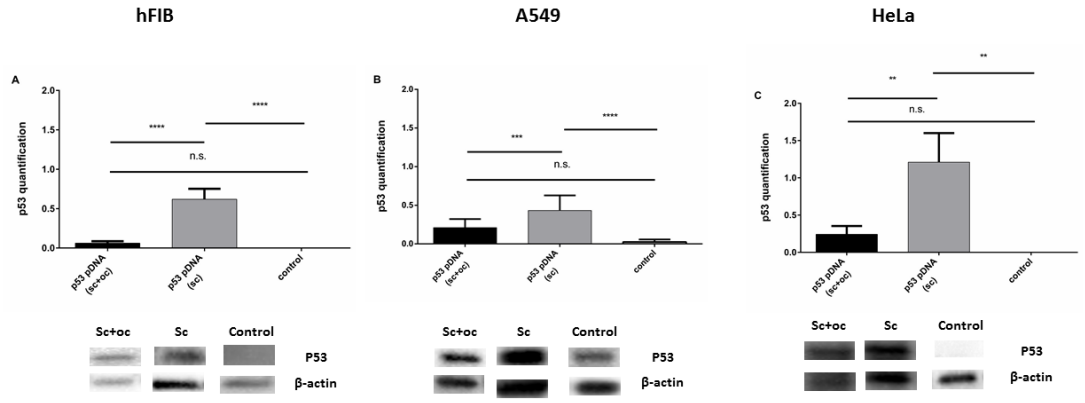


Figure 6 - Evaluation of p53 protein expression following administration of p53-pDNA lipoplexes in different cell lines, after 48h. (A) Human dermal fibroblasts; (B) A549 non-small lung cancer cells; (C) HeLa cervix carcinoma. Data are represented as mean ± S.D., n=3.

Although in previous results we obtained a higher cellular uptake of sc pDNA lipoplexes in hFIB in comparison to the cancer cells, the level of p53 protein expression obtained in western blot analysis was relatively lower. This could be correlated with the mechanisms of p53 expression in hFib and other normal cells [28]. The primary control of the p53 levels is performed through its ubiquitin-mediated proteasomal degradation. Regarding this, there are until now three different studies published, were the Mdm2 was identified as the master endogenous E3-ligase with high specificity for p53 [28]. Thus, the results suggest that in normal conditions, if the cell does not require the p53 function, it can modulate its expression or induce p53 degradation, even after the transgene delivery.

Interestingly, there is a significant difference in the reinstatement of p53 expression in HeLa and A549 cell lines with the latter yielding a lower amount of tumor suppressor protein, what it is in accordance to the previous results of cellular uptake. The lower expression of p53 in A549 cells can be a result of the lower transfection, indicating that these cells are more resistant to the formulation entrance into the cells, making difficult the therapeutic intervention. Also, it should be emphasized that in A549 cell line a negligible p53 gene expression was observed at the basal state when the cells were non-treated. These results are in accordance with the literature since A549 could present some basal levels of wt-p53 protein [29]. This basal level comes from a deletion on the CDKN2A locus that harbors p16 and p14ARF

genes in A549 cells. Without p14ARF, Mdm2 levels are high, which keep p53 low. Even though p53 levels are low, there is still a basal level of the protein [30].

In HeLa cancer cells the oncoproteins E6 and E7 from HPV virus are the responsible for p53 degradation being this, the reason for the nonexistence of a basal level of p53 in these cells [31]. Regarding the p53 expression, it was verified the highest level amongst the cell lines under study, which can be correlated with the pDNA uptake since this cell line is fairly easy to transfect and very receptive to vectors, making it a popular research tool.

Also, it is relevant to underline that for all the cell lines under study, the higher p53 expression level was achieved when the supercoiled isoform was used for transfection, which corroborates other studies describing this specie as the most biologically active [6, 15].

p53-mediated Cell Apoptosis

Apoptosis is measured in terms of binding of externalized phosphatidylserine to phospholipid binding protein Annexin V conjugated with fluorochromes [32]. Concerning that, to further evaluate the influence of p53 tumor suppressor protein expression in cells, the apoptosis was investigated by flow cytometry of different cancer cell lines following transfection with p53-encoding pDNA lipoplexes. Regarding the hFIB, the flow cytometry results are in agreement with those obtained by Resazurin and western blot assays, as only a relatively low number of apoptotic cells were obtained following transfection, with more than 91 % of healthy cells being obtained (Figure 7). These low apoptotic levels could be due to the self-regulation mechanisms presented in non-tumoral cells as previously mentioned [28].

As demonstrated by the results of Figure 8, pDNA transfection promotes A549 cells apoptosis after 48 h, with malignant cells transfected with sc pDNA exhibiting a slightly higher amount of early and late apoptotic cells in comparison to native pDNA formulations. However, it is important to emphasize that no significant difference between sc and native transfected cells were obtained at this time point. In addition, the transfection of HeLa cells with p53 transgene elicits significant cell death with more than 35 % of necrotic/early apoptotic cells (Figure 9). Interestingly, as demonstrated by flow cytometry data the p53 tumor suppressor appears to have a more pronounced therapeutic effect in HeLa cells in comparison with A549 non-small lung cancer cells, which is in agreement with the amount of p53 protein expression obtained in Figure 6. As mention above, these findings could be correlated with the fact that A549 cells are recognized to be more resistant to the action of p53 tumor suppressor since these cells express wild-type p53 [33, 34]. Recent gene therapy experiments using replication-defective adenovirus vectors to express p53 revealed that tumor cells with wt p53 are highly resistant to the apoptotic effect of Ad-p53 both in culture and in tumor xenograft models [35, 36]. Resistance

to p53-mediated apoptosis in A549 may be attributable to tolerance to high-level p53 expression or efficient degradation of exogenous p53. In fact, Lu and collaborators (2002) found that inhibition of Mdm2 expression in A549 using antisense oligonucleotide induces growth arrest and accumulation of p53, suggesting that although Mdm2 is not overexpressed in this cell line, it is still important for regulating endogenous p53. Therefore, they also successfully tested the possibility that Mdm2 may play a role in causing resistance to exogenous p53 expression and consequent function [34].

As revealed by western-blot analysis, the p53 production following gene transfer of sc pDNA is lower than that attained for HeLa cells. This may impose a lower cytotoxic activity from the p53 tumor suppressor, and consequently a lower therapeutic outcome. Likewise, this behavior could also be correlated with the lower cellular uptake of pDNA-liposome formulations as displayed in Figure 4.

Regarding the obtained results, it was verified that the p53-pDNA delivered was not able to promote the desirable apoptosis in this kind of cells. The low cellular uptake is a critical point in this result, what could be improved by using more effective delivery technologies [6], than the used in this research work, the LP200. Concerning that, it is reasonable to associate the lower expression of p53 protein, when compared with HeLa and hFib, to the scarce pDNA cell entrance. with HeLa and hFib, to the scarce pDNA cell entrance.

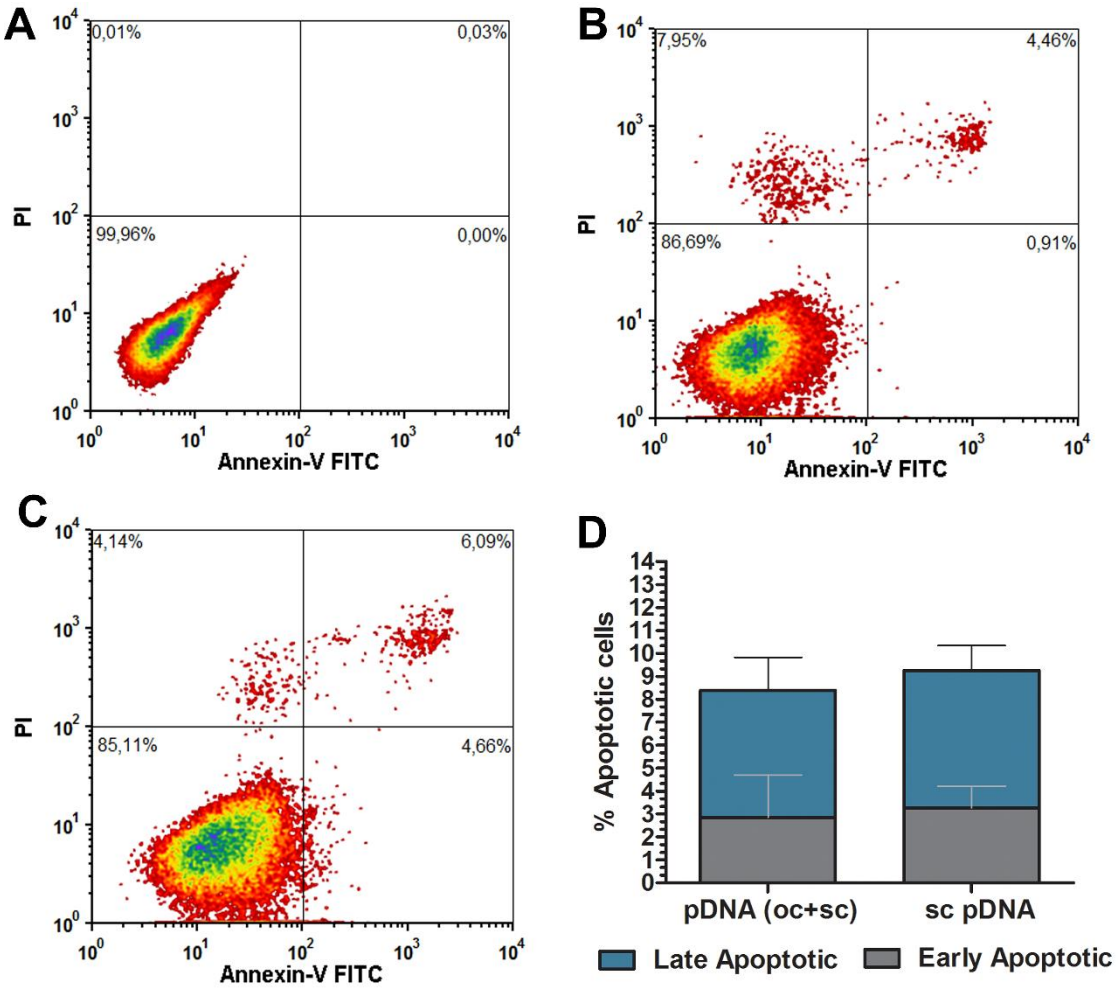


Figure 7 - Flow cytometry analysis of non-tumoral human dermal fibroblast (hFIB) apoptosis following transfection with different formulations of p53-pDNA gene expression vectors. (A) Density dot plot of non-transfected cells, auto-fluorescence. (B) Density dot plot of cells transfected with pDNA formulation containing native pDNA topoisoforms (sc + oc). (C) Density dot plot of cells transfected with sc pDNA topoisoform previously purified by L-methionine affinity chromatography. (D) Analysis of apoptotic cells in hFIB cells transfected with different pDNA biopharmaceuticals. Data are presented as mean ± s.e.m., n=3.

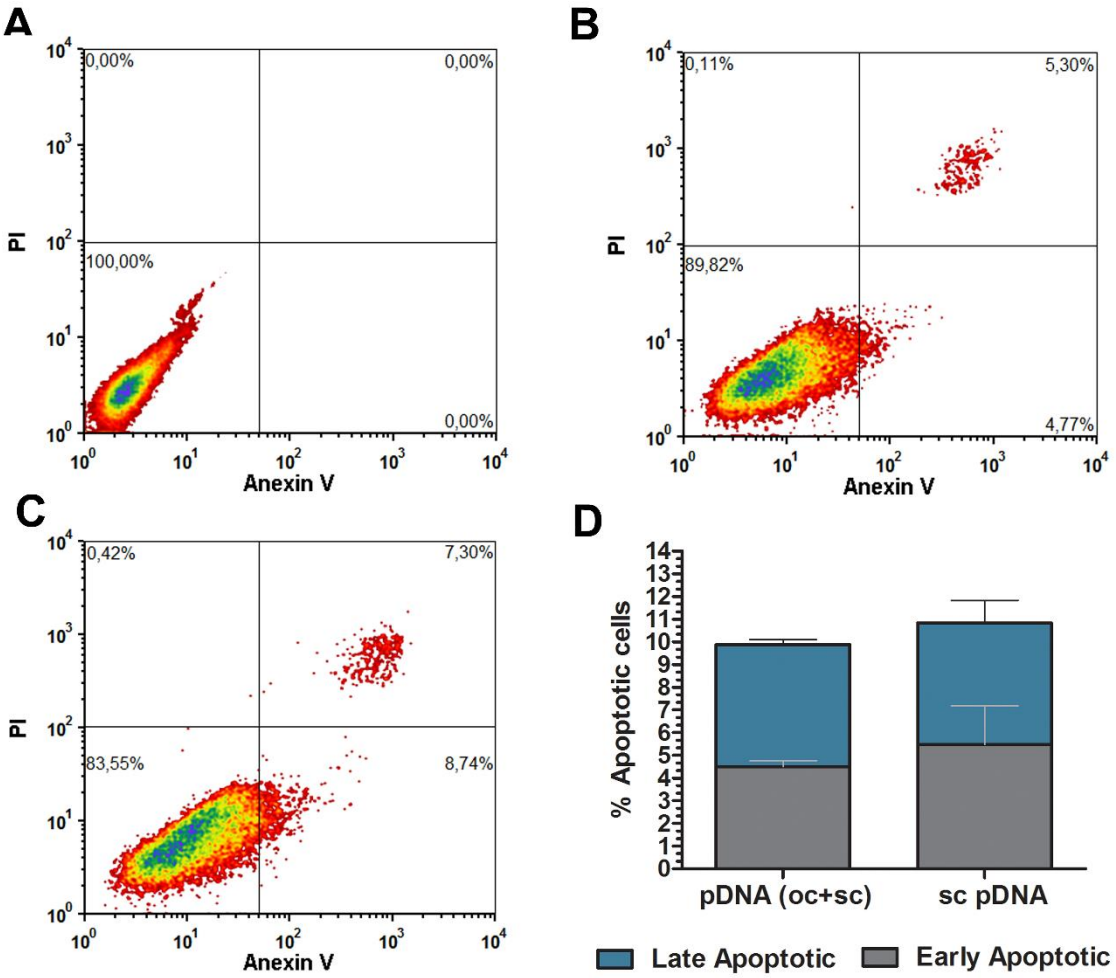


Figure 8 - Flow cytometry analysis of A549 non-small lung cancer cells apoptosis following transfection with different formulations of pDNA gene expression vectors encoding the tumor suppressor p53. (A) Density dot plot of non-transfected cells, auto-fluorescence. (B) Density dot plot of cells transfected with pDNA formulation containing native pDNA topoisoforms (sc + oc). (C) Density dot plot of cells transfected with sc pDNA topoisoform previously purified by affinity chromatography. (D) Analysis of apoptotic cells in A549 cells transfected with different pDNA biopharmaceuticals. Data are presented as mean ± s.e.m., n=3.

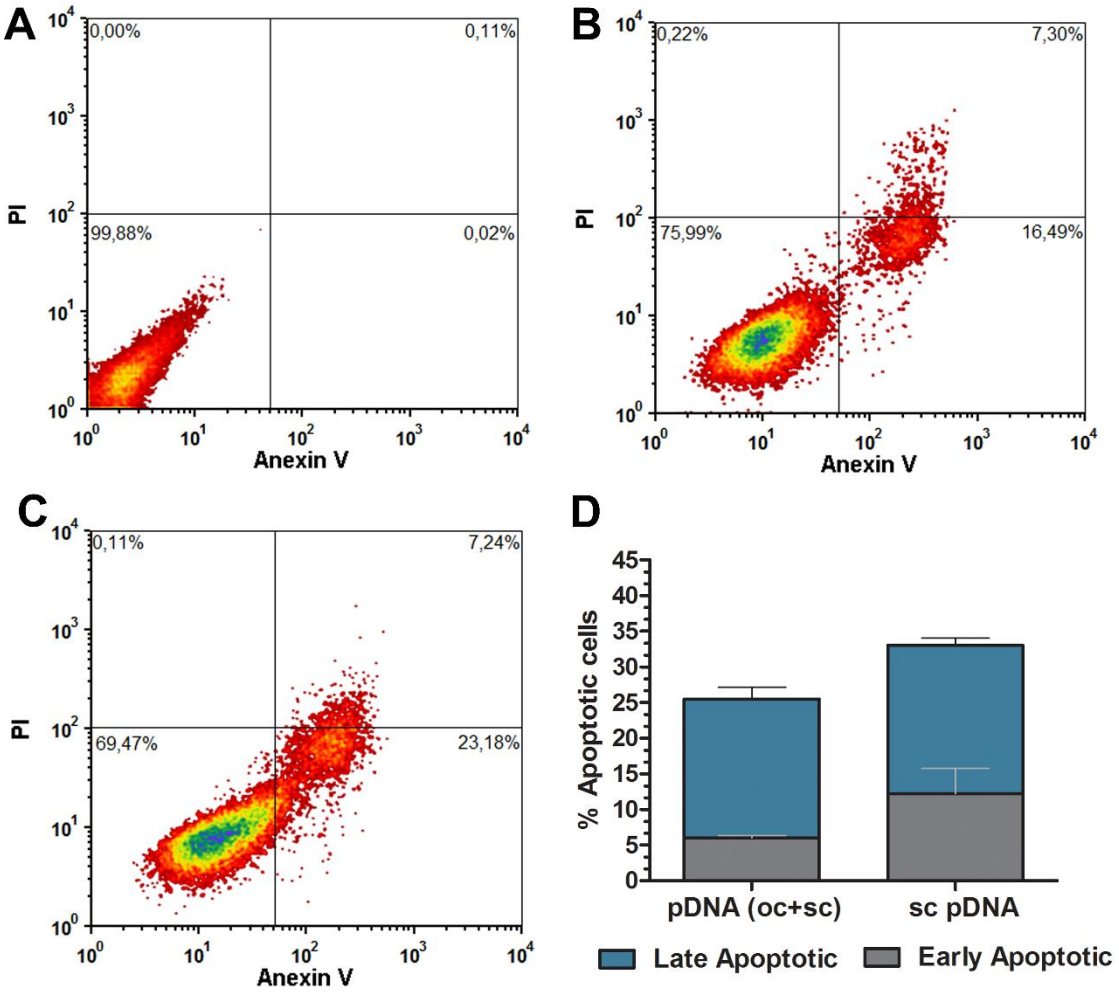


Figure 9 - Flow cytometry analysis of HeLa cells apoptosis following transfection with different formulations of pDNA gene expression vectors encoding the tumor suppressor p53. (A) Density dot plot of non-transfected cells, auto-fluorescence. (B) Density dot plot of cells transfected with pDNA formulation containing native pDNA topoisoforms (sc + oc). (C) Density dot plot of cells transfected with sc pDNA topoisoform previously purified by affinity chromatography. (D) Analysis of apoptotic cells in HeLa cells transfected with different pDNA biopharmaceuticals. Data are presented as mean ± s.e.m., n=3.

Conclusions

p53 is one of the most well-established tumor suppressor proteins and an overwhelming amount of data suggests that p53 inactivation is virtually necessary for tumor development and progression. Without p53 inactivation, oncogenic cells would inevitably undergo rapid cell cycle arrest and/or apoptosis. In roughly half of all tumors, p53 is mutated, which abolishes or greatly inhibits its normal cellular functions. In the remaining tumors, the activities of p53 are most likely inhibited by other means such as inactivation of downstream signalling or increased p53 degradation.

In this study, we took advantage of amino acid-nucleic acid biorecognition affinity chromatography to isolate sc pDNA vectors and evaluated their biological performance in different cell lines, using a commercially available liposomal delivery system. As demonstrated, the biological performance of pure sc p53 pDNA was higher than the native samples which could be seen by the reinstatement of p53 protein expression. Moreover, it was observed that transgene expression is highly dependent on cancer type and on the purity of the pDNA biopharmaceutical. These findings are important for raising awareness on the importance of the topoisoform of pDNA preparations in the development of more effective p53-based cancer therapies. Overall, it was found that the best results were achieved when using the sc pDNA for cells transfection and that the p53 expression was significantly higher in HeLa cancer cells, also followed by a higher apoptosis level, suggesting that p53-based transgene therapy may be particularly effective in this cancer type. In the future, it could be interesting to evaluate the performance of sc p53-pDNA in more cell lines and with different non-viral delivery systems so as to ascertain the realistic potential of this therapy.

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Paper VII

Plasmid DNA nano-complexation with Chitosan and Polyethyleneimine

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Abstract

Gene therapy could be simply defined as the strategy for the introduction of a functional copy of the desired genes in patients. However, this simple definition hides very complex processes related to the design and preparation of the therapeutic gene as well as the difficult search and development of suitable gene delivery vectors. Within several non-viral vectors, nanocarriers have offered an ideal platform for the incorporation of all the desirable characteristics into a single gene delivery system. Concerning this, the present study explores and compares nanocarriers based on the complexation of plasmid DNA with chitosan (CH) or polyethyleneimine (PEI) polymers in order to search for the suitable vector in terms of encapsulation efficiency, zeta potential and size. The cytotoxic effect of the best ratios was accessed and, for p53 encoding plasmid nanoparticles, the ability to promote protein expression was evaluated. Overall, the best properties were found for the ratio 7.5 for CH and 10 for PEI nanoparticles. Also, it was showed that CH nanosystems are more efficient when compared with the PEI polyplexes, resulting in higher P53 protein expression. Actually, cells transfected with CH p53-pDNA nanoparticles presented an increase of about 54.2% of P53 expression against an increase of 31.96% achieved with the PEI p53-pDNA nanocarriers.

Keywords: Chitosan, Gene therapy, Nanocarriers, Polyethyleneimine, Transgene expression.

Introduction

Cancer is originated by genetic alterations caused by inherited or environmental factors and consists of an uncontrollable growth of cells in the body which could lead to organ failure or in a more drastic scenario to death [1]. Concerning this, in the past years, several kinds of treatments have stood for the treatment of solid tumours and haematological malignancies. Currently, treatments like chemotherapy lead patients to a huge diversity of side effects and, in some extreme cases, patients could develop other cancers due to this drastic and longstanding treatment [2]. Regardless, scientists are working on finding more effective alternatives, able to relieve suffering or death caused by cancers. Among these alternative treatments, gene therapy has emerged as a novel and promising approach.

Therefore, cancer gene therapy consists in the introduction of genetic material into the patient´s body being the main goals of this therapy the reduction of tumour burden, the increasing of life expectancy and finally, the improvement of the quality of life of the treated individuals. This therapy intends to initiate tumour self-destruction, down-regulate angiogenesis and/or metastasis, enhance the anti-tumour activity of the immune system, suppress the function of an activated oncogene, or restore expression and/or function of tumour suppressor genes [3]. Between the genes evaluated and applied in cancer gene therapy, p53 is one of the most studied.

p53 is a very important tumour suppressor gene and, it is mutated or deleted in approximately half of all human cancers, showing its crucial role in preventing or control cancer progression [4]. Researchers have demonstrated that in response to stress signals, p53 can induce cell cycle arrest and subsequent DNA repair, senescence or apoptosis, depending on the level of cellular damage and context. When this protein presents some abnormality in its activity, some mutated DNA is not repaired, and this can result in genomic instability and potential cancer development. Also, if cells present mutated or inexistent p53 they are able to escape the apoptosis pathways and proliferate indefinitely. Thus, p53 plays a key role in the prevention of carcinogenesis [5]. Regarding all the mentioned before, the restoration of the functional p53 levels is crucial for the cancer treatment.

The delivery of a gene is a complex operation where several obstacles must be overcome to reach the target human cell nucleus where the transgene should be transcribed correctly. Concerning that, different approaches could be applied in gene delivery. First, the naked DNA could be directly injected into a tumour. This approach is limited to tissues reachable by direct injections (like skin or muscle) and also, it is unsuitable for systemic delivery due to the presence of serum nuclease [6], which would immediately degrade the therapeutic gene. Second, viral vectors could be applied for transferring the genetic material into the host cells. These vectors are biologically effective systems for the achievement of high efficiencies for

both gene delivery and expression. However, the use of viral vectors has some drawbacks associated namely, the possibility to provoke immune responses, the high cost and the difficulty for the preparation and also, the limit to insert only small amounts of genetic material into the cells. Finally, non-viral vectors have stand as safer alternatives to be applied for plasmid DNA (pDNA) transfer [7]. These delivery systems are usually cationic polymers or lipids with the ability to interact with the negatively charged DNA through electrostatic interactions leading to polyplexes and lipoplexes respectively. Non-viral vectors are usually safe (causing a low immune response), are easily prepared, have a low production cost and also, they can be easily produced at large quantities. Other important characteristic about these vectors is the ability to transfer different and large transgenes, being also able to be stored for long periods due to their stability [8].

To eliminate different cancer types, treatments must be applied systematically and therefore, must be targeted to cancer cells. Concerning this and, to enable an easy and safe systemic gene therapy, stable and non-viral gene vectors have been developed to encapsulate and deliver foreign genetic materials in specific cells such as cancerous cells.

Meanwhile, the systemic targeting of the bloodstream is a real challenge for these non-viral vectors since they need to survive in the bloodstream without being degraded or captured by cellular defence mechanisms. Also, when they reach the tumour site, they need to go across the tissue and bind specifically the target cells. After this internalization process, they need to extravasate some intracellular barriers such as the endosomal escape, the cytoplasm traffic and finally they need to enter the nucleus [9]. The ability of these non-viral vectors to overcome these barriers will dictate their efficiency and effectiveness.

Barriers to intracellular trafficking in gene delivery

After the carrier targets the tumour site, there are other major barriers that can limit the movement of the vector within the tissue. These barriers are dependent on the kind of tumour cells since their extracellular composition and structure can vary significantly. In addition, there are also several intracellular hurdles (Figure 1) that need to be overcome for a successful gene delivery.

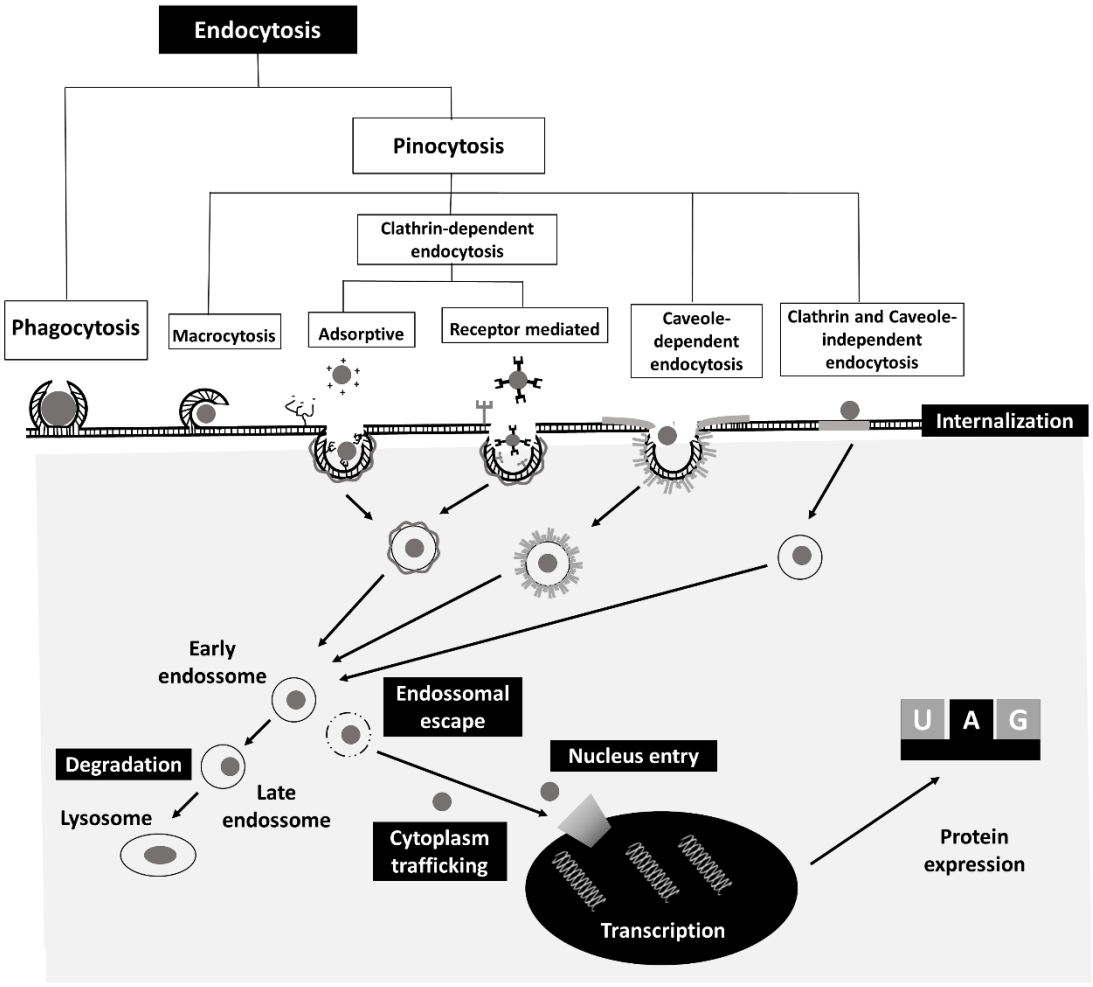


Figure 1 - Schematic representation of the gene delivery process, showing the internalization of a nanocarrier (Adapted from Morille et al. (2008) [10]).

Cellular uptake

The traveling pathway of the polyplexes and lipoplexes is not fully understood, however, the fusion of vesicles with the plasma membrane is perceived as a better route, since it avoids endolysosomal compartments, limiting the contact with the acidic environment which could lead to degradation of the cargo [6]. Several electron and fluorescence microscopy studies have shown the cationic carriers in intracellular vesicles, near the cell membrane, which suggests an entry by endocytosis. Actually, endocytosis is the major path for carriers to cross the cell membrane and is a process generally categorized in phagocytosis or pinocytosis [9].

Phagocytosis is an entry process predominantly used by large particles and it usually happens on phagocytes, macrophages, neutrophils and monocytes. In this case, there is a membrane

extension responsible for enclosing the nanoparticle and then internalize it, creating a phagosome with a diameter that can range from 0.5 to 10 μm. These phagosomes will fuse to lysosomes being the material inside the phagosome destroyed by acidification and enzymatic digestion in the lysosomes. Thus, this cell entrapment process is highly undesirable for gene delivery, due to the final degradation step [11, 12].

Therefore, pinocytosis stands as the most desirable route for the cationic carriers enter in the cell. This process is present in all types of cells in four different ways, such as clathrin-dependent endocytosis, caveolae-dependent endocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis [9]. The most suitable and common entry mechanism for cationic systems seems to be the non-specific adsorptive endocytosis, like clathrin-coated pit mechanisms, since negatively charged glycoproteins, the proteoglycans and the glycerophosphates that are present in the cell membrane, have the ability to interact with the positively charged systems [13]. However, if nanoparticles present specific targeting moieties at the surface, they could be specifically recognized by cell surface receptors which enables them to enter into cells through adsorptive and receptor-mediated endocytosis. Finally, macropinocytosis is the responsible for the carriers internalization through surrounding them with the fluid into large vacuoles [9].

Endosomal escape

As previously referred, endocytosis is the main uptake mechanism for nanoparticles, being the endosome capture, in this case, the major barrier to an efficient transfection. The main drawback associated to the transfer of the endocytic vesicle to lysosomes consists on the drop of the pH inside the endosome from 7 to 5 before the fusion with lysosomes. This lower pH and the hydrolytic enzymes are the main responsible for quick degradation of the DNA [6]. Although the mechanisms for endosomal release of DNA by cationic polymers are not completely understood, there are several hypothesis to explain the process, like “proton-sponge” effect, pore formation in the endosomal membrane, fusion in the endosomal membrane and photochemical disruptions. Among all these processes, “proton-sponge” effect also known as the pH buffering effect, is one of the most trustable ones. This hypothesis is used to explain the disruption of endosomes by cationic polymers with ionizable amine groups. To accomplish that, the ATPase enzymes present in the endosomal membranes transport protons from the cytosol to the vesicle, resulting in an acidification of this compartment. Concerning this, different polymers with a large number of secondary and tertiary amines can buffer the pH which will lead ATPase to transport more protons in order to reach the desired pH. This sudden increase of protons within the vesicle will result in an influx of counter ions that will be responsible for osmotic swelling and rupture of the endosomal membrane. Finally, polyplexes will be released in the cytoplasm [10].

Cytosolic transport and nuclear entry

After the release in the cytoplasm, carriers must be able to travel through cytosol until find nucleus and finally deliver the DNA. However, this process is very slow which makes DNA molecules very susceptible to nucleases. Among the critical parameters during the transport across the cytoplasm there are the rate of mobility, the size and the spherical structure of the carrier [14]. Concerning this, the use of cationic polymers for the DNA delivery offers protection against the cytoplasmic nucleases and also leads to an increase in the nanocarrier mobility. Also, some studies have reported that some cytoskeletal components such as the anionic microtubules could help DNA in a free form or complexed with a carrier to move across the cytoplasm [6].

Finally, DNA vectors must overcome the nuclear membrane and reach the nucleus. In case of non-dividing cells, the vector should enter through the nuclear pores (that have approximately 10 nm). In case of smaller molecules, they should easily diffuse inside the nucleus however, for larger molecules, nuclear localization signals must be activated to enable the transport [15]. In case of dividing cells, it is much easier for carriers enter the nucleus, as this can occur during the mitosis when nuclear permeability is enhanced [16].

Aspects influencing the systemic delivery

It was already reported that only 0.1% of the microinjected DNA in the cytosol is able to reach the nucleus. When nanoparticles are used for the delivery of DNA, the ability to reach the nucleus increases to 1%. In general, the transfection rate and the ability to reach nucleus is still extremely low and is very ineffective. Concerning this and, being this nuclear entry such a huge barrier, there is still the need to create systems able to improve the DNA transport to the nucleus [17]. Scientists proved that the nanoparticles entry is affected by several physicochemical characteristics like the size, charge, shape and also, by the endocytic machinery of the target cells [10].

The size of the carrier will be the main responsible for the entry pathway and, it should range between 10 to 500 nm. For example, large nanoparticles will enter via macropinocytosis while particles with 100 nm will probably be engulfed by clathrin endocytosis and, particles with a size ranging 60-80 nm will use the caveolae-mediated endocytosis as entry mechanism [11]. Moreover, the surface charge of the nanocarriers showed also to be a crucial parameter. Since cell membrane presents negatively charged elements, cationic nanoparticles will strongly interact with cells, which leads to a quick entry [17]. Concerning the neutral nanoparticles, any pH can modulate charges in order to promote the interaction with cells. In these cases, hydrophobic or hydrogen bonds could act to promote the interaction [9]. For anionic

nanoparticles, the endocytosis occurs due to the interaction with the positive domains of proteins present on the membrane. The chemical composition of the nanoparticle surface also influences its hydrophobicity. Hydrophobic nanoparticles present a higher affinity for the cell membrane when compared with hydrophilic nanoparticles. Also, hydrophobicity leads to an improvement of cell uptake in the kinetics and in the amount. On the other hand, hydrophilicity increases the lifetime of the nanoparticles in the blood [18].

It is also important to refer that the success or failure of the gene delivery depends on all these parameters but it is also intrinsically dependent on the target cells. For example, if cells do not present the necessary proteins involved in the specific endocytic pathway it will be very difficult or impossible for carriers to enter in cells by endocytosis. Additionally, some factors such as cell density and hormones, can affect the phenotype of cells and further affect the endocytic pathway. Therefore, normal and tumour cells present notable differences being important to target tumour cells exploring their specificity [19].

Polymers for p53 encoding plasmid delivery

The DNA specific delivery in the target cells to reach the nucleus is a mandatory procedure to achieve a therapeutic effect. Therefore, and summarizing, an ideal DNA carrier should promote a good cargo protection, an excellent colloidal stability, a high cellular uptake efficiency, an efficient lysosome escape and finally, an effective nucleus import and DNA unpacking. Therefore, it is extremely important to use and develop delivery systems that among all the characteristics described, also enable a good endosomal escape, and have the ability to target the cell/tissue of interest using and hydrophilic protective corona for minimizing the non-specific interactions.

Until now, for the delivery of p53-pDNA several polymers have been applied. For example, in 2011 Sharma and collaborators, injected poly(lactic-co-glycolic acid) (PLGA) nanoparticles combined with p53-pDNA in mice. The animals were treated with p53-nanoparticles by either local (intratumoral injection) or systemic (intravenous) administration. During this research work, they found that a single intravenous dose of p53-nanoparticles was enough to reduce tumour growth and improving animal survival [20]. In the past, also linear or branched polyethyleneimine (PEI) was extensively studied in a wide range of molecular weights, as p53-pDNA *in vitro* delivery vector [21, 22]. However, some concerns regarding the high toxic behaviour of high molecular weight PEI have limited the application. On the other hand, low molecular weight PEI demonstrated to be less toxic but showed poor transfection activity [23]. The natural cationic polymer, chitosan (CH), is a linear polysaccharide extensively used for several biomedical and pharmaceutical applications due to the advantageous properties like

biocompatibility and muco-adhesivity [24, 25]. For example, in 2011 Gaspar and co-workers used a chitosan carrier to deliver the p53 encoding plasmid. With the use of this p53-nanoparticle delivery system, they were able to reinstate the levels of the p53 protein expression [21]. However, some limitations to the use of CH have been reported namely its poor solubility in physiological condition due to its pKa value (about 6.3–6.4) [26]. More information about the carriers used until now for the p53-pDNA delivery can be found in Chapter II, namely in paper II. In this chapter it was also described the use of other complementary therapies (like chemotherapy) as well as the different modifications in the carriers (like the use of receptors in the nanocarriers surface) in order to improve the therapeutic effect of the different p53-nanocarriers.

The aim of the study

In this study CH and PEI were used in the production of different plasmid nanocarriers. Therefore, the work started with the optimization of the parameters for complexation between these polymers and plasmids of different sizes, in order to find the suitable conditions in terms of encapsulation efficiency, and nanocarriers zeta potential and size. Then, the best formulations were biologically characterized and, for the p53 encoding plasmid nanoparticles, the ability to promote protein expression was evaluated.

Materials and methods

Materials

The NZYtech Maxi Prep Kit was purchased to NZYTech (Lisbon, Portugal). The 6.07 kbp pcDNA3-FLAG-p53 Addgene plasmid 10838 [27] and the 8.702-kp p1321 HPV-16 E6/E7 Addgene plasmid 8641 [28] were obtained from Addgene (Cambridge, MA, USA). Resazurin sodium salt, CS (Mw = 50–190 kDa) and all the reagents used in bacterial amplification were obtained from Sigma-Aldrich (St. Louis, M.O., USA). PEI (Mw = 10 kDa) was purchased to Polysciences. The DNA ladder was obtained from Bioline (London, UK). The Fluorescein isothiocyanate (FITC) was purchased to Sigma-Aldrich, the Lipofectamine 2000 was purchased to Thermo Fisher Scientific (Lisbon, Portugal) and the Hoechst 33342® was purchased to InvitrogenTM Molecular ProbesTM (Carlsbad, CA, USA). p53 (human) ELISA kit was purchased to Enzo Life Sciences (Farmingdale, USA). All reagents were of research grade and used without further purification.

Methods

Plasmids production and pre-purification

The pcDNA3-FLAG-p53, the p1321 HPV-16 E6/E7 and the PVAX-GFP plasmids were amplified in *E. coli* DH5α. The growth was carried out at 37 ºC, 250 rpm, in Erlenmeyer flasks with 250 mL of Terrific Broth medium (20 g/L of tryptone, 24 g/L of yeast extract, 4 mL/L of glycerol, 0.017 M KH_2HPO_4, 0.072 M K_2HPO_4) supplemented with 30 µg/mL and 100 µg/mL of ampicillin for pcDNA3- FLAG-p53 and for p1321 HPV-16 E6/E7, respectively. For PVAX-GFP, bacterial growth was performed by using 1 L shake flasks with 250 mL of Luria-Bertani medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl), supplemented with kanamycin 30 µg /mL. The cells were grown until the log phase (OD 600nm ± 9). Finally, the cells were collected by centrifugation and stored at -20 °C.

To obtain the pDNA, cells were disrupted by alkaline lysis and the different plasmids were pre-purified with the NZYTech kit according to the supplier protocol in order to obtain the native pDNA (sc and oc isoforms). Basically, after an alkaline lysis the pDNA was bound to the NZYTech anion exchange resin under an appropriate low-salt and pH conditions. Then, the impurities were removed by a medium salt wash and finally the plasmid was eluted through the increase of ionic strength.

Chitosan and PEI polyplexes production

The production of pDNA loaded polyplexes was performed using a complexation method where electrostatic interactions occur due to the positive charge present in the protonated amine groups of each polycation (N), and the negative charge of the phosphate groups of DNA backbone (P). Basically, to determine the specific N/P ratio, the mass of one DNA phosphate group was used and, regarding the pH applied during the formulation of the polyplexes, it was also considered it anionic charge density (near to 1.5 as reported by Mel'nikova and collaborators in 2000 [29]). To the positive charges, the calculations were based on the pKa and the molecular weight of each polycation [30]. Then, 20 µg/mL of pDNA and 10 mg/mL of two different polycations (chitosan and PEI) were prepared in sodium acetate buffer (0.1 M sodium acetate/0.1 M acetic acid, pH 4.5). The desired polymer solution was individually prepared in accordance with the N/P ratio to be tested (the applied ratios varying between 0.1 and 50). After, 100 µL of the previously prepared ratios of the polymer solution were dropwise added, during 1 min, in 400 µL of the previously prepared pDNA solution, under stirring. Then, the polyplexes were incubated at room temperature, for 15 min and then recovered by centrifugation at 14 000 rpm, 10 min. The unbound pDNA was then quantified using UV spectrometry and the encapsulation efficiency (EE) of at least three repetitions was determined through the following expression: EE% = [(Total pDNA amount – pDNA supernatant amount)/Total pDNA amount] × 100.

The different polyplexes were also evaluated through its zeta potential and hydrodynamic diameter. To perform this evaluation dynamic light scattering (DLS) using a Zetasizer Nano ZS particle analyser (Malvern Instruments, Worcestershire, UK), equipped with a He-Ne laser, at 25 °C was used. To perform this analysis particles were resuspended in ultrapure water. All the experiments were performed in triplicate and were analysed through Zetasizer software v 7.03.

Cell culture and transfection

Cell culture experiments were performed with HeLa cancer cell line. DMEM-F12 medium supplemented with 10 % v/v heat activated FBS and with streptomycin (100 µg/mL)) was used for cell culture at 37 °C, under a 5 % CO_2 humidified atmosphere. Initially, cells were seeded in 25 cm3 T-flasks until confluence was reached. Afterwards, the cells were sub-cultivated by incubation on 0.18 % trypsin (1:250) with 5 mM EDTA. The *in vitro* transfection experiments were carried out by seeding 1×104 cells in a 96 well plate with 200 µL of DMEM-F12 complete medium and incubated for 24 h. Then, medium without FBS and antibiotic was used to promote transfection. The transfection of pDNA was then performed with the previously prepared polyplexes. Briefly, the polyplexes were resuspended in 500 µL of Opti-MEM® I medium, and a volume corresponding to 0.14 µg of DNA was used for each well of the 96 well plate. The

nanoparticles were incubated for a period of 6 h after which the medium was exchanged to DMEM-F12 complete medium.

Cytotoxicity

The cytotoxicity of different polyplexes formulations was evaluated by using the resazurin assay. For this evaluation, HeLa and hFIB cells were seeded in 96-well plates as described above. Resazurin (10 µL, 2.5 mM) was added to each well two days after transfection. The plate was then incubated in the dark for 4 h, at 37 °C, in a humidified atmosphere of 5 % CO_2. After incubation, resofurin was measured using a plate reader spectrofluorometer (Spectramax Gemini XS, Molecular Devices LLC, US), at an excitation/emission wavelength of λex = 560 nm and λem = 590 nm. Data represent the mean of three independent experiments.

Cellular Uptake Analysis by Confocal Laser Scanning Microscopy (CLSM)

First of all, the labelling of pDNA biopharmaceuticals with the FITC dye was performed to allow the follow-up of its cellular uptake and intracellular localization. Briefly, 5 µg of pDNA was added to 71 µL of labelling buffer (0.020 g of sodium (di)tetraborate in 1 mL of H2O) and 2 µL of FITC (100 mg of FITC in 200 µL of sterile DMSO). After this, the solution was stirred for 4h at RT, protected from light. Finally, 85 µL of 3 M NaCl and 212.5 µL of absolute ethanol was added to precipitate FITC labelled pDNA by overnight incubation at -20 ºC.

To evaluate pDNA-polyplexes cellular uptake kinetics, 1×103 cells were seeded in complete DMEM-F12 in Ibidi µ-Slide 8-well cell culture treated chambers (Ibidi GmbH, Germany) and cultured overnight. Transfection was performed when 70 % of cells confluence was achieved. Then, cells were incubated for 10 min with Hoechst 33342® (1:1000) (InvitrogenTM Molecular ProbesTM) and subsequently rinsed 3 times with PBS (pH = 7.4). Transfection was performed during 1h, with the different polyplexes produced with the different labelled-plasmids. Following the incubation period, the DMEM-F12 medium was exchanged and 4 % paraformaldehyde in PBS was used for transfected cells fixation (for 20 min, at RT). To enable a better visualization, transfected cells were washed three times with PBS. Visualization was finally performed using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., USA) equipped with a plane-apochromat 63×/DIC objective.

P53 expression by ELISA

The p53 ELISA kit (Enzo Life Sciences) was employed to quantify the P53 protein expression by cells transfection with p53-pDNA vector. Briefly, following transfection with the CH and PEI polyplexes, HeLa cells were rinsed with ice-cold PBS and homogenized in cell lysis buffer: 25 mM Tris-HCl buffer, pH 7.4; 2.5 mM EDTA; 1 % Triton X-100; 2.5 mM EGTA; 25 mM phenylmethylsulfonyl fluoride and complete, EDTA-free protease inhibitor cocktail (Roche).

Cell extracts were then centrifuged at 11500 rpm for 7 min at 4 °C and the supernatant was analysed using Bradford Protein Assay (BioRad) accordingly with the supplier's instructions. Then, the protocol supplied by Enzo life Sciences for the ELISA was applied and the P53 protein expression was finally measured in a plate reader spectrofluorometer (Spectramax Gemini XS, Molecular Devices LLC, US), using an absorbance of 450 nm. Data represent the mean of two independent experiments.

Statistical analysis

Each experience was performed at least two or three times. Data are expressed as a mean ± standard error (S.D.). The statistical analysis performed was one-way analysis of variance (ANOVA), followed by multiple comparison test Turkey. A p-value below 0.05 was considered statistically significant. Data analysis and statistical tests were performed in GraphPad Prism 6 software.

Results and discussion

Although proteins have been used for a long time to treat several diseases, protein therapy have demonstrated to have several drawbacks like the low bioavailability in the body, short life in the blood stream due to high rates of hepatic and renal clearance and *in vivo* instability as it can be degraded in the biological medium. To overcome the last two constrains, it is required to repeat recombinant protein-injection using high doses which can increase its toxicity and also increases the cost of the treatment. Therefore, it was thought that using the gene encoding the protein could be easier and more effective than using the protein itself. In fact, this is the basis of gene therapy, since a single gene can produce many proteins when it enters into a cell [8]. In this study, we take advantage of the p53 encoding gene and conjugate it with synthetic and natural polycationic polymers in order to search for a suitable platform to be applied in cancer gene therapy, based on the re-establishment of the p53 protein expression levels and activity.

Plasmid DNA loading efficiency in polymer nanoparticles

The encapsulation efficiency is one of the parameters that must be evaluated when it is intended to select and optimize nanoparticles for drugs or gene delivery. In fact, an ideal nanocarrier should be able to transport the highest amount of the drug or target biomolecule as possible.

To produce the nanocarriers used in this study, the pDNA/polycation complexes were formed by electrostatic interactions between the negatively charged pDNA phosphate groups and protonated nitrogen atoms of the chosen polycations, as previously mentioned [31]. The work started with a screening of different DNA:CH/PEI ratios in order to evaluate which one could lead to higher pDNA encapsulation. Although the main goal of this research was to evaluate the biological effect of the p53 encoding gene delivered by a CH and a PEI nanocarrier, in the initial screening, different pDNA were used to understand and relate the nanoparticles formation and characteristics with the pDNA length. The sizes of the plasmids under study were 3, 6 and 9 kpb for the PVAX-GFP, pcDNA3-FLAG-p53 and the p1321 HPV-16 E6/E7, respectively.

Through this screening it was verified that for CH nanoparticles higher encapsulation efficiencies were achieved between ratios 1 and 7.5 for the largest and smallest pDNA and, for p53 encoding pDNA the best ratios were between 2.5 and 7.5 (Figure 2). It was also verified an increasing tendency for the EE with the increase on ratios, until reaching the highest EE value, and then a sharp decrease of the EE was achieved for all the pDNA.

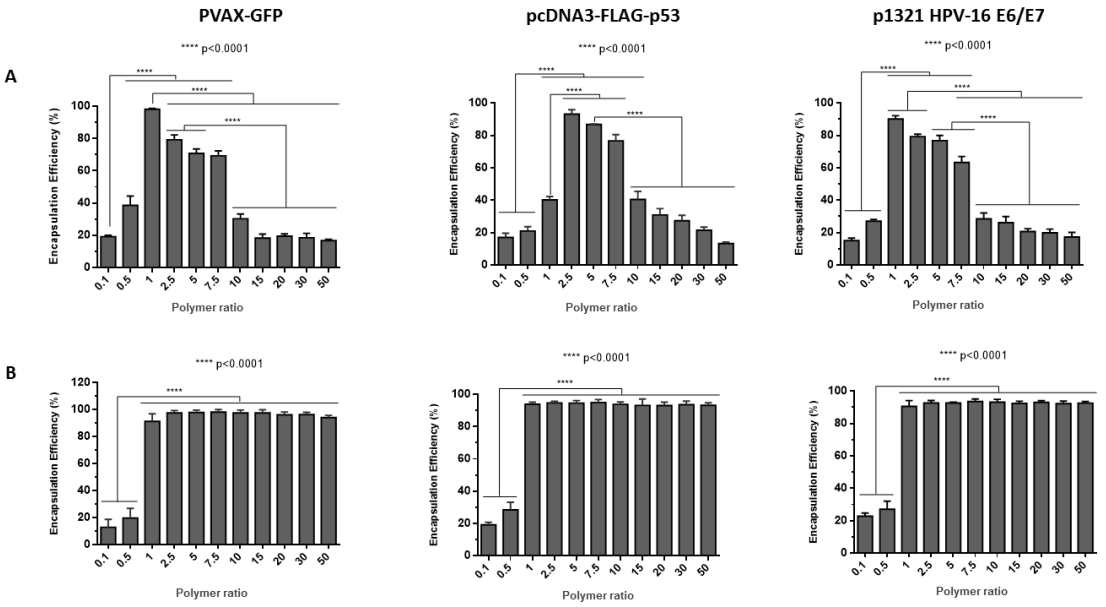


Figure 2 -Percentage of the Encapsulation Efficiency obtained for each formulated nanoparticle. A. CH formulated nanocarriers; B. PEI formulated nanocarriers.

Concerning the encapsulation efficiency achieved for the PEI nanoparticles, it was clear that for all the pDNA in study, the best ratios were above 1, and the maximum EE achieved remained constant for all the higher ratios.

Zeta potential and Hydrodynamic size measurements

The positive surface charge is a very important requirement to be considered for any carrier system to be used as an efficient gene delivery system. Moreover, the entry in the intracellular compartment will be facilitated if the delivery system presents a small size. Thus, the zeta potential and the size of the pDNA-loaded nanocarriers were evaluated. The values obtained from the zeta potential measurements represent the value of the electrostatic potential at the plane of shear and, zeta potential values near ±30 mV are representative of stabilized particles [32]. Figure 3 shows the zeta potential values obtained for each plasmid conjugated with CH or PEI. Through the analysis of these results it was possible to observe that for all the nanocarriers, the zeta potential presented negative values up until the ratio of 1. For the CH nanoparticles the highest value achieved was at ratio 2.5, and then the zeta potential decreased. It was also noticed that for the plasmid with higher molecular weight, the decrease on the zeta potential was more evidenced for ratios above 7.5, while, for the smaller plasmids, a more pronounced difference was achieved for ratios higher than 15. The PEI nanoparticles also presented a decrease on their zeta potential, but only for the highest ratio used (ratio of 50). This behaviour

was previously described for aqueous solutions of two oppositely charged polyelectrolytes. In 2005, Zhang and Shklovskii showed that an increase in the polyion concentration ratio increases the size of the complexes reaching a maximum at the isoelectric point and then decreases again accompanied by a "charge reversal" [33, 34].

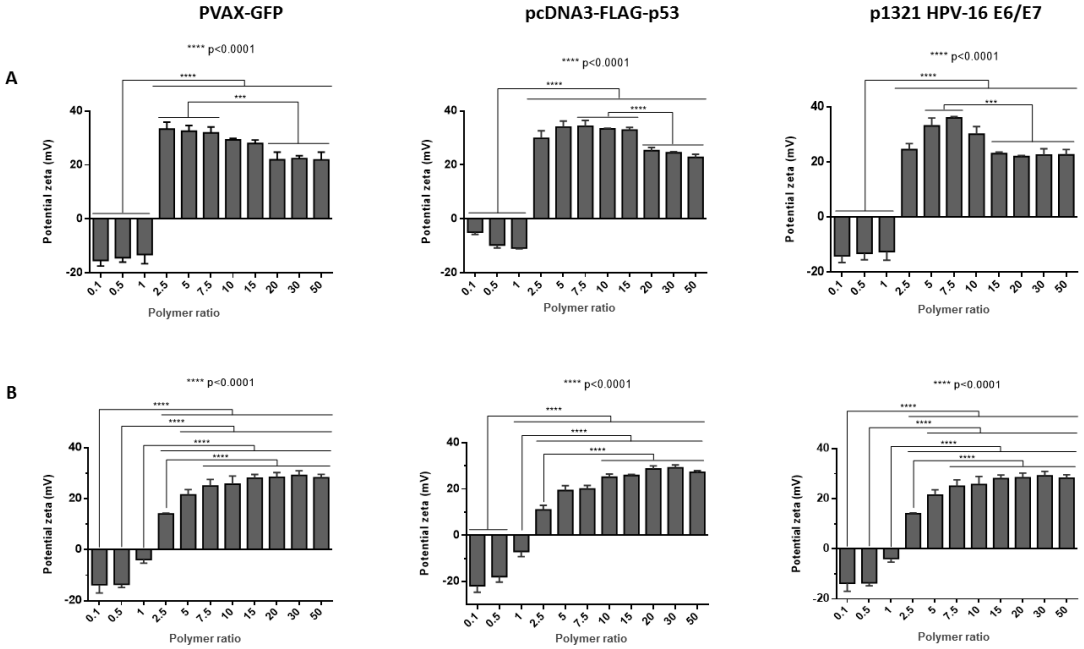
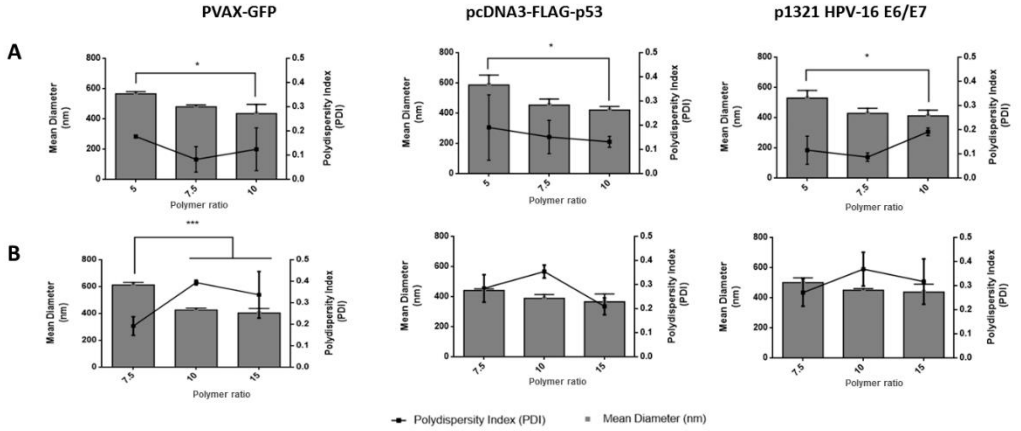


Figure 3 - Zeta potential measurements for the different polyplexes. A. Chitosan polyplexes; B. PEI polyplexes. Data are presented as mean ± S.D., n=3.

For PEI nanocarriers, the highest zeta potential measurements were observed for higher ratios when compared with CH nanoparticles being the values obtained very close to 30 mV. When the PEI values are closely analyzed, it is observed that the EE stabilizes when the maximum value is obtained and, as expected, the zeta graph also presents a stabilization of the charge. Meanwhile, the higher surface charge for CH polyplexes was achieved between ratio 2.5 and 10 and, among these ratios the desirable 30 mV was also found. After reaching this highest charge, the surface charge of the chitosan nanoparticles, suffer a slight decrease and then a stabilization with the increase of the polymer ratio. This decrease could hypothetical be related with a possible rearrangement in the nanoparticle structure enabling a higher presence of the DNA at the particles surface.

Previous literature data repeatedly showed that the DNA is completely complexed with PEI at slightly higher ratios (~3) [35, 36]. To follow the characterization of the nanocarriers, and considering the results achieved for the zeta potential and EE, the assessment of the hydrodynamic size was only performed for the three best ratios. Thus, for Chitosan polyplexes

the ratios of 5, 7.5 and 10 were selected, while the PEI polyplexes characterized were for the ratios of 7.5, 10 and 15.



C

	Chitosan ratio 7.5			PEI ratio 10		
	PVAX-GFP	pcDNA3-FLAG-p53	p1321 HPV-16 E6/E7	PVAX-GFP	pcDNA3-FLAG-p53	p1321 HPV-16 E6/E7
Mean diameter (nm)	478	453	428	425	388	449.5
PDI	0.08	0.15	0.086	0.393	0.354	0.368

Figure 4 - Mean diameter (nm) and Polydispersity (PDI) values of the selected nanocomplexes. A. Chitosan nanocarriers; B. PEI nanocarriers; C. Values obtained for the specific ratios chosen for the biological experiments. Data are represented as mean ± S.D., n=3.

The results achieved for the nanoparticles characterization (Figure 4) showed that the size of CH polyplexes decreased with the increase of the polymer ratio for PVAX-GFP and for p53 encoding pDNA and for p1321 HPV-16 E6/E7 the size decreased between ratio 5 to 7.5 and stabilized between ratio 7.5 to 10. In the case of PEI nanoparticles, de hydrodynamic size decreased between 7.5 to 10 and then stabilizes between 10 to 15, for all the plasmids in study, but with a more pronounced effect for PVAX-GFP. Overall, it was found that the smaller nanocarriers for PEI and CH were obtained at ratio 15 and 10, respectively, and when these polymers were complexed with p53 encoding pDNA. Moreover, the increase of polydispersity, namely in the PEI polyplexes, could be induced by interactions through the accessible unbound DNA chains in one polyplex with the unbound PEI of another polyplex. On the other hand, polydispersity at relatively low ratios (as in the case of CH nanoparticles) could be related with

the initially formed polyplexes since they tend to agglomerate due to the nearly neutral surface charge of the polyplexes [31].

Overall, the PEI nanocomplexes showed to be smaller than the CH nanocomplexes however, for the studied conditions, PEI systems presented a very high PDI when compared with CH.

As mentioned above, it is not easy or favourable the entry of large particles into the cell, and their structure is not stable enough to resist the low pH environment and lysosomal enzymes [37]. Thus, the complete characterization and selection of suitable nanocarriers must include the evaluation of the biological response of the cells transfected with the systems produced. In this case, the biological effect was assessed for the CH and PEI systems that presented the best properties in terms of EE, Zeta potential and hydrodynamic size. These conditions led to the selection of the 7.5 ratio for CH and 10 ratio for PEI.

Cytotoxicity evaluation

CH and PEI have been used in the delivery of DNA, and generally a comparable transfection efficiency is achieved for both polymers. In this work, and as explained above, the protein expression evaluation was performed for the ratio 7.5 for CH and 10 for PEI nanoparticles, due to their characteristics.

In vitro studies using cultured cancer and non-cancer cells to predict a human response typically play a vital role, and thus, besides the biologic effect evaluation it is also required to verify the toxicity levels of the formulations used, in this case the PEI and CH nanoparticles [38, 39]. Therefore, we compared the influence of the different polyplexes in HeLa cancer cells and human dermal fibroblasts (hFib) to search for potential intracellular toxicity often associated with these cationic nanocarriers-mediated uptake. The results obtained (Figure 5) showed that CH nanocarriers loaded with different plasmids did not induced toxicity, as the slight differences are not statistically significant in comparison to the negative controls.

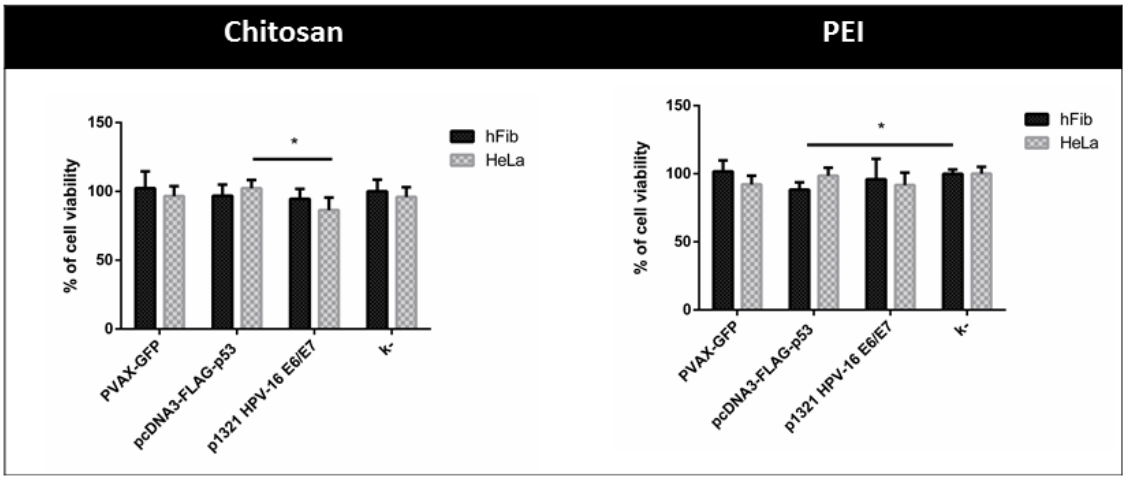


Figure 5 - Evaluation of cell viability following transfection with CH and PEI polyplexes loaded with PVAX-GFP, pcDNA3-FLAG-p53 and the p1321 HPV-16 E6/E7 in different cell lines (human dermal fibroblasts (hFib) and HeLa cervix carcinoma), at 48 h after transfection. Data are represented as mean ± S.D., n=3.

Concerning the PEI polyplexes, statistical relevance was found between the toxicity achieved for p53-encoding pDNA nanoparticles and the negative control but only for the hFib. This result could demonstrate a potential *in vivo* toxicity of this carriers for non-tumour cells which could be a major concern on its future application [40]. Actually, to overcome this drawback it should be required the development of more directed and specific delivery to the target cells.

The toxicity presented by the PEI polyplexes could be explained by the higher polymer ratio applied. In fact, in literature it was demonstrated that cationic polymers have cytotoxic effects at high concentrations because of their strong electrostatic interactions with the cell membrane, which can lead to destabilization and eventually rupture of the cell [41]. Also, some previous studies made attempts to obtain information about the composition of the PEI based polyplexes, demonstrating that large amounts of the PEI remain free and not involved in complex formation [35]. These free PEI molecules presented a higher toxicity than PEI bound as the polyplex [42].

Evaluation of the transfection efficiency

The uptake mechanisms for CH or PEI nanoparticles were already proposed, and these studies indicated that CH nanoparticles are mainly uptake through clathrin-based endocytic mechanism, while PEI polyplexes have presented preference for the caveolar pathway, in HeLa cells [43-45]. As referred, it is commonly accepted that complexes produced between DNA/cationic polymers are taken up by cells via endocytosis, however, further stages of their

endosomal release, namely the transport along the cytoplasm and also, the transfer to the nucleus and further DNA release, are less well understood. One of the mechanisms of endosomal release of the complexes into the cytoplasm is based on the proton sponge hypothesis [17]. In fact, research studies have demonstrated that several cationic polymers, such as PEI and CH have the ability to buffer endosomal acidification causing an accumulation of protons which promotes an influx of chloride anions. This process results in an increase of osmotic pressure which promotes entry of water and thereby the disruption of the endosome. It is worth mentioning that membrane destabilization by free polycation has been proposed as another mechanism that could also contribute to the endosomal release of the highly charged polymers [38].

Concerning this, the DNA delivery process in eukaryotic cells can be divided into three steps. The first step will be the entry of the nanoparticles in cells, the second one will be the transfer of DNA into the cytoplasm and finally, the third one will be the delivery of DNA into the nucleus. In the present work, to explore the DNA delivery process, the behaviour of these molecules was monitored the by confocal laser scanning microscopy. The cell live imaging after 1 hour of transfection (Figure 6) showed the entry into the cell for all the pDNA/cationic polymers studied. The carriers delivering the smaller pDNA promoted faster cell transfection than the ones delivering the largest pDNA for both CH and PEI. In fact, the system that seems to be the faster for transfection was the CH with PVAX-GFP. In this case, the transfection rate was very high and cells also presented a large amount of green inside the nucleus, which can be indicative of the efficiency of these systems entering into the nucleus. Through these results it is predictable that CH nanosystems could be more efficient on transfection when compared with the PEI polyplexes.

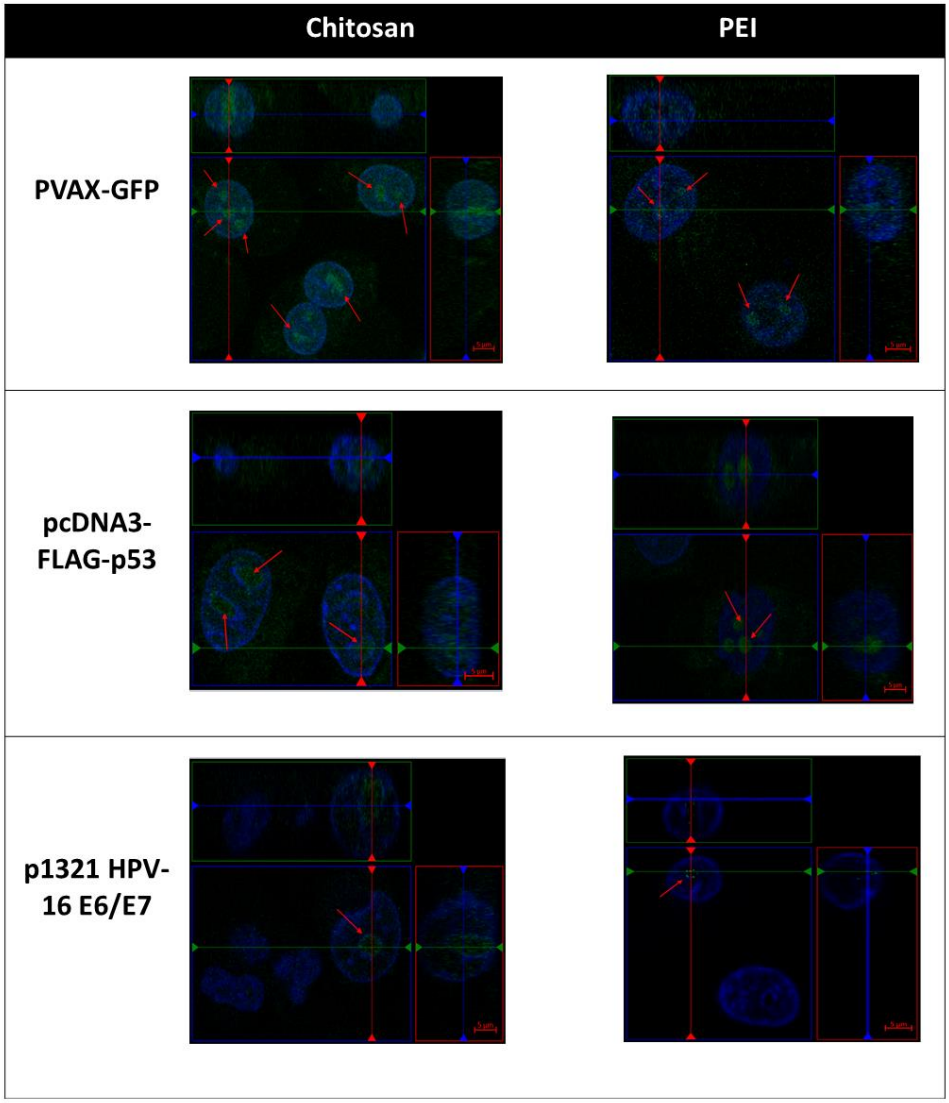


Figure 6 - Orthogonal view of live cell imaging one hour after pDNA CH/PEI complexes addition to the HeLa cervix carcinoa cell line. The blue staining represents the nucleus of cells and the green staining represents FITC-labelled pDNA.

Literature mentioned that lower transfection levels at higher polymer ratios may be induced by competition between the excess of cationic polymer present in the formulation. This extra polymer can bind to the cell surface, preventing complexes from being efficiently internalized [41]. This explanation about the excess of the polymer could be the reason for PEI nanoparticles present a slightly higher cytotoxic behaviour as well as a lower transfection rate, since for this case a higher ratio is applied when compared with the CH ratio chosen. Another reasonable explanation could be the slight difference in the zeta potential presented for both carriers. The CH polyplex presented the highest mV value and this could also favour the interaction with the negative cell membrane.

Concerning the largest pDNA studied, the behaviour for CH and PEI nanosystems was very similar, since in both cases cells presented a low transfection rate and, when transfected, few green was found in the nucleus which can suggest that low protein expression could be

achieved. In fact, different studies have already been performed in order to compare the influence of the pDNA size on the transfection effectiveness, being verified that actually, as short as the pDNA is, as higher is the transfection rate [46].

P53 protein expression

The formation of these polymeric nanoparticles occurs by the entrapment of the genetic material into the polymer matrix and thus, the release rate of pDNA is dependent on cationic polymer biodegradation. In fact, studies suggest that DNA unpacking is one of the major intracellular barriers to effective expression. It has been recognized that a balance between DNA protection and its ability to dissociate from the nanoparticles must be achieved to obtain efficient protein expression [41].

In order to evaluate the real ability of these systems to transfer genetic information across the cell until the nucleus, Hela cancer cells were transfected with both polymeric nanosystems developed on this research work and the P53 protein levels were measured through an ELISA analysis. The cell line was previously used to assess the expression of P53 after a transfection experiment and showed to be very sensitive to the p53 encoding gene treatment (paper VI). From the analysis of Figure 7 it is possible to observe that, although the chosen cells already present a basal expression of P53 protein, when transfected with CH p53 encoding pDNA nanoparticles, this level increases 54.2% and when transfected with PEI loaded with the same pDNA the protein level increases 31.96%, in comparison with the P53 basal level.

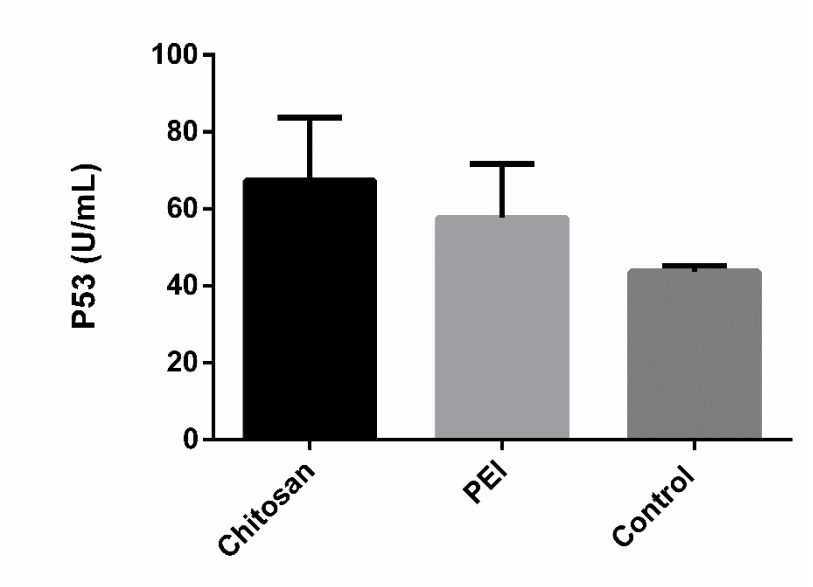


Figure 7 - Evaluation of p53 protein expression following administration of p53-pDNA polyplexes of CH and PEI in HeLa cervix carcinoma. Data are represented as mean ± S.D., n=2.

As mentioned, the free polycation in the DNA/polymer nanoparticles dispersion has shown to be mandatory to an efficient transfection since it helps on the lysosomal escape. In fact, some studies have shown that DNA/polycation complexes alone cannot trigger their endosomal escape through the proton sponge mechanism without a sufficiently high content of free polycation inside the endosome. When nanoparticles find the mechanism to escape from the endosome, the DNA released from the polycation, drives into the nucleus for transcription. However, it was also described that gene expression decreases when DNA was either tightly or loosely bound to the polycations, such as PEI or CH [47, 48]. The tight bound and highly stable polyplexes will be readily endocytosed but possibly not disassembled to access the transcription machinery. On the other hand, DNA weakly bound to the polycation will produce complexes that will dissociate prematurely in the medium and not even be endocytosed by cells [49].

Regarding our results from confocal microscopy analysis, it was possible to observe a slight increase of the p53 nanoparticles entrapment for CH nanoparticles, a behaviour that could suggest a lower P53 protein expression for the PEI nanocarriers. Besides this, we can also suggest that the decreased protein expression could be related with a weak linkage of DNA with PEI, when compared with the CH/DNA nanoparticles, which could lead to a rapid degradation of the delivered gene. This degradation process decreases the amount of viable gene to be used for the transcription machinery which will also decrease the amount of the final P53 protein produced.

It is also important to refer that using the DNA polymer complexation method, we were able to accomplish better P53 protein expression using the CH nanocarriers than Gaspar and co-workers (2011). In that previous study, an ionotropic gelation technique was performed between chitosan and TPP (used as polyanion) and where p53 encoding plasmid vector was added. To accomplish the production of the nanocapsules, TPP+pDNA were dropwise added in the chitosan solution. However, using this method the authors only accomplish 40% of P53 expression [25].

Concerning all the above mentioned, an effective gene delivery nanocomplex should provide an appropriate balance between the DNA and polycation, in order to promote not only binding strength and stability but also, guarantee that DNA has the ability to dissociate intracellularly for gene expression.

Conclusion

In order to promote gene transfer, non-viral systems such as nanoparticles have arisen as efficient and safe delivery tools. Several cationic polymers have been used in the nanocarriers development, and in this work different plasmids were conjugated with CH and PEI in order to search for the suitable nanocomplex combination. Regarding the surface charge and encapsulation efficiency characterization, it was verified that depending on the ratios used it could be possible to define suitable conditions to prepare positively charged nanocarriers highly efficient on DNA loading (around 80 to 100% for the best conditions), which is crucial for the application. Besides, the lowest size nanocarriers prepared with PEI and CH were obtained at ratio 15 and 10 respectively when these polymers were complexed with p53 encoding pDNA, indicating the possibility to promote an effective transfection. The cytotoxic profile showed biocompatibility for CH nanocomplexes however, for p53-encoding pDNA/PEI polyplexes it was verified a slight toxicity in normal cells which could be a handicap for future therapeutic application of these nanocarriers. Regarding the transfection efficiency, it was verified that all the pDNA /cationic polymers systems studied, were able to transfect the cells, but a higher efficiency was reached for the smaller pDNA. Finally, the ability of the polyplexes to promote P53 protein expression was also evaluated using HeLa cancer cells. From the results obtained, the P53 levels increased 54.2% and 32% when CH and PEI nanocarriers were respectively applied. Overall, it is possible to refer that an effective gene delivery nanocomplex should provide an appropriate balance between the DNA and the polycation. The chosen conditions for the polyplexes formulation should consider parameters such as EE, size, zeta potential and polydispersity being also needed to perform a careful polymer selection. Besides, given the presented results and the used complexation conditions, the formulation of nanoparticles using a 7.5 ratio of CH showed to be less cytotoxic and better in promoting transfection and inducing higher P53 protein expression than another PEI nanocarriers.

Acknowledgements

The authors would like to thank Dr. Thomas Roberts for providing the pcDNA3–FLAG–p53 construct through Addgene, ref: 10838, to Dr. Peter Howley for providing the p1321 HPV-16 E6/E7 vector through Addgene, ref: 8641 and also to Professor Miguel Prazeres for providing the PVAX-GFP. This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). J.F.A. Valente and A. Sousa also acknowledge PhD and Postdoctoral fellowships (Ref SFRH/BD/96809/2013 and Ref SFRH/BPD/102716/2014, respectively).

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Chapter IV

Concluding remarks

This work intended to understand the importance of using the sc pDNA isoform in gene therapy approaches and more specifically, highlight the impact of the use of this isoform when applied to restore the p53 levels. Furthermore, it has been reinforced that the use of chromatography using amino acids as specific ligands is a crucial factor in the final quality of the recovered sc pDNA sample being a major parameter to obtain the desired therapeutic results. Finally, efforts have been made to develop suitable nanocarriers, to protect the sc isoform of the plasmid encoding p53 throughout the delivery process.

To accomplish the proposed purification goals several supports/ligands were used in order to recover the sc p53-encoding plasmid isoform. Concerning this, the commercial L-methionine-agarose matrix was the first chromatographic support to be successfully applied. It was proved that this matrix allowed the isolation of the sc pDNA isoform from a native sample (sc + oc) but also enabled the recovery of the desired sc pDNA specie with 97% of purity from a complex lysate made of several nucleic acids like RNA, gDNA, and pDNA, by using a decreasing stepwise gradient of salt. Also, the quality control tests performed with the methodologies recommended by the regulatory agencies showed that the levels of impurities (gDNA, RNA, proteins, and endotoxins) were reduced or in some cases undetectable, when compared with the initial clarified lysate sample.

Then, another commercial matrix with O-Phospho-L-tyrosine ligands was used and CCF design was exploited. To achieve the optimal conditions for the isolation of the sc p53-encoding plasmid, the parameters “binding sc”, “elution sc” and “Temperature” were used and combined. The expected responses from the created model were the recovery yield and also the purity level. Therefore, the main results revealed that the model used was statistically significant and the central point was successful validated. The central point was repeated three times being achieved 49.7% of recovery yield and 98.2% of purity. The quality of the resulting sc pDNA was assessed, being confirmed that the levels of impurities are in agreement with all the parameters required by the regulatory agencies such as EMA and FDA. Overall, this method is faster and cheaper in the screening of the optimal sc pDNA binding/elution conditions. Also, regarding all the statistical results provided by the DoE tool, we can guarantee that we are working with the maximum performance conditions of the L-tyrosine chromatographic matrix.

Meanwhile, a macroporous resin from Tosoh was also modified with L-arginine to evaluate the performance of this support. Contrariwise to L-methionine and to O-Phospho-L-tyrosine supports, in this matrix it was used an increasing stepwise gradient of NaCl to isolate the sc p53-encoding pDNA from a native sample (sc and oc isoforms). The DBC of this new support was also characterized and the results indicated an improvement of more than 50% when compared with the commercial arginine-agarose matrix.

Concerning the above mentioned, several affinity chromatographic matrices/ligands could be successfully applied in the sc p53-encoding plasmid DNA recovery. Besides, adjusting the binding/elution conditions, we were able to favour some particular interactions. Regarding this, it was possible to observe that ionic conditions were predominantly exploited for the macroporous arginine support and hydrophobic conditions for L-methionine and L-tyrosine supports. In general, amongst the supports used under predominant hydrophobic conditions, the O-Phospho-L-tyrosine presented the best recovery yield and purity degree. However, this matrix uses large salt concentrations which from the sustainability point of view of the chromatographic process could lead to some issues. Meanwhile, the previously mentioned L-arginine macroporous support, has the ability to promote pDNA isolation in mild salt conditions. However, this macroporous resin was not yet studied for the sc pDNA isolation from complex lysates what is important to prove its ability for provide p53 sc-encoding pDNA that fulfil the quality requirements (like for example be free RNA, gDNA, proteins and endotoxins) of the regulatory agencies such as FDA or EMA.

Then, the biological activity of the sc-p53 encoding pDNA was evaluated and compared with a native sample. Through that study it was clearly demonstrated that the *in vitro* transfection with pure sc p53-pDNA resulted in a higher expression of the tumour suppressor protein in cancer cells when compared with the native pDNA samples (oc+sc topoisoforms). Also, p53 expression following transfection was significantly higher in HeLa cervix cancer cells in comparison to that obtained in A549 lung cancer cells. These results highlight the need to evaluate the performance of sc p53-pDNA in more cell lines and with different delivery systems so as to ascertain the realistic potential of this therapy. Overall, these findings emphasize the potential of sc pDNA gene-based therapy, also raising awareness of the need to adjust the therapeutics, considering the feature of high heterogeneity of cancer cells.

Finally, different cationic polymers (CH and PEI) where used to be complexed with different plasmids. From the results obtained it was found that PEI polyplexes promote some cytotoxicity in non-cancer cells which could be a major problem for their application *in vivo*. And, was also verified that the smaller pDNA promote higher transfection ability in HeLa cancer cells. Therefore, the ability of the polyplexes to promote P53 protein expression in HeLa cancer cells was assessed and an increase of 54.2% and 32% was verified when CH and PEI nanocarriers were respectively applied. From the obtained results, and regarding the complexation conditions applied, the formulation of nanoparticles using a 7.5 ratio of CH showed to be less cytotoxic and better in promoting transfection and P53 protein expression than another PEI nanocarriers studied. Moreover, from the obtained results, it was verified that an effective gene delivery nanocomplex should provide an appropriate balance between the DNA and polycation, in order to promote an adequate encapsulation efficiency, providing good binding strength and stability for the delivered DNA.

Overall, it was found that the chromatographic methodologies used enabled the successful isolation of the sc p53 encoding pDNA. Also, DoE demonstrated to be a very powerful and quick tool for the establishment of the optimal conditions for the sc p53-encoding plasmid purification. Concerning the biological activity of the sc p53 encoding pDNA it was clearly demonstrated that the biological performance of pure sc was higher than the native samples and also, it was observed that transgene expression is highly dependent on cancer type emphasizing the potential of sc pDNA gene-based therapy. Finally, polyplexes of CH could be used as safe and efficient nanocarriers since non-toxicity, good transfection and P53 protein expression was achieved when they were applied.

In this work, it was demonstrated that the particular use of the sc p53-encoding pDNA isoform is vital for achieving p53-based therapies with a higher success. Moreover, the nanocarriers applied in the delivery of this gene should be carefully chosen and the influence of this sc p53-encoding pDNA must be specifically studied concerning the kind of target cancer cells. Regarding this, if sc p53 encoding pDNA is applied in a completely designed and target therapeutics, it will be possible to be closer from the establishment of a more effective treatment for such complex disease.

Future Trends

Having into account all the work performed until now it could be of interest to perform some additional experiments concerning the two main fields explored in this doctoral thesis: the chromatographic development of new supports in order to optimize the sc p53 encoding pDNA isolation and also, the efficient delivery of this gene inside the nucleus of the cancer cells.

Regarding the exploitation of the chromatographic technic it is suggested the use of combinatorial libraries using as ligands different amino acids. Through this method, it is possible to significantly reduce the time and cost involved in this kind of screenings, rapidly finding the most suitable ligands combination for pDNA isolation. Besides, this conjugation of amino acids could lead to an increase of selectivity or recovery of the desired sc encoding-pDNA extract. In fact, studies have already been carried out using triazine to perform a combinatorial linkage with four amino acids already known to work alone as p53-pDNA adsorbents, namely arginine, histidine, methionine and tyrosine. Promising results were already obtained concerning the binding and the elution ability of some of the studied combinations.

The nanocarriers used in this study should be more developed and optimized, namely by considering the use of some molecules at their surface in order to promote a more target delivery, increasing the efficacy of the therapy. Regarding this, several molecules like AS1411 aptamer, transferrin or folic acid could be used to enhanced the penetration through cancer cells. With this strategy is intended to increase the specificity for cancer cells avoiding the delivery of the drugs in non-cancer cells and also avoiding the cytotoxicity and increasing the efficacy of the therapy. Likewise, the delivery of the p53 encoding pDNA could be conjugated with the power of chemotherapeutical drugs such as Doxorubicin or Gemcitabine in order to perform a more potent, specific and effective anti-tumour formulation. Overall, it is mandatory to perform more studies regarding the specific targeting of the nanocarriers applied mainly because it has been observed during this work that different cancer/non-cancer cells react differently when transfected with the p53-encoding plasmid DNA.