ลักษณะวิกฤติของการผลิตพลาสมิดดีเอ็นเอสำหรับการรักษายีน

CRITICAL ASPECTS OF THE PRODUCTION OF PLASMID DNA FOR GENE THERAPY

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บทคัดย่อ

พลาสมิดเป็น DNA วงแหวนสายคู่ที่นอกเหนือจากโครโมโซมวงใหญ่อยู่ในเซลล์ของแบคทีเรีย พลาสมิดนอกจากใช้เป็นพาหะในการถ่ายยีนในจุลินทรีย์ด้วยกันแล้ว ยังสามารถใช้ถ่ายยีนเข้าสู่เซลล์สัตว์เพื่อการรักษายีนที่บกพร่อง เช่น รักษาโรคพันธุกรรมฆ่าเซลล์มะเร็ง (cancer therapy) หรือเพื่อกระตุ้นให้สร้างระบบภูมิคุ้มกันโดยการใช้ DNA vaccine ซึ่งทั้งสองตัวอย่างหลังนี้เป็นเทคโนโลยีล่าสุด พลาสมิดใช้ได้ง่ายโดยวิธีการฉีดสารละลายพลาสมิดบริสุทธิ์เข้าเนื้อเยื่อของร่างกาย เมื่อเทียบกับการใช้ไวรัสเป็นพาหะในการถ่ายยีนซึ่งอาจจะก่อให้เกิดปัญหา เช่น การสร้างระบบภูมิคุ้มกันเพื่อทำลายไวรัสตัวพายีน การผลิตพลาสมิดในระดับอุตสาหกรรมทำได้ง่ายกว่าการผลิตไวรัส ปัจจุบันกำลังมีการทดลองใช้พลาสมิด สำหรับรักษายีน โดยเฉพาะอย่างยิ่งรักษามะเร็งและการทำ DNA vaccine ในระดับคลินิคมากกว่า 50 แห่ง ทั่วโลกประกอบกับความสำเร็จของ human genome project เพื่อที่จะหาลำดับเบสของดีเอนเอมนุษย์ ส่งผลให้มีความคาดหวังที่จะรักษาโรคพันธุกรรมต่าง ๆ โดยวิธีการรักษาจีน ดังนั้น มีความจำเป็นที่จะต้องผลิตพลาสมิดบริสุทธิ์ให้ได้ปริมาณมากพอกับความต้องการ ปัจจุบันยังไม่มีกระบวนการผลิตที่เป็นการค้า ขั้นตอนการผลิตทำได้โดยการโคลนพลาสมิดที่มี therapeutic gene ใน *E. coli* แล้วเพาะเลี้ยง โดยวิธีการหมัก (aerobie fermentation) ต่อจากนั้นเป็นขั้นตอนการสกัดโดยการใช้

lysis reactor ชนิดที่เป็นถังกวน (stirred tank) ขั้นตอนการสกัดประกอบด้วย การแขวนลอยเซลล์ในบัฟเฟอร์ การสลายเซลล์ โดยการใช้ด่างแก่ และการตกตะกอนโปรตีนและเศษของเซลล์ กระบวนการดังกล่าวทำในถังกวน ดังนั้นการผสม (mixing) จึงเป็นปัจจัยที่สำคัญและต้องทำในสภาวะที่เหมาะสม ความจริงแล้วข้อจำกัดมีมาก เช่น คุณสมบัติของ lysate ซึ่งเป็นสารเหลวที่ viscoelastic เวลาของการทำปฏิกริยา อุณหภูมิ ต้องเหมาะสมเพื่อให้ได้พลาสมิดที่ดีทั้งปริมาณและคุณภาพ สภาวะที่เหมาะสมในการสกัดพลาสมิด วิธีการ ขั้นตอน รวมทั้งการออกแบบ และพัฒนาปฏิกรณ์ชีวภาพสำหรับงานนี้ได้บรรยายรายละเอียดไว้ใน Chamsart (2001)

ABSTRACT

Plasmids are double-stranded circular extrachromosomal DNA molecules that are stably maintained within bacteria. They are normally used as vectors for gene transfer in bacteria and, in addition, spectially, in animal cells which are for gene transfer therapy to treat genetic diseases including cancer, AIDS, and DNA vaccine. Gene transfer therapy is the latest technology to treat incurable inborn diseases. When compared to viral gene transfer system, administration of plasmid DNA can be done readily by injection into body tissue. Moreover, viral vector may cause problems e.g. immumogenicity and virulent reversion. The process for the production of plasmid DNA is much more easily than that for viral gene delivery system. There are about 50 clinical trials to date worldwide using plasmids as gene vehicles, in particular, for cancer therapy and DNA vaccine. With the success of human genome project in order to identify every human DNA sequence (genes), there has been an anticipation to treat genetic diseases using gene transfer therapy systems. This indicates a need for the production of large amount of purely therapeutic plasmid DNA. However, no commercial process for the production of plasmid has been reported. The process steps comprise cloning therapeutic plasmids in bacteria and growing them using fermentation technology. The lysis steps using stirred tank lysis reactor comprises cell suspension, lysis, and lysate neutralisation in order to precipitate proteins and cell debris. For those steps, mixing performance is one of the most important issues in order to operate under optimum lysis conditions. In fact, there are a number

of limitations e.g. lysate rheological properties (i.e. viscoelasticity), reaction (lysis) time, temperature, shear effects on chromosomal DNA and neutralised flocs etc. These affect plasmid yield and quality. However, the optimum conditions for plasmid extraction and specified process steps and especially for lysis reactor design have been defined in Chamsart (2001).

INTRODUCTION

A plasmid is a double-stranded circular extra-chromosomal DNA molecule growing itself up to thousands of copies in a bacterium. A recombinant plasmid can be constructed by genetic engineering (gene splicing) of a foreign gene(s) onto it. Thus, thousand copies of a desired foreign gene can be obtained from a single bacterium. There are a number of foreign gene for genetically therapeutic use, e.g. cancer killer gene, a vaccine gene, a normal gene for a specific function, etc. Those therapeutic genes may be introduced into animal or human tissue using plasmids or viruses as a delivery system in order to try to cure, immunise, or correct a defective gene. This is the essence of gene therapy. Also, a broad definition of gene therapy given is the insertion of a functioning gene into the cells of a patient to correct an inborn error of metabolism or to provide a new function in the cell. The two major methods of gene transfer are (i) DNA mediated gene transfer, in which pure DNA i.e. plasmid DNA (pDNA) is introduced into target cells, and (ii) viral mediated gene transfer in which recombinant viruses are engineered to carry specific foreign genes into target cells.

PLASMID AND GENE THERAPY

Plasmids mediate gene transfer. Administration of plasmids can be done readily by the injection of a pure plasmid solution into body tissue. Plasmid DNA is playing an important role in the development of gene therapy as the latest potential non-viral gene delivery system because of its ease of manufacture and administration compared to viruses. Furthermore, there are safety concerns about the use of viral vectors on virulent reversion and immunogenicity. As regulatory

approval is granted for an increasing number of non-viral human gene therapy clinical trials, there has been a corresponding rise in demand for pharmaceutical grade pDNA. Thus, there is a need to be able to manufacture large quantities of highly purified clinical grade plasmid in a reproducible and scaleable fashion. Nevertheless, no commercial processes have been reported to date though many clinical trials are in progress.

The process for plasmid production is much more easily scaleable than that for viral gene vehicle production. High copy number plasmids of small (~10 kb) relaxed types for gene therapy can be grown and amplified, preferably and advantageously in *E. coli* because of the long history of its safe use and its genetics are completely defined. Plasmids have been used in the laboratory for several decades for certain specific purposes i.e. molecular biological studies. Almost all of those species of recombinant (genetically modified) plasmids used in the laboratory at the moment originally came from *E. coli* plasmids so production in *E. coli* seems appropriate.

In fact, several attempts at gene therapy in humans were made as early as 1979 (Mercola and Cline, 1980). These experiments were unsuccessful and stimulated genetic manipulation in human. Since 1980, the replacement of defective genes has been accomplished in fruit flies and mice and the first clinical trials of gene therapy in humans were expected in 1986 (Olson,1986). However, the first experiments in which genetically manipulated cells were introduced into patients began in 1989, and the first clinical trials of gene therapy, for adenosine deficiency, began in September 1990 (Watson *et al.,* 1992). Various techniques involving non-viral plasmid vectors have been developed for transferring foreign genes into eukaryotic cells (Graham and Van Der Eb,1973; Neumann *et al.*, 1982; Felgner *et al.*, 1987; Tang *et al.*, 1996). The important of pDNA as a pharmaceutical substance has increased considerably since it was shown that naked pDNA with antigen genes injected into muscle tissue result in vaccination (Wolff *et al.*,1991; Vogel and Sarver, 1995; Donnelly *et al.*,1997). Hence, the possibility of using pDNA for human gene therapy and DNA vaccine was highlighted. A number of clinical trials have been started using these products (Alton and Geddes,1998).

With the success of the human genome project for characterising every human gene, there is now an anticipation that many incurable human genetic diseases including several cancers and AIDS can be treated. This idea, in turn, has created the need for the production of pDNA to the standards required by regulatory authorities for clinical products and for the pDNA to be produced in quantities orders of magnitude higher than previous achieved. Ayazi (1999) reported that currently, there are over 200 active clinical trials of gene therapy worldwide involving nearly 2500 specific trials. Prazeres *et al.* (1999) also reported that, in spite of considerable scientific effort over the past few years, no gene therapy product has yet reached the market, even though the first anticipated approvals were expected in the year 2000 with a market for these products exceeding 28 billion sterling pounds by 2010

THE PROCESS FOR THE PRODUCTION OF PLASMID DNA

A typical example of such a process involves the use of DH I *E. coli* as the host cells harbouring therapeutic plasmids, e.g. pTX 0161 plasmid, which has a cancer killer located on it. The development of industrial scale processes for these products offers a unique opportunity for manufacturers. Unlike the majority of bio-therapeutics such as proteins and peptides, pDNA is not itself the therapeutic agent, but a delivery vehicle for a therapeutic gene(s). Production processes are based around the manufacture, recovery and purification of the pDNA. Consequently, a single manufacturing process and facility, appropriately validated, can be used for any number of plasmids each carrying a different therapeutic gene. This approach in turn allows for development time for weeks rather than months or year for new clinical candidates.

Plasmids can be grown and amplify in *E. coli* by aerobic fermentation; for high cell density cultivation, fed-batch fermentation should be employed. For therapeutic use, the plasmids must be extracted and purified to meet the standard required for clinical trials. The production of pDNA creates a number of challenges when developing scaleable purification regimes for which the combined skills for the bioscientist and biochemical engineer are both needed if an efficient solution is to be be obtained. The plasmid has to be purified from the

remaining cell contents, the most relevant of which are chromosomal DNA (chDNA), RNA, lipololysaccharide. These unwanted substances are all large polymeric anions which can have similar electrochemical and physical properties to pDNA which can, therefore, cause problems during purification by, for example, anion exchange chromatography. Being polymers, they also have limited purification handles that can be exploited in high resolution in purification operations.

A typical commercial production processes as defined by Cobra Therapeutis Ltd., (UK first Gene Therapy Company) is given in Fig. 1 (Varley *et al.*, 1999). It involves the production of pDNA in *E.coli* cells using optimised fed-batch fermentation to give a yield in excess of 40 mg L^{-1} of fermentation broth (Chamsart, 2001). The cells harvested by either centrifugation or filtration and then re-suspended, by agitation, in a hypotonic buffer. RNA is then enzymatically degraded, by addition of RNase. The plasmid is then extracted by alkali-detergent lysis whilst mixing. The extraction is followed by weak acid neutralisation, again whilst mixing, which precipitates cell debris, chDNA and proteins. This precipitate is then removed by filtration or centrifugation. The plasmid is then purified from the filtrate using a variety of chromatographic operations. Anion exchange chromatography is the most commonly used capture step, followed by polishing to reduce lipopolysaccharide and RNA levels and to reduce levels of open circular pDNA.

CRITICAL ASPECTS OF THE PRODUCTION OF PLASMID DNA

Critical aspects of the extraction and purification steps are the mixing processes associated with cell lysis. The alkaline lysis is performed on a cell suspension; subsequent neutralization gives a precipitate of structure suitable for filtration. Scale-up of this lysis process presents six specific mixing challenges. The first of these is that significant changes in volume have to be accommodated. The initial cell suspension typically only represents 25 % of the final lysate volume. Secondly, these volumes have to be added and mixed within a limited time

period, in order to prevent the plasmid itself from being irreversibly denatured after extended exposure to the strong alkaline solution. Also, the mixing system must seek to minimise localised "hot spots" of high alkaline concentration, which may cause irrevesrsible denaturation, and degradation of the pDNA. Thirdly, it is observed that, on addition, therefore, it is necessary to mix these two solutions well if the alkaline lysis operation is to be completed after the optimal period of exposure to the alkaline conditions and if precipitation is to be achieved. Fourthly, it is believed that the denatured chDNA obtained from initial lysis, and both chDNA and the flocs generated during the neutralisation, are shear sensitive (Horn *et al.*, 1995; Varley *et al.*,1999). Consequently, mechanical shear should be minimised throughout both steps and particularly during neutralisation to prevent floc break-up which makes subsequent filtration difficult. Fifthly, during these operations, significant changes in the rheological properties of the solution are observed. The cell suspension has a milky-like properties, but on addition of lysis buffer, the solution develops a high apparent viscosity and other rheological complexities i.e. viscoelasticity. Finally, both mixing operations for lysis and neutralisation have to be carried out to completion for subsequent successful downstream operations. If the lysis of the cells in the lysis buffer or the neutralisation of the lysed cells is not complete, it will lead to the presence of viscous material in the final stage and consequent loss of plasmid yield.

Typically, the alkline lysis steps, conducted in a stirred tank, are critically dependent on developing a successful mixing strategy with the optimum lysis conditions. If the mixing is ineffective the lysate becomes almost impossible to filter and/or unprocessable. Such a strategy requires detailed information on the rheological properties of the liquids at various stages. Secondly, the optimised alkaline lysis conditions in terms of alkaline concentration, lysis time, temperature etc. are needed. Thirdly, mixing performance in a designed lysis reactor with a well-defined operation procedure based on fluid dynamic characterisation, is required for further scale-up. Fourthly, a filtration process to separate the unwanted flocculated materials, is needed. Finally it must be shown that all these steps give a satisfactory plasmid yield and do not lead to excessive chDNA and other impurities in the solution obtained from the filtration steps. However, all of these critical aspects have been experimented and then discussed in detail by Chamsart (2001) and Chamsart *et al.* (2001).

CONCLUSION

The possibility of the treatment of incurable inborn diseases including cancers and AIDS by using pDNA as a potential non-viral gene transfer system is introduced. Realisation of this possibility requires plasmids for clinical trials and potentially, for treatment in the next few years. The production processes for pDNA are based on alkaline lysis, which has a number of critical considerations, problems and bottlenecks. These limitations must be overcome to achieve a successfully scalable process. The process involves (i) biological material, i.e. *E. coli* strains, as hosts, and biochemical molecules, especially chDNA and pDNA which must be separated out during purification process, (ii) strong chemical reactions with extreme pH treatments, (iii) changes in fluid properties affecting fluid handling and mixing, etc.

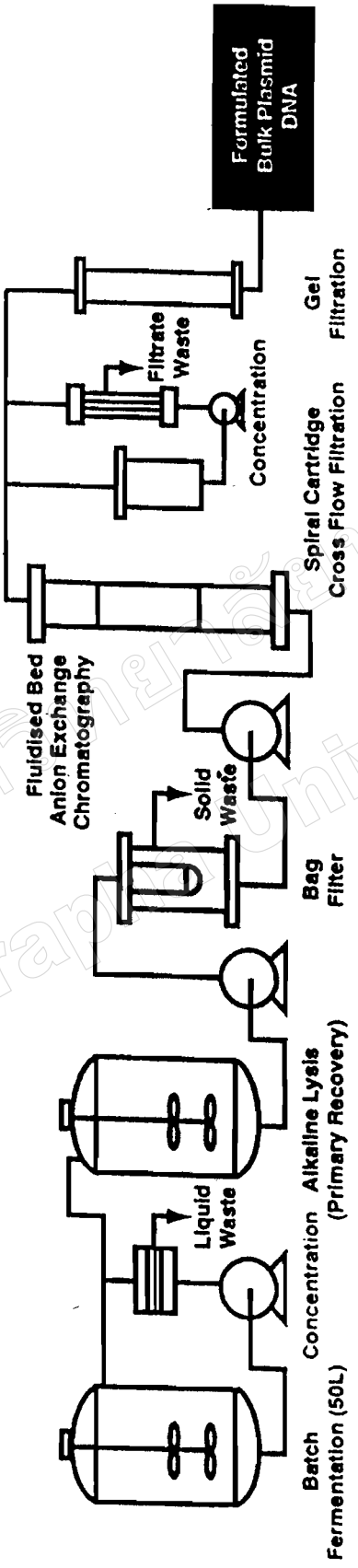

Fig. 1 Large scale process for the production of plasmid DNA (Varley *et al.*, 1999)

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