Advancement in Gene Therapy using Electroporated Microneedle Arraying Technology

Amruta Arun Dandekar*, Shweta S Jogi*, Trisha Saxena*,
*** Department of Pharmaceutical Sciences and Technology,**
Institute of Chemical Technology,
Nathalal Parekh Marg, Matunga,
MUMBAI-400019

Abstract- Introduction of genes into human body in order to replace the missing gene or to correct the defective one is the main goal of gene therapy. Gene therapy is gaining importance and popularity due to its ability of curing various genetic disorders which are otherwise untreatable. Hence, the techniques for insertion of such proteins and macromolecules into the body act as areas for intensive research. One of the techniques known as electroporation uses electric field for greater permeation but has some major drawbacks. Microneedling array is another promising technique to increase permeation. Thus, these two techniques when used in combination can be very effective in gene therapy. This paper not only reviews such systems but also provides a comparative study amongst the proposed systems, concluding that indeed gene therapy can be developed at a multifold rate by utilizing electroporated microneedle arrays.

Keywords: Electroporation; Microneedle Array; Gene Therapy; Macromolecule Permeation.

1. Introduction

The Skin is an easily accessible organ both at the molecular as well as the cellular levels and hence, it turns out to be attractive as far as the development of therapeutic and genetic medicines is concerned. The ability to target genes directly to the skin can be used extensively in Gene Therapy. Despite the potential applications for gene therapy, the stratum corneum proves to be a major barrier to the penetration of macromolecules and genes. [1]

Electroporation, a technique in which cells are exposed to an electric field so as to increase the permeability of the cell membrane [2] has been broadly applied to enhance the skin permeation of drugs. This technique creates short living hydrophilic pathways in the lipophilic transcellular region in the stratum corneum barrier which provide an alternative pathway for the drug to permeate. [3] Microneedle arrays are minimally invasive devices that bypass the Stratum Corneum, thus achieve systemic delivery by the transdermal route. [4,5]Microneedle Arrays are applied to the skin surface and their length is decided in such a way that they are long enough to penetrate to the dermis, but are miniscule enough to avoid puncture of blood vessels and sensitization of nerve endings. [6,7]

This review paper talks about the latest technology which combines the above procedures of Electroporation and Microneedle Arrays wherein the DNA coated microneedles are not only minimally invasive but also act as microelectrodes for electroporation (Shown in Fig. 1). Electroporated microneedle array is a thereby, one stop solution which is non-invasive and painless technique for introducing DNA in the skin with additional benefits of localized electric field due to its structure and composition. The paper further talks about an electroporation electrode based on flexible microneedle array for in vivo nucleic acid delivery, demonstrating its fabrication, modifications to the previous design to increase the efficiency of transfection and its potential future applications. Thus, the paper concludes by saying that electroporation using microneedle arrays as electrodes is one of the most technologically advanced and promising technique for gene therapy.

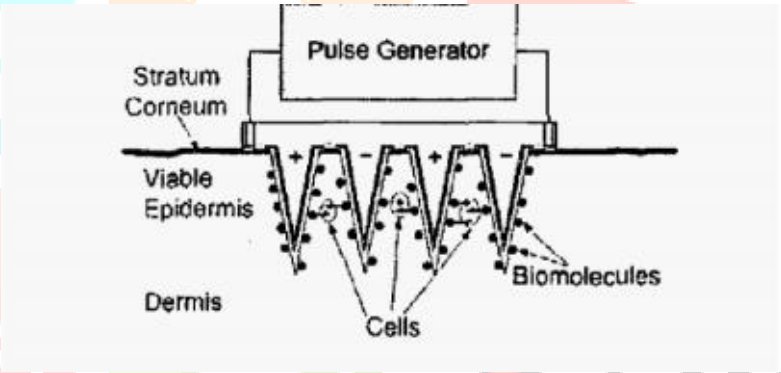


Figure 1 Conceptual use of microneedle array to electroporate cells in epidermis and thereby increasing the expression of DNA vaccine [8]

2. Latest Research:-

Studies in the past have declared electroporation as a promising technique for in vivo delivery of macromolecules like DNA proteins. [9] However, this technique has its own drawbacks like muscle twitching, immense pain and permanent damages like tendinitis. [10] Tackling these issues can be relatively easier if microneedles are used as electrodes for electroporation. A tightly packed arrangement of solid or hollow needles is inserted into the skin which electroporates it and subtly introduces the macromolecule into the dermis. To attain this result, the needles should be rigid, mechanically strong and electrically conductive

to achieve electroporation. Many recent studies have suggested various types and fabrication of such microneedles which would be effective for gene therapeutics.

1. One of the leading microdevices for electroporation is based on a technique known as metal transfer process for patterning the microneedle. The traditionally accepted process for metal patterning is photolithography which is usually employed for high precision devices. However, this microneedle employs bilayer micromolds which effectively creates polymer based electrically conductive microneedles which could easily permeate through the Stratum Corneum without mechanical failure.

   i) Fabrication:

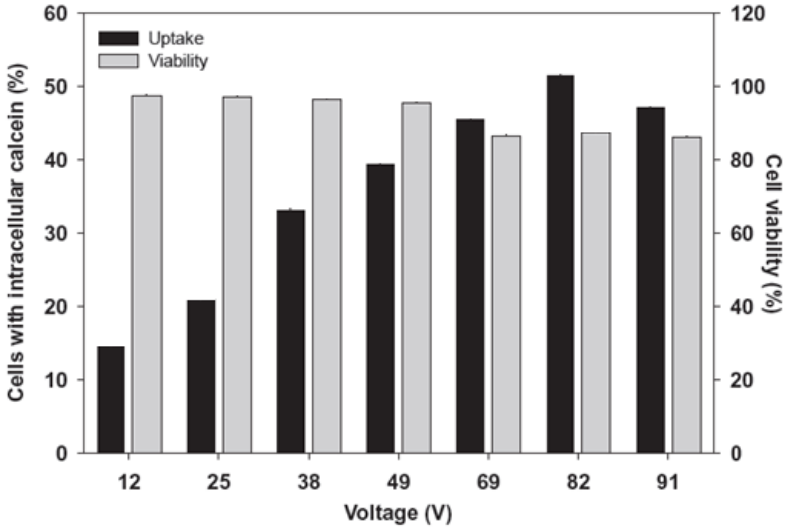
   There are four steps in the fabrication of the metal patterned microneedles. These are:-

   - Fabrication of the Rigid Mould: As told earlier, this device is fabricated using two layers. The first layer is formed by a polymer called SU-8. This polymer is, typically a negative-tone photoresist i.e. only the parts exposed to UV are cross linked and the remainder stays back in the solution. The fabrication of the second layer of this microneedle is done using the conventional methods of photolithography.
   - Fabrication of the Secondary Structure: The secondary structure fabrication includes depositing a Titanium or Copper layer onto the rigid mould which helped in the electrical conductivity of the needle.
   - Fabrication of the Metal patterned Microneedle Array: In this step, metal transfer micromoulding technique is employed to form hollow space within the needle. The target polymer (usually, a biocompatible polymer like polylactic acid) is then casted into the mould.
   - Fabrication of needles capable of electroporation: In this step, the microneedles are kept under laser light. This step forms microchannels which establish electrical connections necessary for electroporating live skin cells.

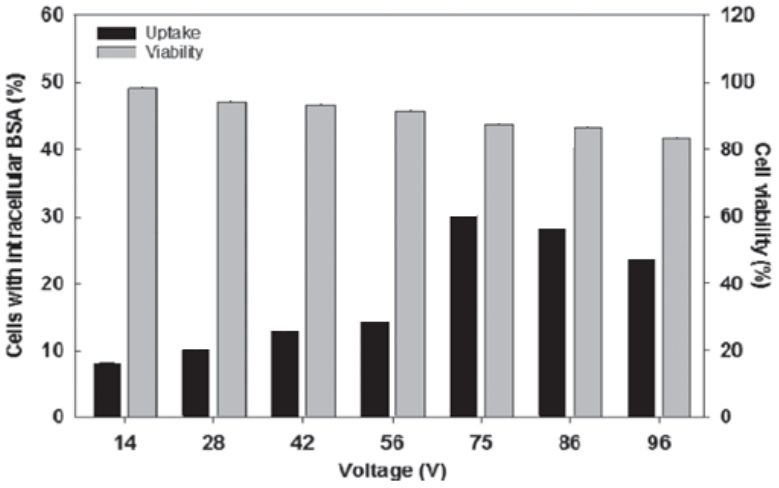
ii) Experimental validation of this microneedle device:-

- Insertion Test: Microneedles are subjected to insertion tests to ensure the mechanical strength of the microneedle. This particular microneedle successfully pierced the Pig Cadaver Skin and showed desirable results of electroporation even after multiple insertions.
- Extent of electroporation: The extent of electroporation i.e. the depth to which the protein had penetrated the skin was known by using a green fluorescent protein. The needle was coated with Calcien (Low Molecular Weight Protein) and a plasmid DNA encoded this protein (Shown in Fig.3a). The uptake of Calcien during this study increased till a certain voltage after which it slightly declined. The second parameter that was assessed was cell viability. It was observed that cell viability remained unaffected till a certain voltage and then significantly dropped. The cells were also subjected to a pulse voltage, but no change to cell viability was observed.

  Similar tests were repeated for a macromolecule called BSA as the applications of this therapy may involve the insertion of high molecular weight proteins (Shown in Fig.3b). Initially, the uptake graph was a weak function of voltage but it shot up and a certain voltage after which it almost remained constant. However, cell viability decreased with an increase of voltage, in cases of macromolecules. [11]



(a)



(b)

Figure 2 Experimental validation of metal patterned microneedle. (a) Graph depicting the effect of voltage on uptake of Calcien and subsequent cell viability. (b) Graph depicting the effect of voltage on uptake of BSA and subsequent cell viability. [11]

Advancements to the above needle were done to eliminate its short coming. SU-8 was replaced by PoIymethylmethacrylate (PMMA). The fabrication was done by micromoulding technology the steps for whom are as follows:-

Step 1: Release from the master PDMS mould

Step 2: Metal deposition

Step 3: Patterning using laser causing electrodeposition.[12] The process of fabrication of the microneedle is shown in the Fig.3.

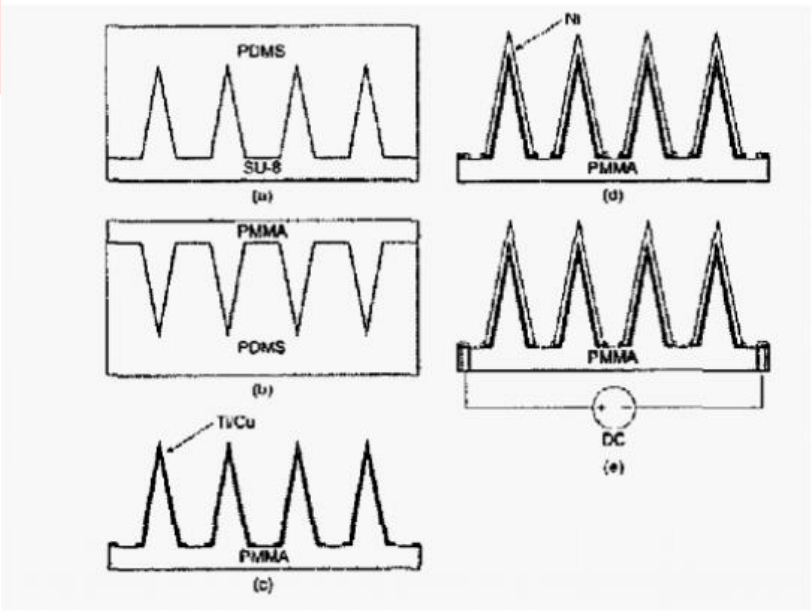


Figure 3 Schematic Diagram of the fabrication process.

i) Experimental validation of this microneedle device:-

- Insertion Tests:

This study was unique due to its approach of determining the extent

of electroporation. To test the electroporation ability of the microneedle array, an in-vitro assay was performed. This RBC lysis assay provides an easy way to quantify the electroporation effect. On electroporation, the RBCs rupture and release their haemoglobin into solution which was easily quantified using absorption spectroscopy. A RBC pellet was formed onto which the

proposed microneedles were connected. For each electroporation experiment, 25 microliters of concentrated RBC pellet was pipetted into the microneedle array which after pulse application was sent to a centrifuge tube. After centrifugation, 700 microliters of the supernatant was collected for quantifying the amount of haemoglobin using absorption spectroscopy at 575 nm absorption wavelength. The amount of haemoglobin in the samples was then calculated as a percentage of positive control which is reported as RBC lysis (%) the results of which are shown in figure 4.

- Extent of electroporation: To test the electroporation ability of the microneedle array, an in-vitro assay was performed. This RBC lysis assay provides an easy way to quantify the electroporation effect.

  On electroporation, the RBCs rupture and release their haemoglobin into solution which was easily quantified using absorption spectroscopy. A RBC pellet was formed onto which the proposed microneedles were connected. For each electroporation experiment, 25 microliters of concentrated RBC pellet was pipetted into the microneedle array which after pulse application was sent to a centrifuge tube. After centrifugation, 700 microliters of the supernatant was collected for quantifying the amount of haemoglobin using absorption spectroscopy at 575 nm absorption

wavelength. The amount of haemoglobin in the samples was then calculated as a percentage of positive control which is reported as RBC lysis (%).

The ultimate application of these microneedle electrode arrays is to cause reversible electroporation of living cells in the skin and thereby transfect those cells with DNA. Fig.5 shows electroporation of RBCs to cause cell lysis. The reason behind this observation is that irreversible electroporation of RBCs is much related to reversible electroporation of skin cells.

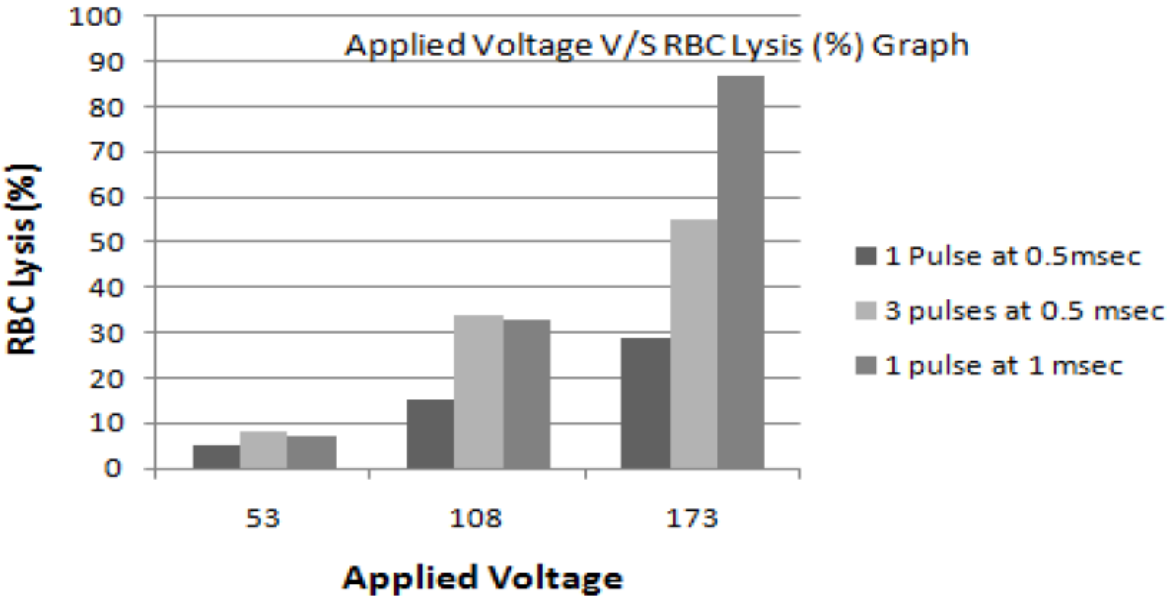


Figure 4 Haemoglobin released from red blood cells after electroporation using the microneedle electrode array.

- Electric Field Simulation: A 3-D finite element (FE) model was created using FEMLAB 3.1 to examine the electric field distribution around the microneedle array. The simulation results demonstrated that with 100V applied between two adjacent rows, maximum field strength of approximately 10kV/cm near the microneedle electrodes was generated which was too high for electroporation for delivering biomolecules, while it was suitable for cell lysis. At the middle of two adjacent rows, the field strength was about 4kV/cm. These field strengths scale with the applied voltage and can therefore be optimized for specific applications.

2. As the research continued, electroporation coupled with microneedling array technology established itself as a technique with promising future.[13,14,15] An effort was made to introduce a hollow microneedle array with small volume of liquid into the dermis, which is summarized below:- [16].

i) Fabrication- Matrices made of Radel R .Gold was deposited on the needles for better conductivity along with biocompatibility. Radel, being insulating material was given a conductive layer of gold by sputtering deposit method to make microneedles conductive. Titanium was used as adhesive for gold deposition. As Radel is water adsorbing material, components were heated before deposition. Milling of gold and titanium

layers was done to have electrical tracks. In order to insulate electrical tracks from skin silicon oxide was deposited. Finally microneedles were rinsed in deionised water, bathed in TFTN titanium etchant and again rinsed. To apply electrical pulses, a modified Cliniporator was connected to microneedles. The microfluidics reservoir was attached to hollow microneedles to allow fluid injection. [17]. A polymethylmethacrylate (PMMA) box containing the microneedle array, the Microfluidic system and the electrical connections was equipped with a semi rigid polyamide capillary for the pneumatic activation of the micro reservoir. A conventional syringe was attached with 20 kPa pressure for fluid infusion. The system was made more efficient with addition of vibratory inserter. Along with this a PIC 16F877 Microcontroller, was used to control vibration and injection duration. [16] The schematic of array is as shown in fig 5.

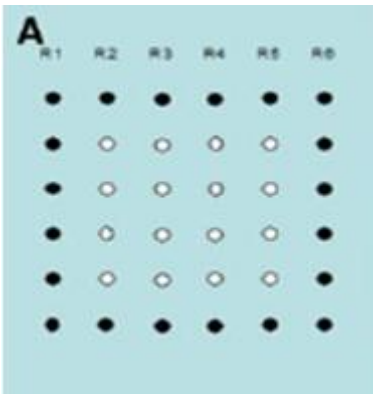


Figure 5 Microneedle array representation (where filled circle is plain microneedle and hollow circle is hollow microneedle) [16]

ii) Experimental validation of this microneedle device: - For checking DNA transfer (1) a model protein luciferase and (2) a model antigen ovalbumin was delivered using the system as described earlier into mice skin in vivo. But it was seen that DNA transfer was not that

significant by using this system which can be seen by bio illuminance imaging graphs and from the summary of table. [18]

This can be due to following reasons –

1. Volume of material that can be injected which is limited due volume of reservoir and also in this case due to high viscosity of DNA.

2. Improper electric field distribution which is due to needle shape causing unequal electroporation of cells. It was also found that the application of electric field should be between each row of microneedles with lower electrical fields in order to have cell permeabilization without the danger of having irreversible electroporation threshold.

Table 1 Luciferase expression in skin. [18]

Injection means	Type of electrodes	DNA amount (in micro grams)	Electrical parameters	RLU
30 G needle	Plate	50	700 V/cm,100 microseconds 150 V/cm,400 milliseconds	884
30 G needle	Microneedles	50	700 V/cm,100 microseconds 150 V/cm,400 milliseconds, 1Hz	53.5
Microneedles	Microneedles	10	700 V/cm,100 microseconds 150 V/cm,400 milliseconds, 1Hz	n.d.
Microneedles	Plate	10	700 V/cm,100 microseconds 150 V/cm,150 milliseconds	n.d
30 G needle	None	50	Not applicable	100

4. Electroporation Chip Based On Flexible Microneedle- with advance research in this area it was found that flexible microneedle could reduce the electroporation voltage [19] thus becoming more useful for practical purposes. This study proposes such a flexible microneedle array.

Fabrication[20,21]- This fabrication employed 400 micrometre-thick Silicon wafer containing 100 nm SiO2 and 100 nm Si3N4 on the front used with glass bonded to it. Pattern of SiO2/Si3N4 was done using

lithography and reactive ion technique. Silicon was used due to its good strength. KOH was used for etching 320 nm deep silicon. After etching ,dicing was done for remained 80 micrometre thickness of silicon in order to have lesser applied voltage for electroporation by reducing the interval of needles(here kept 340μm apart). Independent silicon microneedle array was formed by etching 80 micrometre-deep silicon by KOH .Metal deposition followed by lift-off process was done to produce connection lines. Gold layer was patterned as electrode and gold was selected due to its good conductivity and biocompatibility. Sequential isotopic etching of glass was done, resulting in 10 micrometre-wide metal lines formation undercut beneath silicon needles. This formed rigid microneedle array hereafter mentioned as MNA without parylene. The 8 micrometre-thick parylene deposition and patternization was done using lithography and oxygen plasma resulting in rigid MNA with parylene. Glass substrate removal by HF and packaging of silicon needles and metal lines by parylene layer obtained flexible MNA. Parylene film was used as substrate due to its good flexibility, providing tight attachment between proposed chip and living tissues with different profiles and sizes.

It also acts as insulator between electrodes. Electroplating was employed for thickening Au layer to 6μm for withstanding high electroporation current and parylene layer (2μm) coating was done on the bottom side of MNA for the purpose of encapsulation. Schematic of the fabrication process is as shown in fig 6.

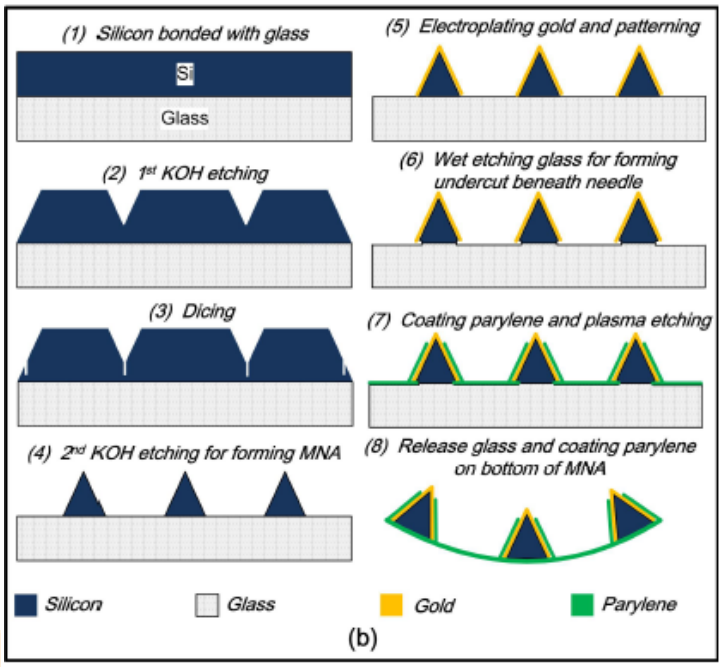


Figure 6 Fabrication process [21]

i) Experimental validation of this microneedle device:-

- Imaging technique: In this experiment in vivo delivery of plasmid DNA and siRNA to mouse muscle tissue using the flexible microneedle array fabricated was done and efficiency was observed. [21] siRNA was successfully delivered by MNA chip. The delivery with electroporation shown better delivery in the sense that siRNA was delivered to myocytes without getting metabolized whereas the delivery without electroporation shown undesirable metabolization of siRNA. The detection here was done with fluorescence imaging by using Cy-5 labelled siRNA. Plasmid DNA (pmRFP-C1) was used for checking nucleic acid delivery which proved successful in vivo electroporation as DNA was expressed in myocytes as detected by fluorescence imaging.
- Extent of electroporation: For removing difficulty of high voltage in in vivo electroporation optimized electroporation voltage was determined by plotting the graph of RFP delivery efficiency and applied voltage. The peak value of voltage for highest efficiency was 40V which is comparatively small and non-hazardous to skin.

In both of these deliveries of siRNA and plasmid DNA no severe injury to the skin was observed in fact the mouse maintained their physical health well and microneedle array fabricated was capable of withstanding the current without any damage as confirmed by SEM.

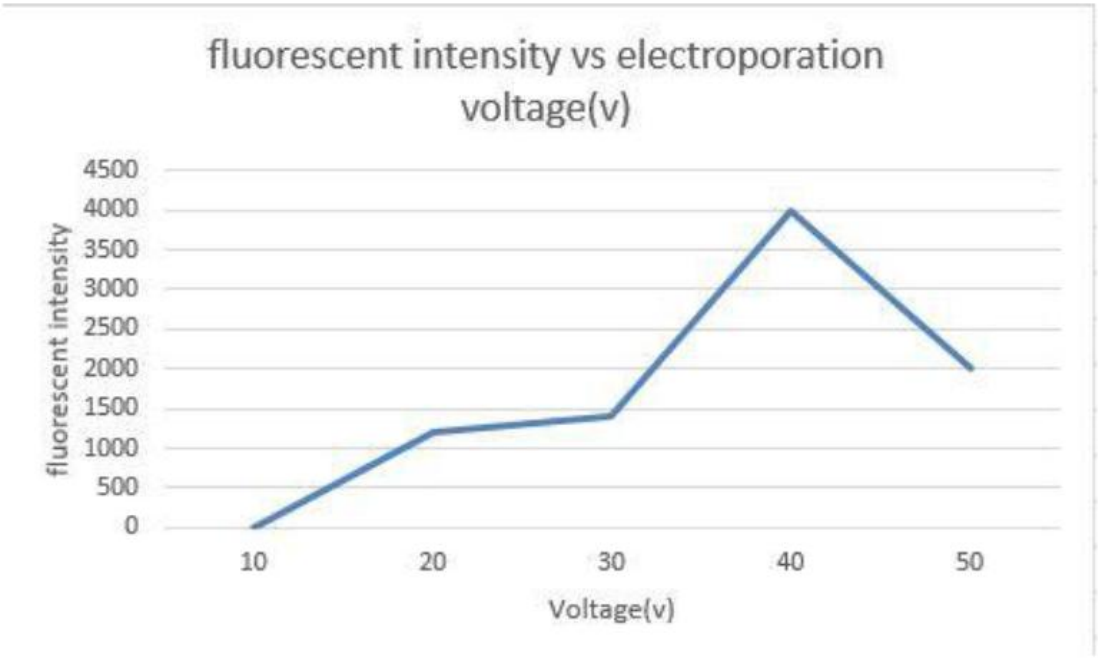


Figure 7 Graph of RFP expression and applied voltage showing highest efficiency at 40V. [21]

3. Results:-

Previous studies have proved gene therapy to be one of the promising methodologies to treat hereditary genetic disorders. However, the difficulty in introduction of these molecules (proteins in chemical nature) into the human body posed as a serious drawback. While the oral route of administration would lead to the protein degradation, parenteral route was found to be extremely painful and inconvenient to administer. In such a scenario, electroporated microneedle arrays proved to be extremely useful as far as painlessness ease of administration and extent of gene permeation was concerned.

4. **Discussions:-**

This study gave an insight into various researches made by people all over the world. The first research employed latest metal patterning technique instead of traditional photolithography technique whereas the second research went a step ahead to test a similar fabrication on human subjects for insertion tests and conducted an in-vitro RBC lysis assay. The third research bought a paradigm shift by switching from solid microneedles to hollow needles. Though the concept seemed very gripping, the results weren't as satisfying and hence some modifications for greater volume holding capacity and better electric field distribution should be incorporated in the proposed structure to have efficient DNA transfer. The fourth study proved to be the most promising model amongst the four discussed above with the benefits of non-invasiveness and low voltage application. This was proved using an in-vivo experiment in mice using bioluminescence imaging techniques.

Post reviewing the researches made by the scientists in the field of electroporated microneedle arraying technology, it appears to be a highly recommended way to administer genes and macromolecules through this method considering the enhanced permeation with painless delivery. Though the methods discussed above show some benefits in delivery over other methods, there are challenges associated with their implementation. First of all, the treatments must be simple to use i.e. the use of a single device for all the methods without continual intervention by the patient or physician must be achieved. Also, fitting various parameters like applied voltage, penetration depth, contact time, dosing etc and optimization of the same is unique for each type of molecule. Safety studies are needed to assess the potential for pain, especially for those methods utilizing electrical modalities. Furthermore, validation and costing of such devices is also a major concern. Thus, more and more molecules should be explored for administration via this way in order to achieve full potential of this technique.

With recent advances, these technologies are getting smaller and less expensive to manufacture, resulting in considerable commercial interest, particularly in the area of protein delivery, vaccination, and sensing. Despite the maturity of these methods, there is still a need for

further study and development, particularly in the areas of device miniaturization and the permeabilization.

4. References:-

[1]F.Chabri,K.Bouris,T.Jones,D.Barrow,A.Hann,C.Allender, K.Brain, J.Birchall,"Microfabricated silicon microneedles for nonviral cutaneous gene delivery", DOI: 10.1111/j.0007-0963.2004.05921.

[2] Neumann, E; Schaefer-Rider, M; Wang, Y; Hofschneider, PH,"Gene transfer into mouse lyoma cells by electroporation in high electric fields". The EMBO Journal, Vol.1 (7), pp.841-845. PMCID: PMC553119, 1982

[3] Keshu Yan, Hiroaki Todo, Kenji Sugibayashi,"Transdermal drug delivery by in-skin electroporation using a microneedle array", International Journal of Pharmaceutics.

[4]R. F. Donnelly, M. J. Garland, D. I. J. Morrow, K. Migalska, R. R. S. Thakur, R. Majithiya, A. D. Woolfson, Microneedle-Mediated Transdermal and Intradermal Drug Delivery, Wiley.

[5]R.F. Donnelly, T.R.R. Singh, D.I.J. Morrow, A.D. Woolfson, Microneedle-Mediated Transdermal and Intradermal Drug Delivery, Wiley, 2012, chapter 5.

[6]]M.R. Prausnitz,"Advanced drug delivery reviews",ELSEVIER,vol,56,pg no. 581–587, doi:10.1016/j.addr.2003.10.023,2004.

[7] T.M. Tuan-Mahmood, M.T. McCrudden, B.M. Torrisi, E. McAlister, M.J. Garland, T.R. Singh, R.F. Donnelly, Eur. J. Pharm. Sci. ,Volume 50,pp 623–637,2013.

[8] A. D. King, R. E. Walters, "Delivery of macromolecules into cells," U.S.Patent 6,603,998,2003

[9] M. J. Jaroszeski, R. Heller, R. A. Gilbert, "Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery", Humana Press, Totowa, NJ, 2000

[10] A. Gothelf, L. M. Mir, J. Gehl, "Cancer Treat. Rev. 2003"

[11]Seong-O Choi, Yeu-Chun Kim, Jeong Woo Lee et all, "Intracellular Protein Delivery and Gene Transfection By Electroporation Using a Microneedle Electrode Array", 2012, DOI: 10.1002/.201101747.

[12] Seong-0 Choi, Jung-Hwan Park, Yoonsu Choi, Yeuchun Kim, Harvinder S. 3, Yong-Kyu Yoon et all an "Electrically Active Microneedle Array for Electroporation of Skin for Gene Delivery" discussed in the 15th International Conference on Solid-State Sensors, Actuators and Microsystems, Korea, June 5-9.

[13] Hooper JW, Golden JW, Ferro AM, King AD, " Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge."

[14]-Kaushik S, Hord AH, Denson DD et al; "Lack of pain associated with microfabricated microneedles."

[15]Prausnitz MR, Langer R (2008) Transdermal drug delivery. Nat Biotechnology 26:1261–1268

[16] Lie´vin Daugimont, Nolwenn Baron et al; "Hollow Microneedle Arrays for Intradermal Drug Delivery and DNA Electroporation Membrane Biology"; 2010; 236:117–125; DOI 10.1007/s00232-010-9283-0

[17]-Hoel A, Baron N, Cabodevila G et al (2008) Microfluidic distribution system for an array of hollow microneedles. J Micromech Microeng 18:065019

[18] Vandermeulen G, Daugimont L, Richiardi H et al (2009) Effect of tape stripping and adjuvants on immune response after intradermal DNA electroporation. Pharm Res 26:1745–1751

[19]-H. Aihara, J. Miyazaki, "Gene transfer into muscle by electroporation in vivo", Nat. Biotechnology., vol.16,pp 867-870, 1998.

[20]-R. Wang, Z. Wei, W. Wang, Zh. Li, "Flexible microneedle electrode array based-on parylene substrate", The 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences, Okinawa, Japan, 28/10/2012 to 1/11/2012

[21] Zewen Wei1*, Renxing Wang2*, Shuquan Zheng3*, Zicai Liang3 and Zhihong Li4,"AN ELECTROPORATION CHIP BASED ON FLEXIBLE MICRONEEDLE

ARRAY FOR IN VIVO NUCLEIC ACID DELIVERY", IEEE 27th International Conference on Micro Electro Mechanical Systems (MEMS) Pages: 817 - 820, DOI: 10.1109, (2014).