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Novel procedures for the production of multi-compartmental biodegradable polymeric Microneedles

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NOVEL PROCEDURES FOR THE PRODUCTION OF MULTI-COMPARTMENTAL BIODEGRADABLE POLYMERIC MICRONEEDLES

A THESIS SUBMITTED IN PARTIAL FUL FILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
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to me,
to Rosa,
to my family,
particularly maternal grandfather.

Table of contents

GENERAL ABSTRACT

Skin delivery of drugs, which means delivery directly into the epidermis, represents today an interesting alternative to hypodermic injection and cutaneous administration. The main difficulty in current techniques of skin release, for example patches, is linked to the poor permeability of the horny layer of the skin, which obstructs the passage of large, charged and hydrophilic molecules.

Microneedles, inserted into the skin in order to cross the stratum corneum, which is the outer layer of the epidermis, can solve the permeability issue without causing pain or harm to the patient. Among the many advantages of the use of microneedles as compared to the conventional methods: it's possible introduce high molecular weight; it can be mentioned reducing the patient's pain during administration of the active principles compared to hypodermal injections and the possibility of self-administration of the drug by the patient without the need of the intervention of specialized personnel. Furthermore, when integrated into suitable miniaturized systems, the use of microneedles can allow a controlled release of small amounts of molecules, at the predetermined site, in a correct countable concentration and in a determinate time.

Microneedles are well known in the pharmaceutical and cosmeceutical field as effective and pain-free micro-scale devices employed for skin release, transdermal vaccination and drug delivery. Preliminary results show that microneedles represent a promising technology to deliver therapeutic compounds into the skin for a range of possible applications, in fact microneedles have been studied *in vitro*, *in vivo* for a variety of applications. Many researchers have produced microneedles with different designs and different materials by means of various fabrication processes. However, the majority of the known techniques are not suitable for sensitive drugs and active molecules, in particular those thermo-labile, since they

often involve high temperatures, while the processes carried out at room temperature usually involve hazardous photoinitiators.

The aim of this work is a new stamp based method to fabricate multi-compartmental polymeric microneedles containing a model drug. This approach overcomes the limitations deriving from the known methods for the production of polymer microneedles, e.g. micro-casting and drawing lithography; since no hazardous temperatures; no multi-step filling process and no UV are required.

The process is divided in two parts to obtain microneedles with two types of possible release. Two different processes were used to produce microneedles.

In the first process microparticles were placed in the cavities of the elastomeric PDMS mold obtained from the previously produced master and subsequently softened by means of a suitable vapor mixture in order to produce the microneedles, then a polymeric solution of PVP was cast on the mold. In the second process, a polymeric solution of PVP was cast on the mold to create a tip array, then microspheres were placed in the cavities and subsequently softened by means of a suitable vapor mixture in order to produce the microneedles body. Later, the microneedles were collected from the mold with the aid of a PVP covered medical tape and to remove the microneedles array from the mold.

The microneedles' porous structure allows to modulate the degradation of the polymer and provides a better control of the release profile of the embedded drug. This method allows to preserve both the microstructure and molecule distribution of the starting microspheres, providing shaped microparticles that can be released over time. In particular, in this work it is demonstrated that the shaped microparticles keep a porous microstructure and cargo distribution similar to that of the starting microspheres, whereby the release of enzyme embedded in the shaped microparticles and starting microspheres is equivalent.

The microneedles porous structure allows modulating the degradation of the polymer and a better control of the profile release of the drug contained in it. This process overcomes several limitations of the known methods for the production of

polymer microneedles and has very promising applications in the pharmaceutical and cosmetceutical industry.

CHAPTER I

STATE OF ART

1. Introduction

The ultimate aim of every therapy is to restore the normalcy of life, but ironically sometimes, the requirements of treatment are such that the normal rhythm of life is disturbed. Most drugs are administered in the form of pills or injections, but these methods of delivery are not always optimal.[1, 2] Medication taken orally must not only be absorbed successfully out of the intestine into the bloodstream, but must also survive the harsh and enzyme-rich environments of the gastrointestinal tract or the first pass through the liver. Drugs that cannot be taken as pills are typically administered by injection, which introduces the problems of pain, infection, and expertise required to carry out an injection. Both the methods of delivery have limitations, as the full dose of drug is introduced into the body at one time. An approach which is more appealing to patients and offers the possibility of controlled release over time is transdermal drug delivery. Clinical benefits, industry interest and regulatory precedence had predicted a strong market for transdermal drug products due to the limitations of conventional drug deliveries. However, transdermal delivery is severely limited by the inability of the large majority of drugs to cross skin at therapeutic rates due to the barrier imposed by the stratum corneum, the outer 10/15 μm of skin, that generally allows the transport only of small molecules (<400 Da).[3] In order to address this limitation, a variety of methods to increase transdermal transport have been studied, including chemical enhancers, electric fielders, ultrasound, and thermal methods.[4-6] Although still under active investigation, these enhancement methods have made only limited impact on medical practice to date. Recently several research groups have focused

the attention on overcoming problems associated with the barrier properties of the skin, reducing skin irritation rates and improving the aesthetics associated with passive patch systems. A new approach to transdermal drug delivery that acts as a bridge between the user-friendliness of patches and the broad effectiveness of hypodermic needles has recently received attention.[4, 5, 7] By using needles of micrometer dimensions, termed microneedles, skin can be pierced to effectively deliver drugs, but with a minimally invasive and painless manner that lends itself to self-administration and slow delivery over time. It is proposed that micron-scale holes in the skin are likely to be safe, given that they are smaller than holes made by hypodermic needles or minor skin abrasions encountered in daily life.[1]

1.1 Control of drug delivery

The control of drug concentration in the blood or in a specific area of the body is very important, for this reason there are already numerous pharmaceutical forms that allow for a delayed release, or by which the speed and duration of release of the active ingredient can be “programmed”. Such formulations are defined “controlled release systems” and allow for the setting of the blood level of the drug, so as to avoid phenomena of sub-or over dosage and minimize unwanted side effects. Conventional methods of administration of drugs normally do not allow to obtain a controlled release over time of active ingredient: in many cases, there is a sudden release of the drug from the pharmaceutical form and this can lead in a short time to reach the limit of toxicity, namely the concentration at which the active principle involves side effects or toxicity.

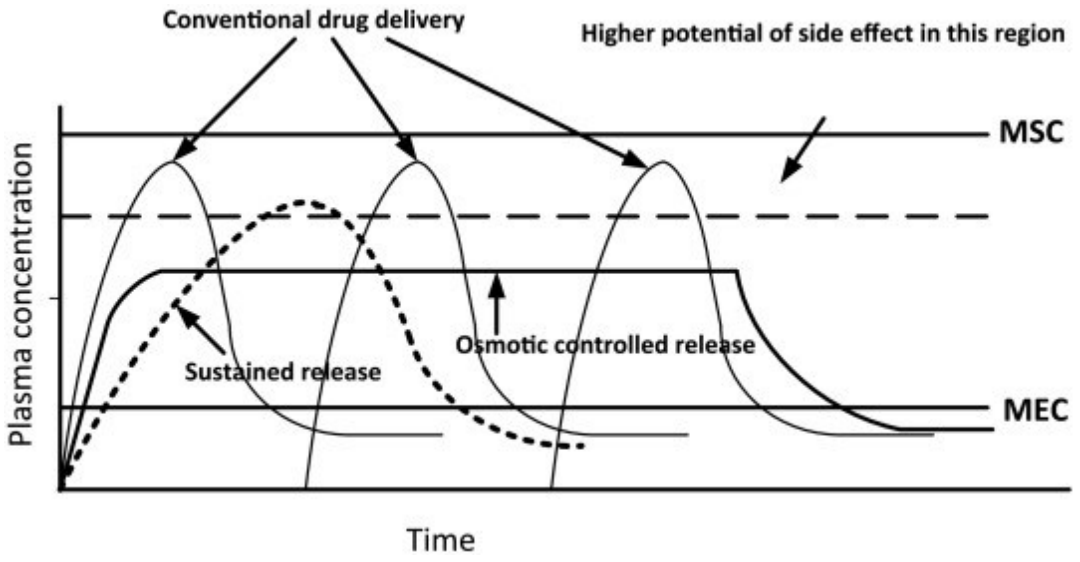


Fig. 1.1. Comparison between a conventional delivery system and a system of controlled release.[8]

Once given the drug, it keeps to a relatively short therapeutic limit and subsequently its blood levels decrease slowly until the next administration. Therefore, the desired therapeutic effects may be obtained only by repeated administration of the drug, which are often not well accepted by the patient in addition to being very expensive.

The modern pharmaceutical technology has successfully attempted to overcome these problems with the creation of systems for controlled release of drugs, namely of pharmaceutical preparations which, once administered to the patient, are capable to release over time the active principle contained in them, ensuring its constant concentration in the blood. In this way the frequency of administration can be reduced with advantages both for the patient and the cost of therapy.

The result of the research carried out in this area is also represented by the innovative use of particular polymers as a means of releasing and targeting biologically active molecules. These polymers are able to allow an increase in the residence time of the drug in the bloodstream, reduce antigenicity, and increase stability against enzymes and a greater bioavailability.

In order to achieve a gradual or prolonged release of the active ingredient, various pharmaceutical formulations have been developed which can be divided into three groups:

- sustained release: the medicine administered dissolves quickly providing an initial dose of active ingredient within the therapeutic range and is able to release for a certain time a lower dose. In fact, the concentration is not maintained constant but fluctuates within the therapeutic band;
- long-acting: the amount of drug administered is slightly greater than that required to reach the desired therapeutic response. In this way, without producing hazardous pudding, a prolonged effect is achieved, as compared to the normal dose since the body uses more time to eliminate or metabolize the drug;
- repeat-action: provides an initial dose of medicine which is released later when the effect of the first is fading.

For the production of medicines of this type various techniques can be employed, which are distinguished on the basis of the method by which the active principle is locked inside the pharmaceutical preparation.

This can happen by means of:

- physical locking by external coatings and excipients (currently the most common method);
- entrapment by ion-exchange resins;
- encapsulation using biodegradable polymers.

According to a relatively new technique, the drug is embedded in a biodegradable polymer matrix, and the active substance is released through diffusion, degradation and/or erosion processes of the matrix itself.

The controlled release systems that use polymeric substances are classified according to the mechanism that controls the release of the drug.

Pharmaceutical forms aiming to control the diffusional mechanisms by which the release speed is determined are the most used in the pharmaceutical industry and

can be divided into systems and reservoir matrix systems. For what concerns the systems, the drug is present as a core coated by a diffusional barrier of the polymeric type and this involves a release rate that follows a zero-order kinetics, at least for a certain time. Fig. 1.2 shows a typical profile of the rate of release of this system.

In matrix systems the active substance is dissolved or dispersed in an inert polymer matrix. A different approach involves the use of an erodible polymer, namely control chemical, in which the drug is ideally distributed evenly over the entire polymer matrix and its release is generally governed by a combined effect of polymer degradation and diffusion. In this way a kinetic release of the first order is usually achieved, even though more complicated kinetics has also been observed, since the diffusion of the drug is affected by the degradation of the matrix (fig. 1.2).

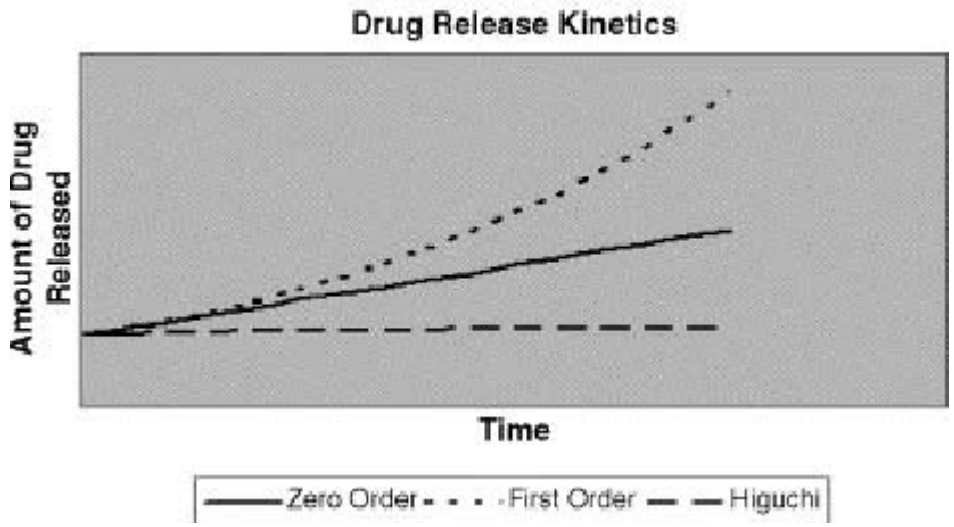


Fig. 1.2. Kinetics of drug release.[9]

1.1.1 Materials for controlled release

There are numerous techniques that allow to obtain gradual drug release using polymers. The easiest method to realize monolithic systems with predetermined geometric shape and dimensions, consists in creating an environment adequately concentrating organic solutions of polymers containing the drug of interest, and then proceed with the slow evaporation of the solvent. With this method, polymers that are soluble in organic solvents must be used. The evaporation must take place under well controlled conditions to avoid the formation of sediment, bubbles, or clusters. Other methods for preparing polymer matrices involve the use of UV. All these techniques have the drawback of providing a final product which is polluted by unwanted residual substances, in some cases even toxic, so it is necessary to perform downstream operations of separation and purification, such as filtration and washing, thus affecting the final cost of the preparation and creating products which are difficult to dispose.

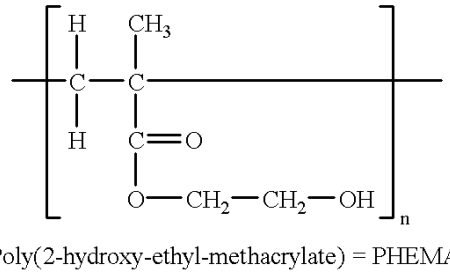
The choice of the polymer used to realize the device has to be made carefully.

An ideal material should be inert and without impurities, biocompatible and easily to process. The type of material also depends on the kind of issues that one would like to accomplish and the type of drug incorporated.

After the work,[10] hydrogels have gained an increasing interest for their hydrophilic character and their biocompatibility, so that recently some hydrogels have become particularly suitable for tissue engineering.

Hydrogels are hydrophilic polymers characterized by a network capable of absorbing water up to one hundred times their dry weight, and can be chemically stable or can degrade and eventually dissolve. Gels are called “reversible” or “physical” when the network is held together by secondary forces, such as ionic forces or hydrogen bridges. On the other hand, gels are called “permanent” or “chemical” when covalently cross-linked.

Fig. 1.3 shows the formula of the chemical hydrogel Poly (2-hydroxyethyl-methacrylate) (PHEMA).



Poly(2-hydroxy-ethyl-methacrylate) = PHEMA

Fig. 1.3. Structural formula of poly (2-hydroxyethyl-methacrylate), PHEMA.

Due to its high biocompatibility, PHEMA has found wide use in the biomedical field, especially in those areas where a good interaction with biological fluids is needed, such as contact lenses. PHEMA is a type of hydrogel that commonly has a three-dimensional network and is able to consider a large amount of water without dissolving.

For this class of materials, the kinetics of water absorption is very relevant: the drug, immobilized in the dry hydrogel, is released when the water absorbed swells the polymer matrix. For this reason, a controlled swelling of the hydrogel implies a controlled release of the drug.

In recent years, other alternative material usable for forming microneedles were used. Carboxymethyl-cellulose (CMC) (fig. 1.4), is an organic polymer obtained by reacting cellulose with caustic soda (NaOH) and monochloroacetic acid (MCAA) ($ClCH_2COOH$) (fig. 1.5).[3]

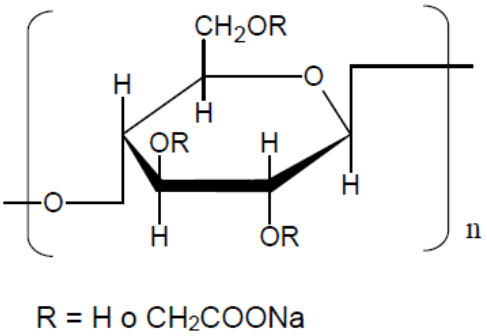


Fig. 1.4. Structural formula of CMC.

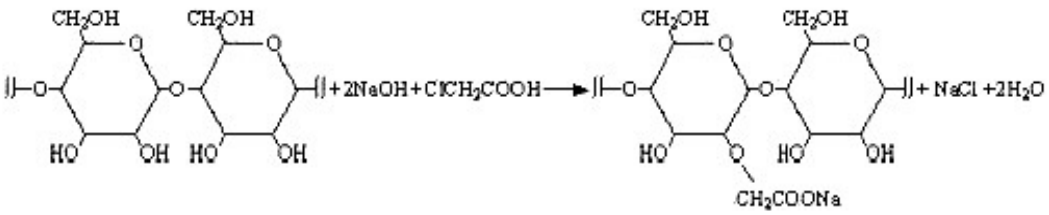


Fig. 1.5. Exemplified reaction scheme of carboxymethylcellulose.

It is a kind of material already used in many different areas due to its ability to stabilize the solutions. It can also be used in the biomedical field since it is biocompatible, readily biodegradable in water and approved by FDA. Moreover, it is expected to be mechanically strong due to its relatively high Young's modulus (1 GPa).[11]

Another material that is arising interest in the development of biodegradable devices is poly (hydroxybutyrate) – PHB (fig. 1.6), biodegradable thermoplastic polyester produced by bacterial fermentation, whose biodegradation time is short.

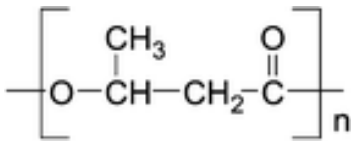


Fig. 1.6. Structural formula of polyhydroxy butyrate (PHB).

PHB is a polymer with high crystallinity (50-70%) and an elasticity modulus of 3GPa; however PHB has some disadvantages, such as high fragility, showing 3-5% tensile elongation at break, and low thermal stability above its melting point.[11]

An alternative to improve these properties and PHB processability is to mix it with another polymer. Very recently, Scandola *et al*. have disclosed that polyhyroxybutyrate (PHB) forms miscible blends with cellulose esters,[12] as cellulose acetate propionates (CAP) and cellulose acetate butyrate (CAB).

From a study by Buchanan, that used a copolymer poly(hydroxybutyrate-co-valerate) (PHBV) (fig. 1.7).

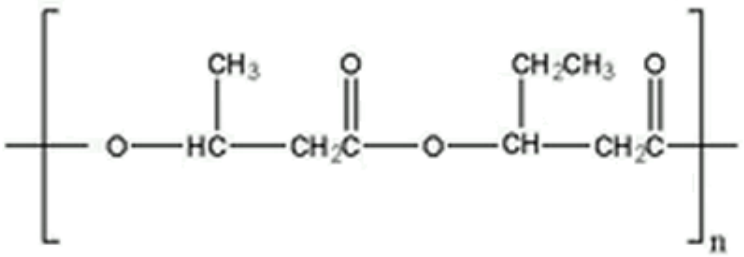


Fig. 1.7. Structural formula of polyhydroxy butyrate-co-valerate (PHBV).

It is clear that mixing PHBV and CAB, in the range 20-50% PHBV, can improve elongation at break, while the tear strength is largely unaffected, as shown in fig. 1.8.[13]

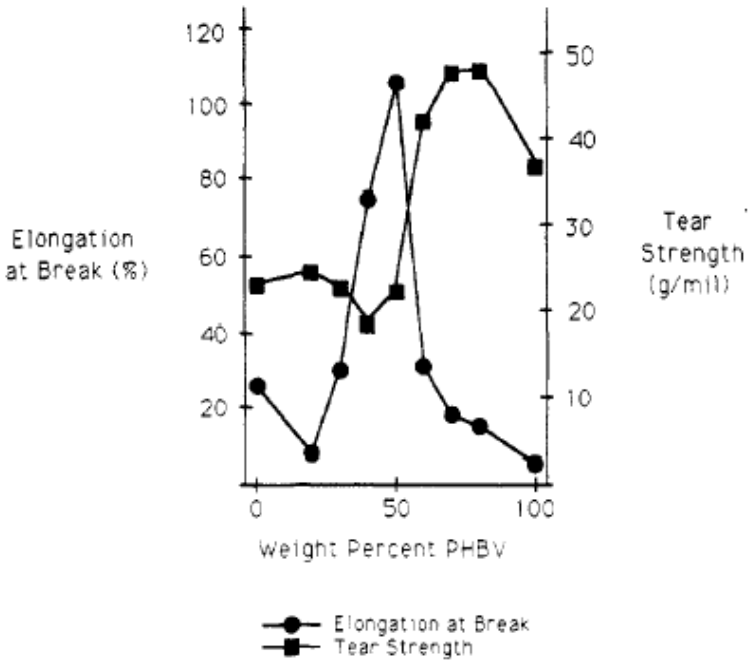


Fig. 1.8. Change in tear strength and elongation at break of the CAB/PHBV blends containing 0-100% PHBV[12].

However, polymers of lactic acid and glycolic acid and their copolymers, such as lactic-co-glycolic acid, are so far the most interesting and widely used in the release of drugs and in the development of biomaterials. These polymers were originally used as materials for absorbable sutures, and thereafter have been used for manufacturing biodegradable release systems. Polylactides are polyesters that have the advantage to decompose into fragments, biologically acceptable by the host, which are then metabolized and removed through normal metabolic processes. These polymers are, in fact, essentially non-toxic and biodegradable (fig.1.9).

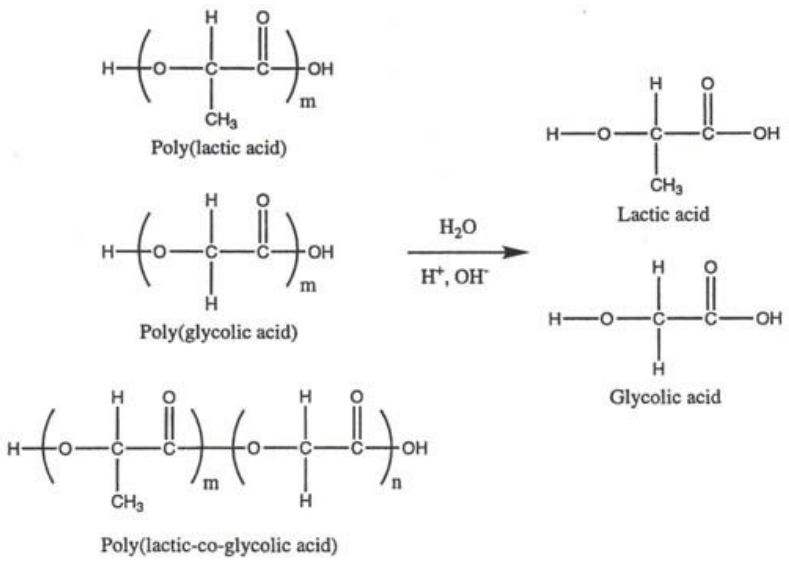


Fig. 1.9. Structural formula of PLA, PGA and their copolymer PLGA.

The mechanism of degradation for all polyesters seems to be a simple homogeneous autocatalytic reaction. The rate of erosion depends on the chemical structure, the morphology of the polymer matrix and the crystallinity.

1.2 Transdermal drug delivery

Transdermal drug delivery systems are topically administered medicaments in the form of patches that deliver drugs for systemic effects at a predetermined and controlled rate. Often, this promotes healing to an injured area of the body. An advantage of a transdermal drug delivery route over other types such as oral and topical, is that it provides a controlled release of the medicament into the patient. Drug is meanly delivered through the skin with the aid of transdermal patch. A transdermal patch is a medicament adhesive patch that is placed on the skin to deliver a specific dose of medication through the skin into the bloodstream over time. However, a disadvantage related to the development of transdermal patches, stems from the fact that the skin is a very effective barrier. The transdermal route is

indeed desirable, but there is one small obstacle: the skin's function is to keep things out of the body.

1.2.1 Skin structure

In order to design effective transdermal drug delivery systems, it is necessary to have a proper knowledge of the skin structure. Indeed, if the barrier provided by the stratum corneum is not crossed, active molecules cannot be properly delivered.

The integument or skin is the largest organ of the body, making up 16% of bodyweight, with a surface area of 1.8 m^2. It has several functions, the most important being to form a physical barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against micro-organisms, ultraviolet radiation, toxic agents and mechanical injures. There are three structural layers of the skin: the epidermis, the dermis and subcutis (fig.1.10).

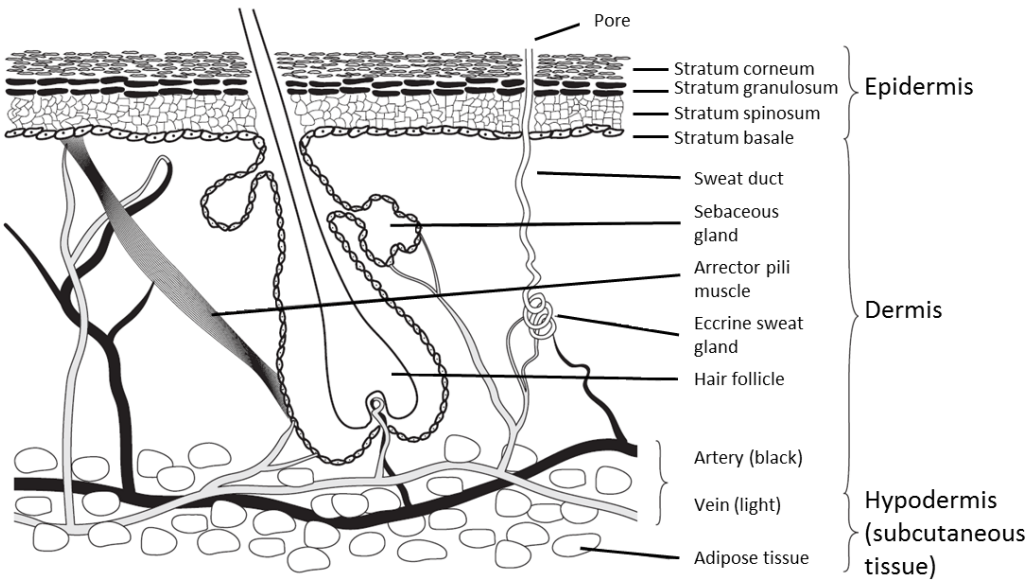


Fig. 1.10. Structure of the skin.

The epidermis is the outer layer, serving as the physical and chemical barrier between the interior body and exterior environment; the dermis is the deepest layer providing the structural support of the skin, below which is a loose connective tissue layer, the subcutis or hypodermis which is an important depot of fat.

The epidermis is a stratified squamous epithelium.[14] The main cells of the epidermis are the keratinocytes, which synthesizes the protein keratin epithelium.[14] Protein bridges called desmosomes connect the keratinocytes, which are in a constant state of transition from the deeper layers to the superficial one. The four separate layers of the epidermis are formed by the differing stages of keratin maturation. The epidermis varies in thickness from 0.05 mm on the eyelids to 0.8±1.5 mm on the soles of the feet and palms of the hand. Moving from the lowest layers upwards to the surface, the four layers of the epidermis are: stratum basal (basal or germinativum cell layer)

- stratum spinosum (spinous or prickle cell layer)
- stratum granulosum (granular cell layer)
- stratum corneum (horny layer).

The final outcome of keratinocyte maturation is found in the stratum corneum, which is made up of layers of hexagonal-shaped, non-viable cornified cells known as corneocytes. In most areas of the skin, there are 10±30 layers of stacked corneocytes with the palms and soles having the most. Each corneocyte is surrounded by a protein envelope and is filled with water-retaining keratin proteins. The cellular shape and orientation of the keratin proteins add strength to the stratum corneum. The cells are surrounded in the extracellular space by stacked layers of lipid bilayers (fig 1.11).

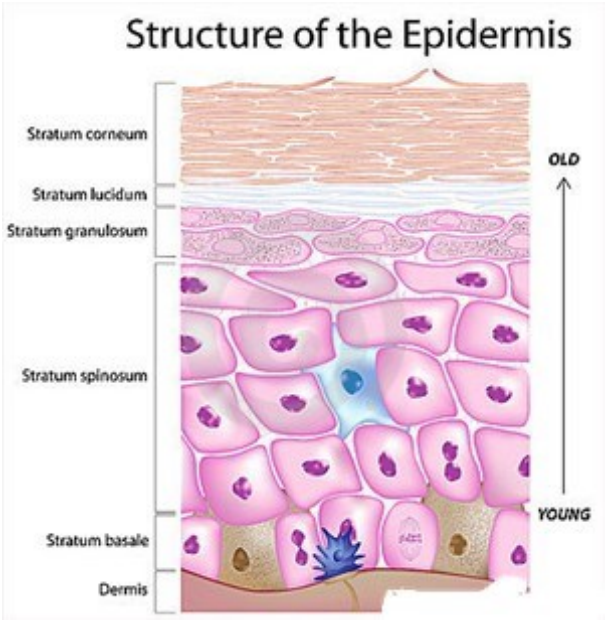


Fig 1.11. Structure of the epidermis.

The resulting structure provides the natural physical and water-retaining barrier of the skin. The corneocyte layer can absorb three times its weight of water but if its water content drops below 10% it no longer remains pliable and cracks. The movement of epidermal cells to this layer usually takes about 28 days and is known as the epidermal transit time.

The dermis is a complex structure composed of two layers: thin papillary layer, and the thicker reticular layer. The structure is highly irregular, whit dermal papillae from the papillary dermis projecting perpendicular to the skin surface. It is via diffusion at this junction that the epidermis obtains nutrients and disposes waste. The dermo-epidermal junction flattens during ageing which accounts in part for some of the visual signs of ageing. The subcutis is composed by loose connective tissue and fat, which can be up to 3 cm thick on the abdomen.

1.2.2 Devices and technologies for transdermal delivery

From a global perspective, advances in transdermal delivery systems can be categorized in three different generations. The first generation is mainly based on today's patches produced by judicious selection of drugs that can cross the skin at therapeutic rates with little or no enhancement. In addition to patches, in this class are included liquid spray, gel and other topical formulation. The second generation is characterized by additional advances for the delivery of small-molecules by means of an increase of skin permeability and the use of driving forces for transdermal transport; an example is provided by chemical enhancers conjugated to the drug. The third generation is meant to enable transdermal delivery of small-molecule drugs, macromolecules (including proteins and DNA) and virus based and other vaccines through targeted permeabilization of the skin's stratum corneum.[15]

The kind of technology used by transdermal devices can be divided into passive or active methods based on whether or not an external source of energy is used for skin permeation enhancement. Passive methods include use of chemical enhancers, emulsions and lipid assemblies that increase stratum corneum permeability through different mechanisms. They may act on the desmosomes, a kind of proteic junctional complex localized spot-like on the lateral sides of the plasma membranes, forming cohesion between keratinocytes, or modify the intercellular lipid domains to reduce the barrier resistance of the bilayer lipids [16] (fig 1.12) but are often associated with higher skin irritation.

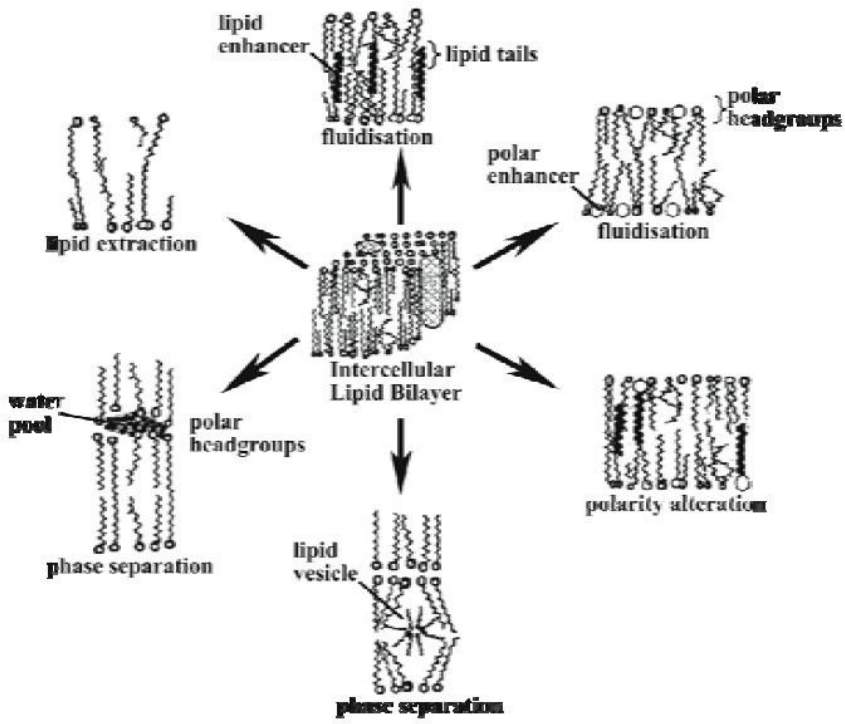


Fig. 1.12. Action of penetration enhancers within the intercellular lipid domain.[16]

Chemical approaches have emphasized formulations that selectively disrupt lipid bilayer structures in the stratum corneum to avoid effects in the viable epidermis in order to prevent skin irritation. In this way, the increase of permeability is limited to SC but does not address the barrier of the full epidermis, reducing release efficiency.[17]

Active methods, like electroporation and iontophoresis, increase the transport across the skin by physically disrupting the barrier or using a driving force for drug transport, through an external energy source.

In particular, electroporation creates a transitory structural perturbation of lipid bilayer membranes due to the application of high voltage impulses. The electrical stimulus causes a reorganization in cellular membrane, whereby phospholipids shift their position opening pores which act as conductive pathway through the

bilayer as they are filled with water (fig. 1.13). The creation of pores induces a high but reversible increase in transmembrane transport.

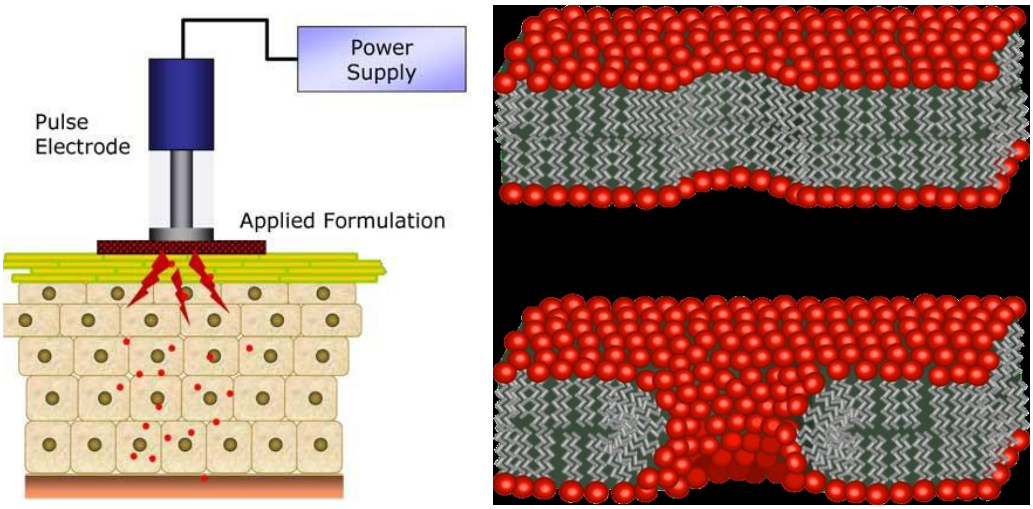


Fig. 1.13. (Left) Schematic representation of electroporation system; (Right) Theoretical arrangement of lipids after electrical pulse showing a hydrophobic pre-pore (up) and a hydrophilic pore (down).

Iontophoresis, instead, uses an electric field to move charged substances, usually a medication or a bioactive agent, through the skin by repulsive electromotive force. A small electric current is applied to an iontophoresis chamber placed on the skin, containing a charged active agent and its solvent; another electrode carries the return current (fig 1.14). The positively charged chamber, the cathode, will repel a positively charged chemical species into the skin, whereas negatively charged substances are collected on the anode. These devices are equipped with a power supply, which adds to the cost and the complexity of the treatment, and requires the passage of current in the patient's body, resulting annoying or even painful for them.[18] Furthermore, both these methods need the presence of trained sanitary personnel.

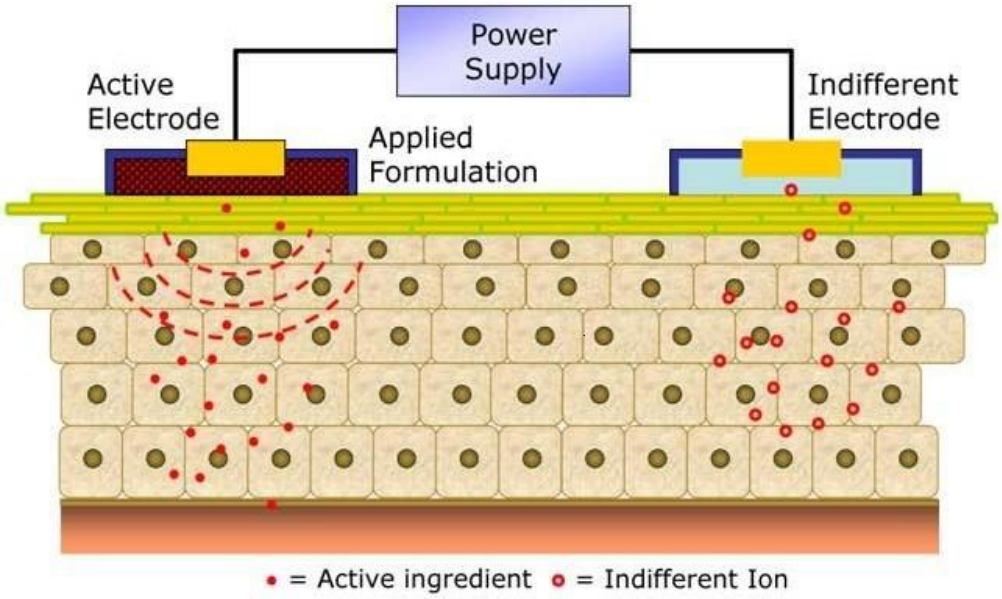


Fig 1.14. Scheme of the iontophoresis system.

Another active method is liquid jet injection, which use a high-speed jet to puncture the skin and deliver drugs without the use of a needle (fig. 1.15).

Jet injectors can be broadly classified into multi-use nozzle jet injectors (MUNJIs) and disposable cartridge jet injectors (DCJIs), depending on the number of injections carried out with a single device.

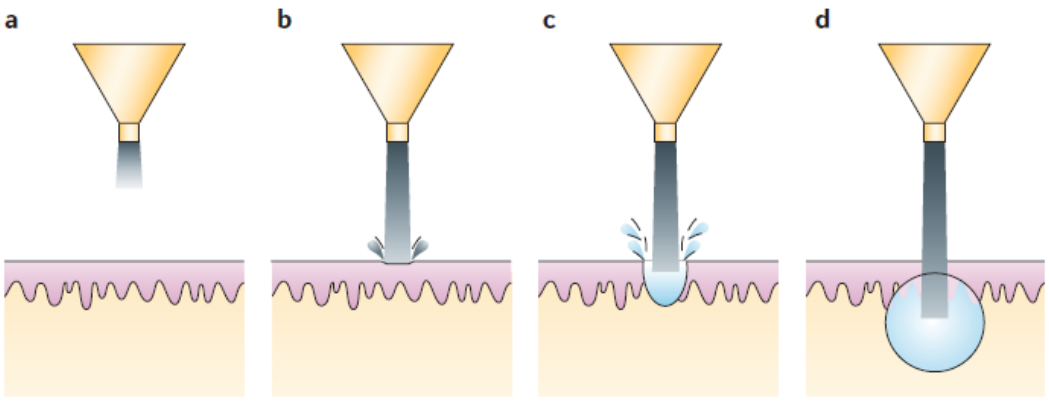


Fig 1.15. Schematic depiction of the jet injection process. A) Impact of a piston on a liquid reservoir in the nozzle increases the pressure, which shoots the jet out of the nozzle at high velocity (velocity >100 m s–1). B) Impact of the jet on the skin surface initiates formation of a hole in the skin through erosion, fracture or other skin failure modes. C) Continued impingement of the jet increases the depth of the hole in the skin. If the volumetric rate of hole formation is less than the volumetric rate of jet impinging the skin, then some of the liquid splashes back towards the injector. D) As the hole in the skin becomes deeper, the liquid that has accumulated in the hole slows down the incoming jet, and the progression of the hole in the skin is stopped.

Liquid jet injections for immunization were first carried out using MUNJIs, which allowed repeated injections of vaccine from the same nozzle and reservoir at a rate of up to 1,000 immunizations per hour. They were successfully adopted for rapid mass immunization using vaccines against a large number of diseases, including measles, smallpox, cholera, HBV, influenza and polio.[19] But because the problems of cross-contamination, due to splash back of interstitial liquid from the skin onto the nozzle, the kind of treatment moved to the use of DCJIs that have sterile orifice and nozzle and is discarded between patients. All these systems increase skin permeability by disrupting or altering temporary structure of stratum corneum. Unfortunately, once the drug delivery is completed, it takes several hours or days to recover its integrity and this cause discomfort to the patient and can increase risk of infections specially if the treated area is extended.[20]

Therefore, there is still a need for an ideal transdermal drug delivery system that a) is safe by maintaining skin permeability only during the desired period of drug delivery, b) can create sustained or bolus delivery profiles, c) can deliver

therapeutic volumes/doses of drug quickly with minimal discomfort, d) has rapidly responsive pharmacokinetics and pharmacodynamics, e) causes minimal pain and irritation, and f) is simple, inexpensive, and self-administrable.[21]
All the requirements listed above seem to be satisfied by using microneedles. These devices are needles of micron dimension, able to puncture the stratum corneum, as to create microchannel that allow also the passage of hydrophilic macromolecules, without stimulation of nerves ending in dermis. Details concerning microneedles properties, materials and fabrication techniques are reported in the following chapter.

1.3 Microneedles

A microstructured transdermal drug system, consists of an array of microstructured projections, called microneedles, that are applied to the skin to provide intradermal delivery of active agents, which otherwise would not cross the stratum corneum. Such microneedles can be used for different kinds of drug delivery. One of the first developed microneedle based devices – and is still used nowadays - was mainly intended to be applied to the skin prior to topical administration of a medicament. This device comprises microneedles typically made of metal, with the aim to provoke a temporary mechanical disruption of the skin so that the stratum corneum can be effectively crossed by the medicament. Microneedles are somewhat like traditional needles, but are fabricated on the micro scale. They are generally hundreds microns in length. Microneedles have been fabricated with various materials mainly including polymers. A microneedle is thus smaller than a hypodermic needle, does not hurt when it pierces the skin and offers several advantages when compared to conventional needle technologies. Microneedles are specifically designed in order to cross the stratum corneum, which as mentioned is the outer layer of the skin, but being short enough to avoid the puncture of nerve endings. Conventional needles which do pass this layer of skin may effectively

transmit the drug but may lead to infection and pain, since they are much longer. Various types of microneedles have been fabricated such as solid (straight, bent, filtered), and hollow. Microneedles can also be coated with a drug typically using a water-soluble formulation. In this case, after insertion of microneedles into the skin, the drug coating is dissolved off into the skin, after which the microneedles are removed. Alternatively, microneedles can be made completely out of water-soluble or biodegradable polymer which encapsulates the drug within the microneedle matrix. In this way, the microneedles completely dissolve or degrade in the skin, thereby releasing the encapsulated drug payload and leaving behind no sharps waste, since microneedles have dissolved away.

Different active molecules, such as vaccines or diagnostic agents, can be delivered. In addition, other tissues besides the skin can be targeted, such as the eye.

Hollow microneedles can be used with drug patches and timed pumps to deliver drugs at specific times. Arrays of hollow microneedles can be used to continuously carry drugs into the body using simple diffusion or a pump system. Hollow microneedles can also be used to remove fluid from the body for analysis – such as blood glucose measurements – and to supply microliter volumes of insulin or other drug as required.

The hollow needle designs include tapered and beveled tips, and could eventually be used to deliver microliter quantities of drugs to very specific locations. An array of 400 microneedles has recently been used to pierce human skin for delivering macromolecular drugs. Very small microneedles could provide highly targeted drug administration to individual cells. These are capable of very accurate dosing, complex release patterns, local delivery and biological drug stability enhancement by storing in a micro volume that can be precisely controlled.

1.3.1 Solid microneedles

The fabrication of solid microneedles has been pursued to provide sufficient mechanical strength through choice of the proper material and geometry and to reduce the force needed to insert microneedles into tissue by increasing tip sharpness.

1.3.1.1 Silicon microneedles

Short silicon microneedles have been prepared using a silicon dry-etching process based on reactive ion etching with a chromium mask,[22, 23] as well as isotropic etching in an inductively coupled plasma etcher.[24] An isotropic wet etching of crystalline silicon using an alkaline solution has also been utilized to obtain solid microneedles.[25-27] Wet etching methods can lower fabrication costs compared to dry etching, but the geometry of microneedles is restricted by anisotropic etching along crystal planes by the KOH etchant.[28] To overcome this limit of wet etching, isotropic dry etching and anisotropic wet etching methods have been combined.[24] As an alternative approach, microneedles have been fabricated to serve as neural probes by dicing a silicon substrate to create a grid pattern of deep grooves and then acid etching the resulting pillars to create sharpened probe tips.[29-32]

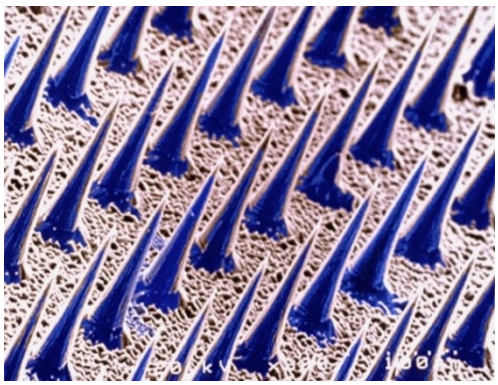


Fig 1.16. Silicon microneedles [26]

1.3.1.2 Metal microneedles

Metal microneedles have been prepared by three-dimensional laser ablation,[33, 34] laser cutting,[35] wet etching,[36, 37] and metal electroplating methods.[38, 39] Rows of solid metal microneedles were fabricated directly, whereas two-dimensional arrays of microneedles have been made by cutting microneedles into stainless steel and titanium metal sheets and then bending them at a 90° angle out of the plane of the sheet. Two-dimensional metal microneedles have also been prepared by depositing metal onto positive or negative microneedle molds by means of electroplating or electro less plating.[38]

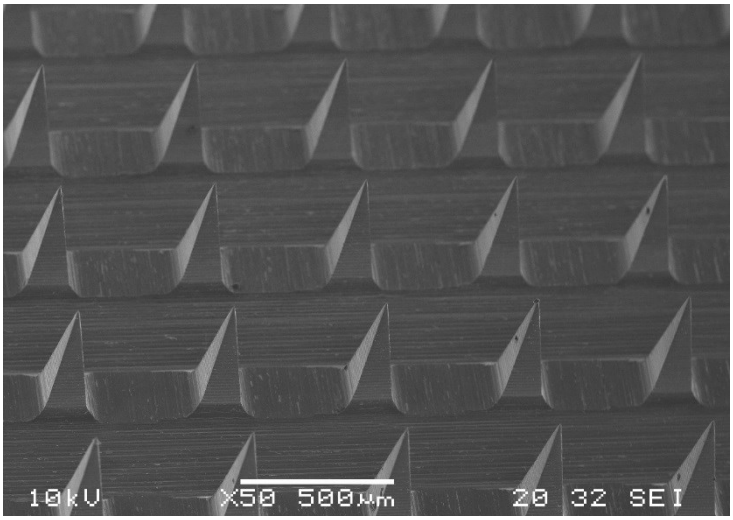


Fig.1.17. SEM image of Metal microneedles Micropoint Technologies.

1.3.1.3 Ceramic microneedles

Ceramic microneedles have been fabricated using ceramic micro molding and sintering. Solid ceramic microneedles were prepared by micromolding an alumina slurry using a PDMS microneedle mold and ceramic sintering.[40] Ceramic microneedles have also been made lithographically using a two-photon induced polymerization approach. A focused laser was scanned within a photosensitive

polymer–ceramic hybrid resin using a galvano scanner and a micro positioning system to induce polymerization locally in the shape of the microneedles.[41-43]

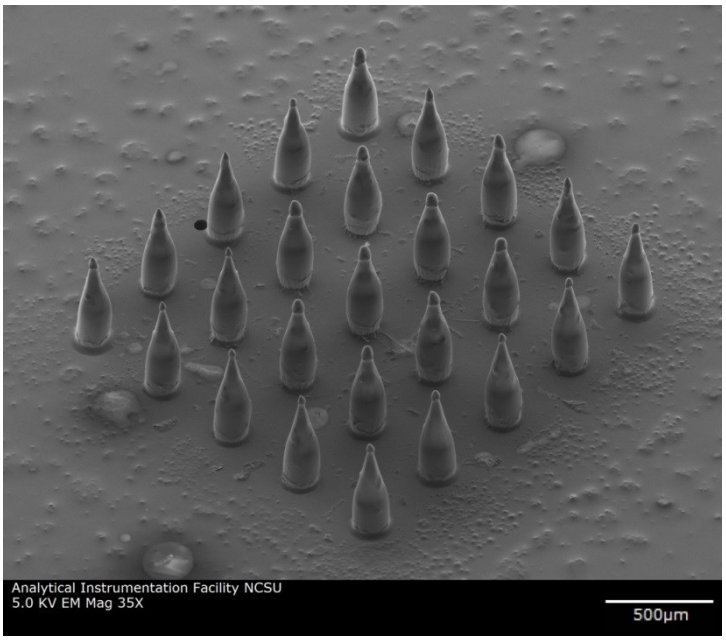


Fig.1.18. SEM image of Ceramic microneedles[44]

1.3.1.4 Polymeric microneedles

One of the main problems with metallic and silicon microneedles is the accidental breakage of the needle inside the skin, since the organism is not able to remove them. For this reason, it is preferred to use biodegradable or dissolvable materials. Certain types of polymers have optimal properties, as biocompatibility, biodegradability and mechanical strength, to be used for realization of microneedles. Some of these, like poly(lactic-co-glycolic) acid (PLGA), poly-lactic acid (PLA),[45] carboxymethyl cellulose (CMC),[46] polyvinylpyrrolidone (PVP),[47] are already widely used for these applications. By changing polymer matrix, or using a double inclusion, i.e. drug in nano/microparticles incorporated in microneedles, it is possible to manage drug release kinetics and drive the drug to

the target site. Generally, polymer microneedles are prepared by replica molding in PDMS stamps obtained on polymer masters typically made by photolithography using optically curable polymers, which are then typically employed as master structures for replication by molding.

The classical method to produce polymeric microneedles is a multistep process. There are three phases: 1) manufacturing of a master; 2) mold production; 3) replica molding of the final polymeric device.

An example is the ultraviolet (UV)-curable polymer SU-8 which has been used extensively to fabricate microneedles.[48-50]

A common UV lithography process, using SU-8, is illustrated in fig. 1.19. The substrate is coated with few hundred microns of photoresist deposited with the spin coating technique that is afterwards soft baked in order to remove the solvent and improve resist-substrate adhesion. UV lithography consists in radiating a photoresist through a chrome mask. In particular, using a mask with an array of circular or square dots it is possible to obtain microstructures having the shape of needles. After irradiation, a post-exposure bake is performed to increase the cross-linking degree of the irradiated areas and stabilize them against the action of solvents during the development step. The development is performed immersing the substrate in propylene glycol methyl ether acetate at room temperature, followed by a rinsing step in water or isopropanol.[51]

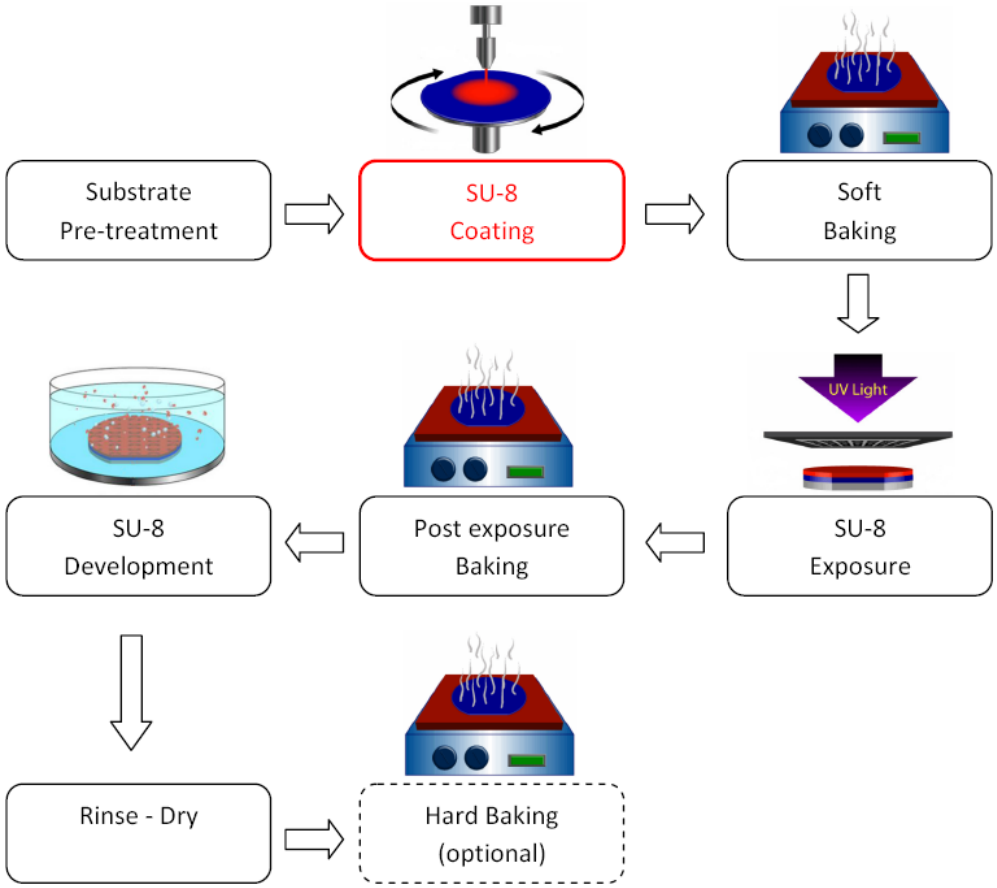


Fig. 1.19. Photolithography standard protocol to create SU-8 master molds.[52]

By using a combination of microlenses on a glass substrate and inclined rotation of the substrate, the path of UV light through the SU-8 was made to taper, thereby defining microneedle structures with sharp tips. PMMA has also been used to make microneedle arrays employing an inclined deep X-ray exposure and vertical X-ray exposure known as the lithography, electroplating and molding (LIGA) technique.[53, 54] Using these approaches, microneedles can be fabricated as tall as a few millimeters with a high aspect ratio using standard contact lithography equipment due to the low optical adsorption of the polymers in the UV range of 390 nm.

A two photon initiated polymerization method has also been used to fabricate three-dimensional microstructures including microneedles. In this approach, a near

infrared ultra-short pulsed laser was focused into a photocurable resin to form three-dimensional microstructures using as sequential layer-by-layer fabrication technique.[55-57]

These UV-curable polymers have been used primarily as master structures for making to create molds with successive preparation of the PDMS molds. Polyvinylalcohol (PVA), silicon and aluminum have also been used as mold materials to replicate polymer microneedles.[58, 59] The mold can then be filled at elevated temperatures with a molten thermoplastic material such as polycarbonate [54] and PMMA [60] to produce non degradable microneedles upon cooling and solidification.

Usually, if a thermo-plastic polymer like PLGA chosen, first PDMS stamp is loaded with a solution containing the drug or the encapsulated drug. Evaporation of the solvent leaves solid drug particles partially filling the mold; residual particles remaining on the surface of the mold can be removed using adhesive tape, and then, after drying, the polymer is melted on the stamp, that is filled with the help of vacuum. The mold is afterwards recovered with powder of biocompatible polymer and placed in a vacuum oven. Vacuum is necessary to remove entrapped bubble and helps to pull the polymer melt into the grooves of the mold (fig 1.20).

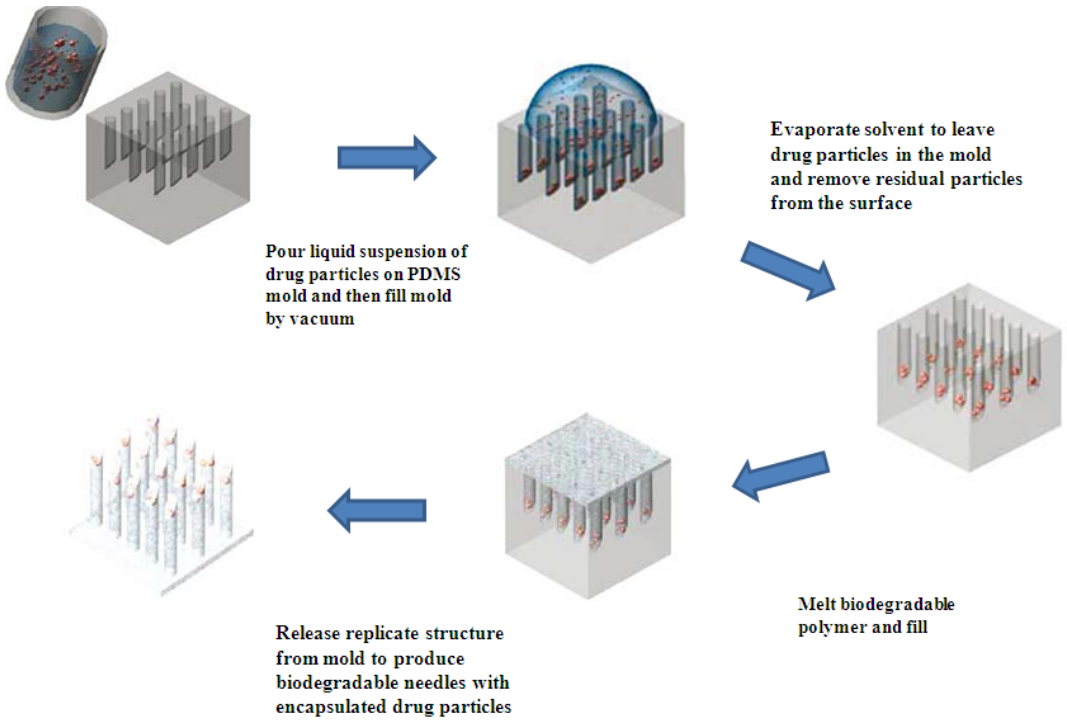


Fig. 1.20. Method to fabricate polymer microneedles that encapsulate drug for controlled release.[61]

As shown in fig 1.21, this procedure gives an inhomogeneous distribution of drug inside the needle. Indeed, the most of it is accumulated towards the tip, and this, even if entail release of entire amount of drug, implies a fast release.

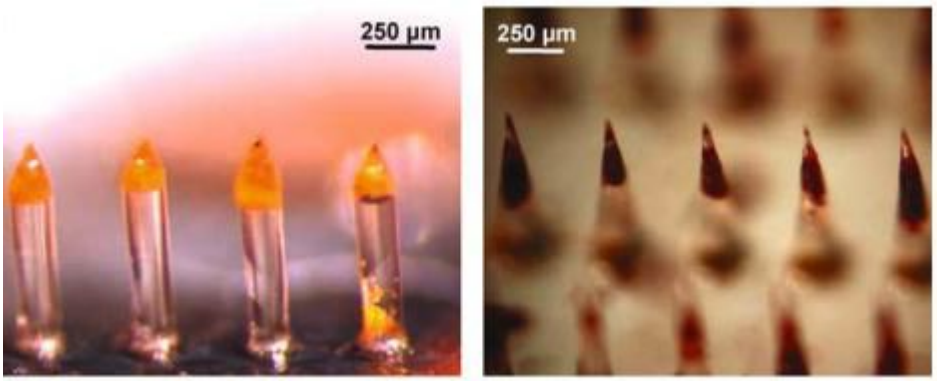


Fig. 1.21. Polymer microneedles bevel-tip (left) and tapered-cone (right) made of PLGA and encapsulating calcein within their tips.[61]

Although this fabrication method is very simple and cheap, it is not suitable for all drugs. Indeed, generally, thermoplastic polymers with enough mechanical strength for this application, require a high melting temperature, that is incompatible with a wide drug's variety.

On the other hand, photocurable polymers, like PEG and PVP, liquid before reticulation, or hydrogels modeled starting from aqueous solutions can also be used. In this case it is easier to mix the drug directly in the polymer matrix, obtaining a homogeneous distribution. By loading model drug into dissolving microneedles in different ways, one is able to design systems that can achieve rapid or extended release from a microneedle patch. Drug can be selectively incorporated into the microneedles themselves and not into the backing layer. A small volume of solution with drug is cast into the holes of the micromold to form microneedles. After wiping off excess solution from the micromold surface, polymer without drug is added onto the micromold and solidified. To administer larger drug doses as an extended release over at least hours, drug can be incorporated into both the microneedles and backing layer or, alternatively, just the backing layer[46] (fig 1.22).

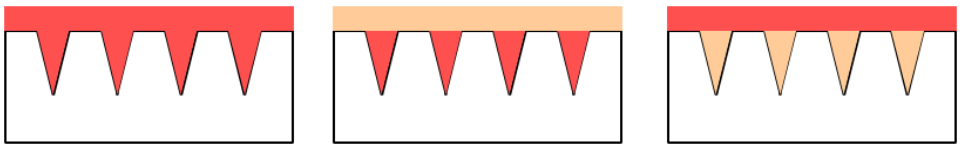


Fig. 1.22. (a) Schematic representation of drug loaded in the entire device's volume, (b) into microneedles and (c) in back layer but not in microneedles[46].

The advantage of this approach is to obtain in a very simple way an array with hundreds of microneedles, all with the same shape. This process is also easily scalable to an industrial level further lowering the cost, for this reason several research groups have focused their attention on these methods, trying to improve efficiency. Chu, Choi and Prausnitz have developed a system to insert an air bubble

in the base of microneedles to concentrate drug towards needle's tip, as to minimize drug wastage. Moreover, they have incorporated a pedestal at the base of microneedles in order to insert tips more fully in the skin. More complete insertion of the microneedles allowed a higher fraction of the encapsulated drug to be delivered.[62] In some cases, because of long degradation time of PLGA, microneedles arrays are designed in order to leave tips inside skin, so drug release can continue after removal of the supporting patch, to reduce patient nuisance.

This is possible coupling PLGA needles with a polymeric base quickly dissolvable,[63, 64] pushing arrowhead needles inside skin with metal shaft[64] or creating complex structure that rest stuck in tissue.[65] In other cases they are used hydrogel microparticles that by means of a swelling mechanism, cause microneedles breakage and acceleration of drug release.[66] Recently a novel drawing lithography technique has been proposed,[67, 68] such process free from replica moulding creates 3D microstructures from 2D thermosetting polymers, the difficulties of this technique are the high temperature, and the poor reproducibility due to the dynamic interaction between frame and fluid.

Another novel non-contact and room temperature electro-drawing (ED) process for the direct and rapid fabrication of biodegradable microneedles by means of an electrohydrodynamic (EHD) process has been proposed,[69] this technique consists of drawing the microneedles from a sessile drop of a biopolymer solution by using EHD forces.

In general, a variety of biodegradable and water-soluble polymers have been micro molded primarily to encapsulate drugs within the microneedle matrix for subsequent release in the skin. Polymer microneedles should be developed to completely degrade/dissolve in the skin and thereby leave behind no biohazardous sharps waste after use.

These microneedles are typically made solely of safe, inert, water-soluble materials, such as polymers and sugars that will degrade/dissolve in the skin after

insertion. The most frequently used matrix materials are sodium hyaluronate, that is naturally present in the skin, and sodium carboxymethylcellulose.[70-75]

Both are approved as inactive materials by FDA for parenteral drug products. Other materials include poly(vinylalcohol) (PVA),[76] poly(vinylpyrrolidone) (PVP),[77] methylvinylether-co-maleic anhydride (PMVE/MA) (Gantrez AN-139®) [78, 79] and low molecular weight sugars like maltose[80, 81] and trehalose [82]. Microneedles have also been prepared from biodegradable polymers such as polylactic-co-glycolic acid (PLGA)[80] polylactic acid (PLA) [83] and polyglycolic acid (PGA).[45]

However, due to their slow dissolution rate in skin and a preparation method using high temperatures[63] or organic solvents, these polymers are less suitable as matrix material. They can be used as a skin pretreatment to increase permeability.

Most of the dissolving microneedles in the literature need to be inserted into skin for at least 5 min to fully dissolve. In contrast, biodegradable polymer microneedles must be inserted and remain in the skin for at least several days to effectively utilize their controlled-release degradation properties to provide controlled release delivery in skin for up to months.[61] To shorten this time, arrow head microneedles were designed to separate from the shaft within seconds and remain embedded in the skin for subsequent dissolution.[64]

Microneedles encapsulating hydrogel microparticles were also designed for successful separation of microneedles within less than 1 h of insertion into skin by swelling of the hydrogel microparticles.[66]

Because microneedles may not insert fully into skin, it is sometimes desirable to encapsulate drugs only in the microneedle tips. For this purpose, drug has been localized in microneedle tips by forming multi-layered microneedles using sequential applications of different compositions of polymer solutions [84] and using a particle-based molding method.[65] Drug was also localized in tips by the addition of an air bubble at the base of each microneedle during fabrication, which prevented drug diffusion from the microneedles into the patch backing.[62]

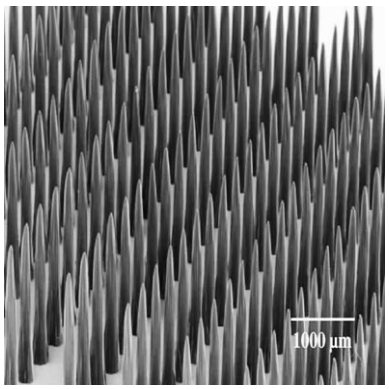


Fig. 1.23. SEM image Polymeric microneedles. [45]

Different types of cargos have been used including almost all vaccine types, ranging from peptides and proteins [70-72] to DNA vectors encoding antigenic proteins [75, 85, 86] and attenuated or inactivated viruses.[73, 87, 88] Antigens are generally dispersed directly in the Microneedles matrix[70-74] but they can also be encapsulated in nanoparticles or in a cross-linked structure [63, 89, 90] to potentiate or alter the immune response.[63, 89, 91] Furthermore, adjuvant can be incorporated in the Microneedles [92].

1.4 Applications

The ability of microneedles to release efficiently both small and large molecules has encouraged studies for various applications.

Several studies, regarding vaccination through microneedles for influenza,[64, 93] and Hepatitis B,[94] have demonstrated that with this new method is obtained a clinical response higher than hypodermal injection [95] using a lower drug dosage.[96] Also for other bioactive macromolecules, like insulin, heparin and growth hormone, that cannot be delivered orally because of proteolitic degradation, microneedles represented a valuable alternative. Zosano Pharmahas developed a parathyroid hormone coated microneedle patch system that is now under phase-3

clinical trial. These patches show an ideal plasma profile, indicative of efficient parathyroid hormone therapy in osteoporosis using microneedles.[97]

Another, less common, application is drug delivery into eyes through sclera, fibrous membrane containing eyeball, for glaucoma treatment. This administration route has been proved to be more effective than topical administration or systemic delivery.[98]

Also in cosmetic and cosmeceutical fields the majority of products can lend themselves to microneedles technology. So it is possible to apply release through microneedles for treatment against ageing (wrinkles, lax skin), scarring (acne, surgical), photodamage and hyperpigmentation (age/brown spots).[98, 99]

Microneedles can be used, not only to administer drugs, but also to withdraw body fluids for diagnostic purposes. An example is blood withdrawal for glucose estimation: in this way it is possible to reduce blood sample required while making the procedure painless.[100]

1.5 Limits of most diffused fabrication methods

The production of polymeric microneedles has not diffused yet because the techniques illustrate beforehand show many limits and difficulties.

The polymer microneedles produced with stamp-based methods have high costs, because are multi steps process, that require much time for the production.

Also the selection of polymer is important because the microneedles must have a sufficient strength to penetrate the skin,[45, 101] and for this reason only high molecular weight polymers can be used.

The production of polymer microneedles usually involves high temperature and that limit the use of thermolabile drugs.[45, 61]

The solvent casting at room temperature is a complex and long procedure.[46, 102]

Casting at room temperature of photocurable polymers has been proposed,[47, 103] but UV can impair the activity of the incorporated drug, and photoiniziators are potentially toxic.[104]

In this wide scenario is set the present work, aimed at the production of polymeric microneedles that can overcome all the limitations deriving from the micro-casting and the drawing lithography approach, avoiding the use of high temperatures, multi- step filling process and hazardous UV are required.

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Chapter II

Experimental section

In this work a new fabricated-mold-based method to create polymeric and biodegradable microneedles is discussed. The fabrication of microneedles starts with the realization of a master with different microfabrication techniques, followed by the production of polymeric microparticles. In particular, the attention has been focused on PLGA as polymer for the production of microparticles. The innovation is to produce microneedles deforming the fabricated microspheres under gentle process conditions (i.e. at room temperature by using a solvent/non-solvent vapor mixture).

2.1 Material

Poly (lactic-co-glycolic acid) 50:50 (PLGA RESOMER® RG 504H), 38000 - 54000 Dalton, was purchased by Boeringer Ingelheim and used as received. Polyvinylpyrrolidone (PVP 856568 Mw 55 KDa) Dimethyl Carbonate (DMC, D152927), as solvent of the PLGA, as well as all the chromophores used as model drug, Sulpho Rhodamine (SulphoRh6G, S470899) and Paraffin wax (327204) were purchased by Sigma Aldrich. Poly (dimethyl-siloxane) (PDMS), used as flexible support, was provided by Sylgard® (184 Silicone Elastomer Kit, Dow Corning). Pig skin was kindly provided by dott.ssa Antonelli Carmela of ASL Napoli 2 Nord, taken from the butchery implant ICS (Industria Carni Sud) of Caivano, Naples (IT).

2.1.1 Poly (lactic-co-glycolic acid)

PLGA is one of the most successfully developed biodegradable polymers. Among the different polymers developed to formulate polymeric microparticles, PLGA has

attracted considerable attention due to its attractive properties: (i) biodegradability and biocompatibility, (ii) FDA and European Medicine Agency approval in drug delivery systems for parenteral administration, (iii) well described formulations and methods of production adapted to various types of drugs e.g. hydrophilic or hydrophobic small molecules or macromolecules, (iv) protection of drug from degradation, (v) possibility of sustained release, (vi) possibility to modify surface properties and/or better interaction with biological materials and (vii) possibility to target nanoparticles to specific organs or cells. The high biodegradability of PLGA is related to its hydrolysis that leads to metabolic monomers, lactic acid and glycolic acid (fig.1) since these two monomers are endogenous and easily metabolized by the body *via* the Krebs cycle, and a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications. The polymers are commercially available with different molecular weights and copolymer compositions. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio.[1, 2] The forms of PLGA are usually identified by the monomers ratio used. For example, PLGA 50:50 identifies a copolymer whose composition is 50% lactic acid and 50% glycolic acid. Poly (lactic acid) (PLA) has also been used to a lesser extent than PLGA due to the lower degradation rate.

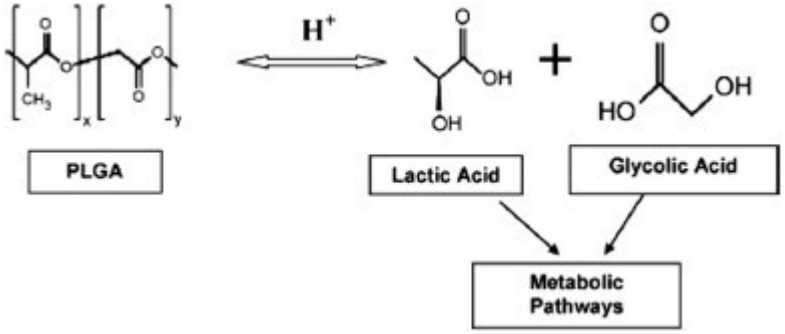

Fig. 2.1. Structural formula of PLA, PGA and their copolymer PLGA.

According to the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained: these are usually identified in regard to the monomers' ratio used (*i.e.*, PLGA 75:25 identifies a copolymer consisted of 75% lactic acid and 25% glycolic acid). Different synthesis mechanisms are used to obtain PLGA and the process parameters influence strongly the physical-chemical characteristics of the end product.

The PLGA can be used in various biomedical applications, *i.e.*, in drug delivery, where the drug/biomolecule kinetic release is dramatically influenced by the polymeric degradation rate.

PLGA degrades by hydrolysis of its ester group, through bulk or heterogeneous erosion, in aqueous environments.

In details, the degradation path can be divided in four steps:

- hydration: water penetrates into the amorphous region and disrupts the van der Waals forces and hydrogen bonds, causing a decrease in the glass transition temperature (Tg);
- initial degradation: cleavage of covalent bonds, with a decrease in the molecular weight;
- constant degradation: carboxylic end groups auto-catalyze the degradation process, and mass loss begins by massive cleavage of the backbone covalent bonds, resulting in loss of integrity;
- solubilization: the fragments are further cleaved to molecules that are soluble in the aqueous environment.[3]

The Tg of PLGA is reported to be above 37 °C and, hence, PLGA has a glassy behavior in nature, showing fairly rigid chain structure.

2.1.2 Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP), also commonly called polyvidone or povidone, is a water-soluble made from the monomer *N*-vinylpyrrolidone.[4]

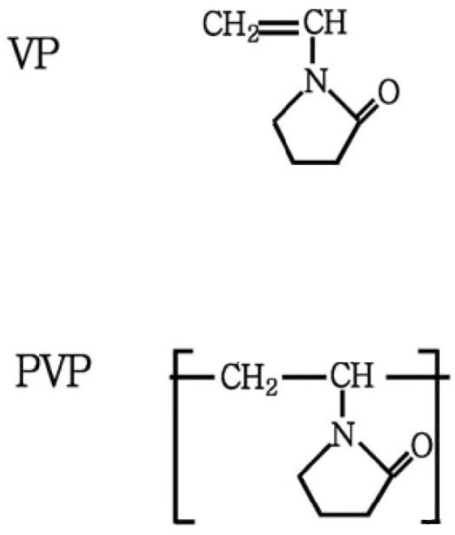

Fig. 2.2. Structural formula of VP and PVP.

PVP is also used in many technical applications:

- as an adhesive in glue stick and hot-melt adhesives
- as a special additive for batteries, ceramics, fiberglass, inks, and inkjet paper, and in the chemical-mechanical planarization process
- as an emulsifier for solution polymerization
- to increase resolution in photoresists for cathode ray tubes (CRT)
- in aqueous metal quenching
- for production of membranes, such as dialysis and water purification filters
- as a binder and complexation agent in agro applications such as crop protection, seed treatment and coating

- as a thickening agent in tooth whitening gels[5]
- as an aid for increasing the solubility of drugs in liquid and semi-liquid dosage forms (syrups, soft gelatin capsules) and as an inhibitor of recrystallization
- as an additive to Doro's RNA extraction buffer
- as a liquid-phase dispersion enhancing agent in DOSY NMR [6]
- as a surfactant, reducing agent, shape controlling agent and dispersant in nanoparticle synthesis and their self-assembly[7].

The U.S. Food and Drug Administration (FDA) has approved this chemical for many uses, and it is generally considered safe.

PVP is soluble in water and other polar solvents. For example, it is soluble in various alcohols, such as methanol and ethanol,[8][as well as in more exotic solvents like the deep eutectic solvent formed by choline chloride and urea (Relin).[9] When dry it is a light flaky hygroscopic powder, readily absorbing up to 40% of its weight in atmospheric water. In solution, it has excellent wetting properties and readily forms films. This makes it good as a coating or an additive to coatings.

PVP was first synthesized by Walter Reppe and a patent was filed in 1939 for one of the most interesting derivatives of acetylene chemistry. PVP was initially used as a blood plasma substitute and later in a wide variety of applications in medicine, pharmacy, cosmetics and industrial production.[7, 10]

2.2 Microspheres of PLGA

Single emulsion process: Oil-in-water emulsification processes are examples of single emulsion processes. Polymer in the appropriate amount is first dissolved in a water immiscible, volatile organic solvent (e.g, dichloromethane (DCM)) in order to prepare a single phase solution. The drug of particle size around 20–30 μm is added to the solution to produce a dispersion in the solution. This polymer dissolved drug dispersed solution is then emulsified in large volume of water in presence of emulsifier (polyvinyl alcohol (PVA) *etc*.) in appropriate temperature with stirring. The organic solvent is then allowed to evaporate or extracted to harden the oil droplets under applicable conditions. In former case, the emulsion is maintained at reduced or atmospheric pressure with controlling the stir rate as solvent evaporates. In the latter case, the emulsion is transferred to a large quantity of water (with or without surfactant) or other quench medium to diffuse out the solvent associated with the oil droplets. The resultant solid microspheres are then washed and dried under appropriate conditions to give a final injectable microsphere formulation.[11-14] **Double (Multiple) emulsion process:** Water-in-oil-in-water emulsion methods are best suited to encapsulate water-soluble drugs like peptides, proteins, and vaccines, unlike single emulsion methods which is ideal for water-insoluble drugs like steroids. First, an appropriate amount of drug is dissolved in aqueous phase (deionized water) and then this drug solution is added to organic phase consisting of PLGA and/or PLA solution in DCM or chloroform with vigorous stirring to yield a water-in-oil emulsion. Next, the water-in-oil primary emulsion is added to PVA aqueous solution and further emulsified for around a minute at appropriate stress mixing conditions. The organic solvent is then allowed to evaporate or is extracted in the same manner as oil-in-water emulsion techniques. In double emulsion processes, choice of solvents and stirring rate predominantly affects the encapsulation efficiency and final particle size.[11, 15, 16]

In this work PLGA microspheres were obtained through the double emulsion process fig.2.3.

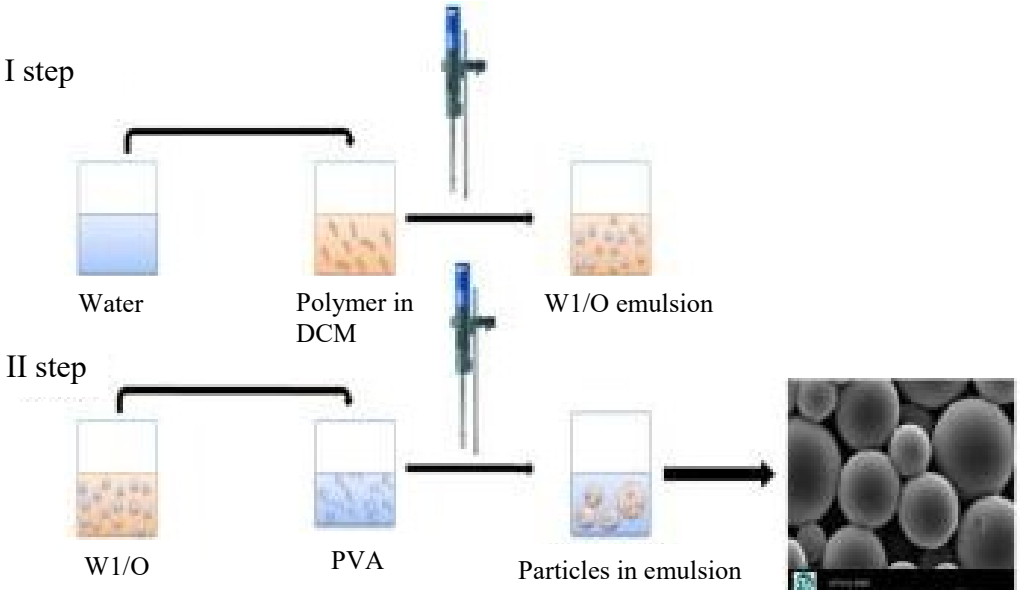

Fig.2.3. Schematic diagram of double emulsion techniques.[17]

The polymer was dissolved in DCM, in order to obtain the dispersed phase of emulsion, while for the continuous phase it was used the (polyvinyl alcol) PVA.

2.2.1 Production process

For the preparation of the solution consisting of the discontinuous phase, 100 mg of polymer (83 mg of PLGA and 17 mg of Pluronic F68) were dissolved in 1 ml of solvent dichloromethane (DCM), while 0.55 mg of Sulforhodamine B was dissolved 1 ml of water. 100 µl of the latter solution was added to the former. The emulsification process was performed with the Ultra-turrax that is a homogenizer. The first emulsion has been prepared for 30 s at 15000 rpm and added to the continuous aqueous phase based on 10 ml of PVA solution (at 2% w/v). Second emulsion has been carried out always with the same instrument for 1 min at 20000 rpm. After that, the solution is kept under mechanical stirring for about 2 h and 30

min, at a speed of 450 rpm, to remove completely the polymeric solvent trough evaporation method. The microparticles are rinsed with deionized water to remove the PVA from the solution with 3 centrifuges of 10 min at 15000 rpm. For their conservation, they are filtered from the water, frozen at – 80°C for 15 min and then subjected to freeze-drying over-night.

2.3 Master production

The techniques used for the production of microneedle is a stamp based method, for this reason an important aspect is to create a master with a good shape to allow the indentation in the skin. Microfabrication technology has traditionally been used to produce master of microneedles. In the early years, the fabrication technology for silicon based structures has been focused on lithography, etching, and deposition. Besides IC based methods, other fabrication processes such as micromolding, wire electro discharge machining, laser machining, ion and electro-beam machining and dicing were also exploited for miniaturization.[18] The surface micromachining and bulk micromachining are the two branches of microfabrication techniques. Surface micromachining is an addictive process, which consists of the fabrication of microstructure from deposited thin films.[19] The bulk micromachining is a subtractive process that uses the selective removal of materials from substrate to form microstructures.[20]
More recently, the microfabrication technology has been increasingly used to machine micro-scale devices related to biological applications.[21, 22]

Compared to the numerous biological applications such as biosensors and fluidic microdevices for sample separation, the use of microfabrication in the drug delivery has been limited. One potential approach was to use microneedles to achieve optimum therapeutic effect for new drugs. With the application of advanced microfabrication technology, novel microdevices may be fabricated to fulfill the requirements for drug delivery. One example is based on microneedles

with a good shape and a sharp tip (1÷10 μm), which increases the mechanical strength needed to support polymer microneedles.[23, 24]

2.3.1 2Photon Laser

In recent years, two-photon lithography (TPL) has emerged as a powerful tool to create complex, small-scale 3D materials.[25-30] By focusing a femtosecond laser into a negative-tone photoresist, polymerization can be locally induced within the focal region of the beam. Rastering the laser focus throughout the photoresist in three dimensions then enables the creation of polymer structures with virtually any geometry.[31-33] This architectural versatility renders these 3D polymer structures useful for many technological applications, including drug delivery,[34-36] tissue engineering,[37-39] micro-nano-optics,[40, 41] and photonics.[42, 43]

2.3.1.1 Photolithography process

2PP was performed on a Nanoscribe Photonic Professional GT system (Nanoscribe GmbH). The Nanoscribe system uses a 780 nm Ti-Sapphire laser emitting ≈100 fs pulses at 80 MHz with a maximum power of 150 mW and is equipped with a 63x, 1.4 NA oil immersion objective. The substrate is placed in a holder that fits into a piezoelectric x/y/z stage. A galvo scanner determines the laser trajectories. The oil-immersion configuration the lens is immersed in oil with a refractive index matching that of the glass coated with the photoresist; here, because of the refractive index difference between the glass and the photoresist, a laser scattering takes place at the glass-photoresist interface then worsening the fabrication performances of the system. The negative tone IP-DIP photoresists assures a high resolution combined with simplicity of use and is optimized for the process of multiphoton polymerization. The IP photoresists are exclusively available to users of the Nanoscribe systems. In the fig. 2.4 a representation of geometrical array produced with 2 Photon laser different masters have been developed with same

shape but with different dimension.

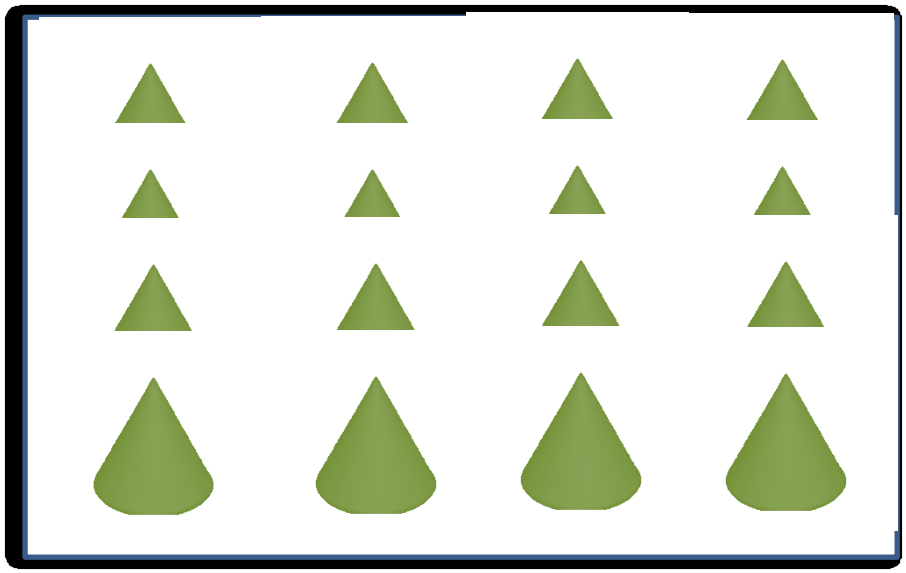

Fig. 2.4. Schematic representation of Microneedle array with conical shape.

2.3.2 Laser 2D and ICP-RIE

Less than 10 years ago, inductively coupled plasma (ICP) reactors have been introduced for silicon reactive ion etching (RIE) process leading to the deep reactive ion etching (DRIE) technique. The main novelties on these rather new ICP-RIE systems are the following:

- separation of the main plasma from the wafer
- a higher plasma density
- improved Radio Frequency RF-power supply
- improved performance for pumping and mass-flow systems
- pulsed Low Frequency LF substrate biasing
- new chemistry and new process (Bosch and cryogenic)

These hardware and process novelties led to improved performances, for instance:

- Higher selectivity for deep etching
- Higher aspect ratio (AR)
- Higher etching rate, either for anisotropic or isotropic etching
- Reduction of parasitic effects: notching, aspect ratio dependent Etching (ARDE), etc.

The microfabrication of high aspect ratio microstructures (HARMS) is the main benefit of these technologies with numerous applications in the field of MEMS. There are two main ways of achieving HARMS by DRIE. The most popular way is the 'Bosch process', a patented process developed by[44], which is based on alternating multiple steps of etching and sidewall passivation. The main alternative is the 'cryogenic process', relying on cooling the stage and silicon to cryogenic temperatures using liquid nitrogen.[45, 46]

2.3.2.1 Production master

Silicon wafers with resistivity ranging from 1 to 10 Ω·cm, and thickness of 450 μm were used in this study. With use of a spin coater a layer of AZ4562 positive photoresist was deposited on the silicon wafer. Using a speed of 3000 rpm a photoresist layer of 8 μm was obtained. The spincoater employed was the WS-650 Series (Laurell Technologies Corporation, USA). Then, after a postback at 100°C for 3 min, the wafer was used for photolithography treatment. The thickness was measured with a contact profilometer (Dektak Veeco 150).

For the 2D lithography process we used a mask-less 2D lithography system (Heidelberg DLW-66FS) equipped with a Diode Laser, 405nm, 100mW. 2D laser was performed on a Heidelberg instrument. With 2D laser an array of circle with 150 μm of diameter was fabricated. Then the sample was developed in a 4:1 water-developer solution for 60 s, rinsed with water and dried with nitrogen; as developer we used the AZ 351-B (Microchemicals GmbH).

The patterned sample was etched with the inductively coupled plasma reactive ion etching technology (ICP-RIE); the system used here was the PlasmaPro 100 Cobra (OXFORD Instruments).

Etching was performed until the oxide mask fell off due to the under etching, which generated the pyramidal needle structure.

The structure of microneedle was fabricated by the mixed anisotropic and isotropic plasma etching in inductively coupled plasma (ICP) etcher.[47] The ICP high density plasma etcher is well known to carry out deep reactive ion etching (DRIE), implemented with BOSCH process, which utilizes an etching cycle using SF_6 gas and then switches to a sidewall passivation cycle using C_4F_8 gas.[48]

After the dry etching, the sample was immersed in acetone for the photoresist stripping, rinsed with 2-propanol and dried with nitrogen. To characterize the etching results the sample was analyzed by SEM (FESEM ULTRAPLUS ZEISS).

In the fig. 2.5 a representation of geometrical array produced with 2D Laser and ICP-RIE different masters have been developed with same shape but with different dimension.

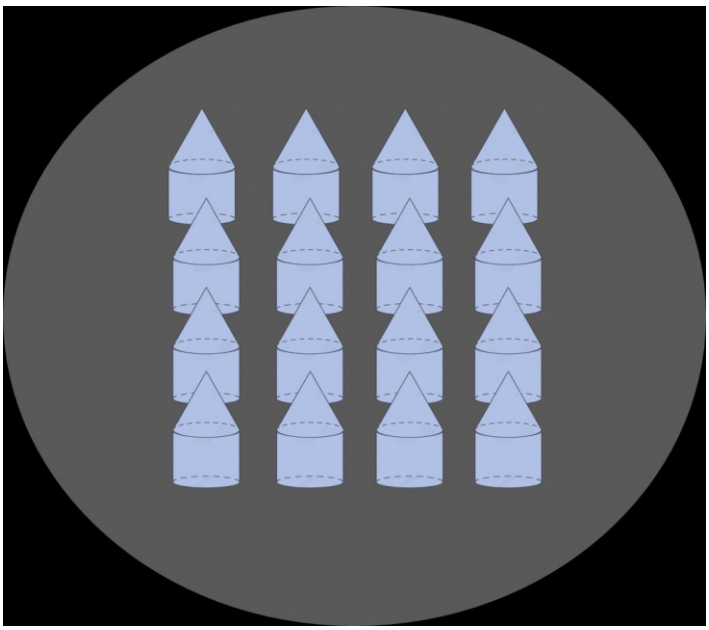

Fig.2.5. Schematic representation of Microneedles array with pyramidal shaped tip cylinder.

2.4 PDMS mold

Poly (dimethyl siloxane) (PDMS) is a silicon elastomeric polymer with many properties that make it excellent as material for biomedical application, namely optical transparency, chemical and biologically inertness, non-toxicity, permeability to gases and excellent mechanical resistance.
The structure of PDMS is:

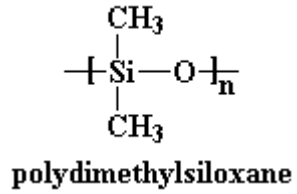

Fig. 2.6. Structural formula PDMS.

Several properties of PDMS are instrumental in the formation of high quality patterns:

- PDMS is an elastomer and conforms to the surface of the substrate over a relatively large area
- it is deformable enough such that conformable contact can even be achieved on surfaces that are non-planar on a micrometer scale
- elastic characteristics of PDMS allows it to be released easily from complex and fragile structures
- PDMS provides a surface with low interfacial free energy ($21.6 * 10^{-3}$ Jm^{-2}) and chemically inert
- PDMS is homogeneous, isotropic and optically transparent down to 300 nm
- PDMS is durable and the stamps can be used up to about 100 times over a period of several months without noticeable degradation.

- surface properties of PDMS can be modified by plasma treatment followed by the formation of SAMs (self-assembled monolayers) to give appropriate interfacial interactions with materials with a wide range of interfacial free energies.

The reparation of PDMS start with the mixing of the precursor with the curing agent, generally in ratio 10:1, and kept under vacuum to remove entrapped air bubbles. To make microneedles molds, master structure arrays of microneedles in relief are coated with liquid PDMS precursor and allowed to cure in oven; from a single master it is possible to get several molds fig 2.7.

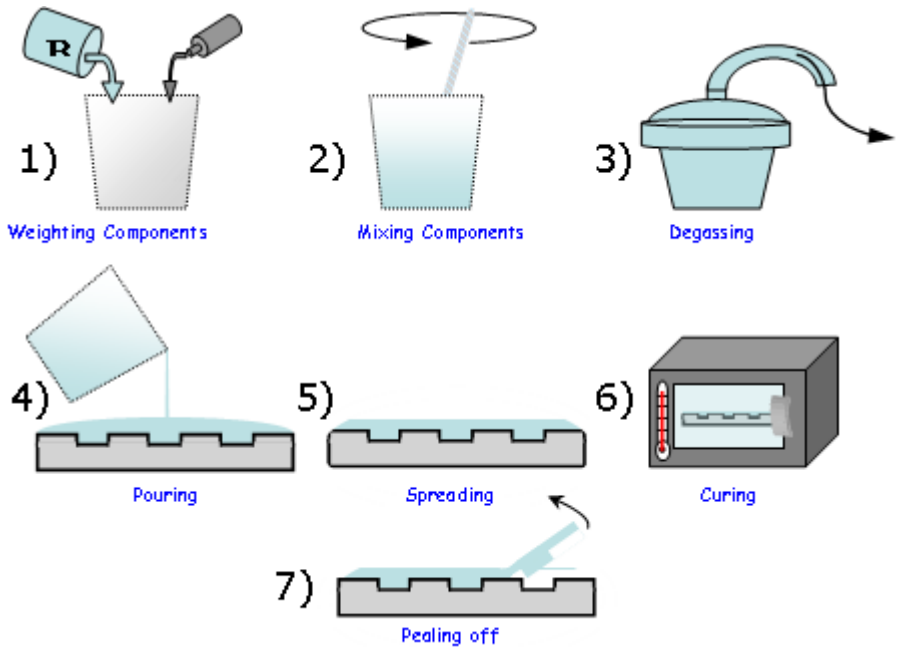

Fig. 2.7. Schematic procedure for fabrication of PDMS stamp.

After polymerization and cross-linking, solid PDMS presents a hydrophobic surface and this facilitates the separation from hydrophilic materials.

It is possible to make PDMS hydrophilic by adding to the PDMS precursor and treating agent mixture a surfactant i.e. SILVET in ratio 100:1.

Thus PDMS structure can be used in combination with water and alcohol solvent without material deformation.

2.5 Polymeric microneedles

Two different processes have been used for the production of microneedles array, obtained through the use of a stamp based process assisted by solvent.
The first process can be divided in three steps:

1. microparticles inserted in the stamp;
2. PLGA microparticles sintered with a room temperature process, with a solvent not solvent mixture;
3. hydrophilic matrix deposition.

The second process can be divided in four steps:

1. production of microneedles tips with hydrophilic polymer through degasing and spinning;
2. microparticles inserted in the stamp;
3. PLGA microparticles sintered with a room temperature process, with a mixture of solvent not solvent;
4. deposition of hydrophilic polymer on the medical tape.

2.5.1 Solvent evaporation

Solvent evaporation was performed with a Drechsel Bottle.

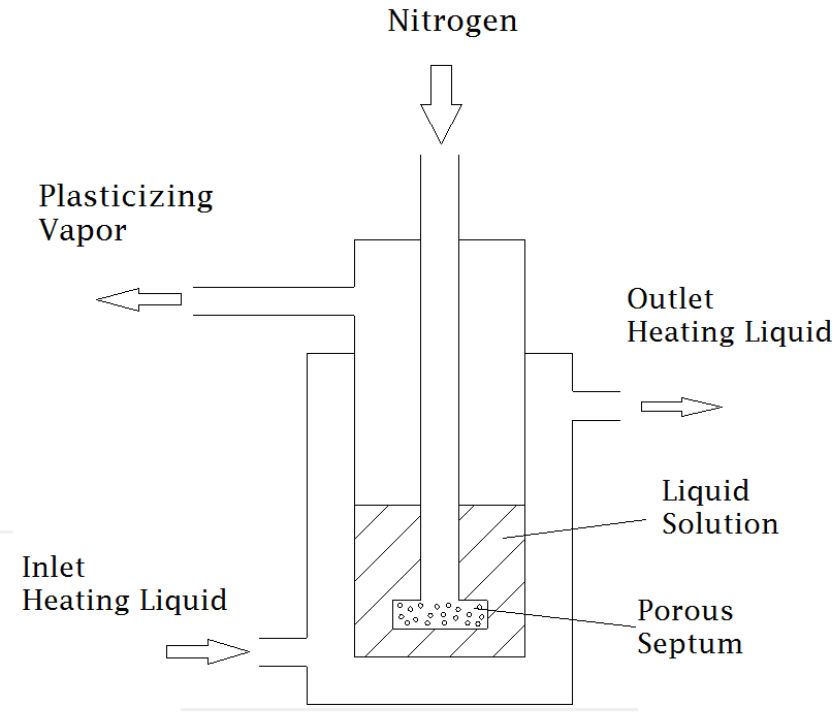

Fig. 2.8. Scheme of the Drechsel Bottle.

The mixture of solvent is put inside the bottle with a stream of nitrogen flow that produces bubbles. It was possible to control the pressure of nitrogen flow through a pressure regulator.

The plasticizing vapor product arrived on the substrate through a tube. The drechesl bottle was connected to a thermostatic bath, which is used to maintain constant the temperature of the inlet heating liquid.

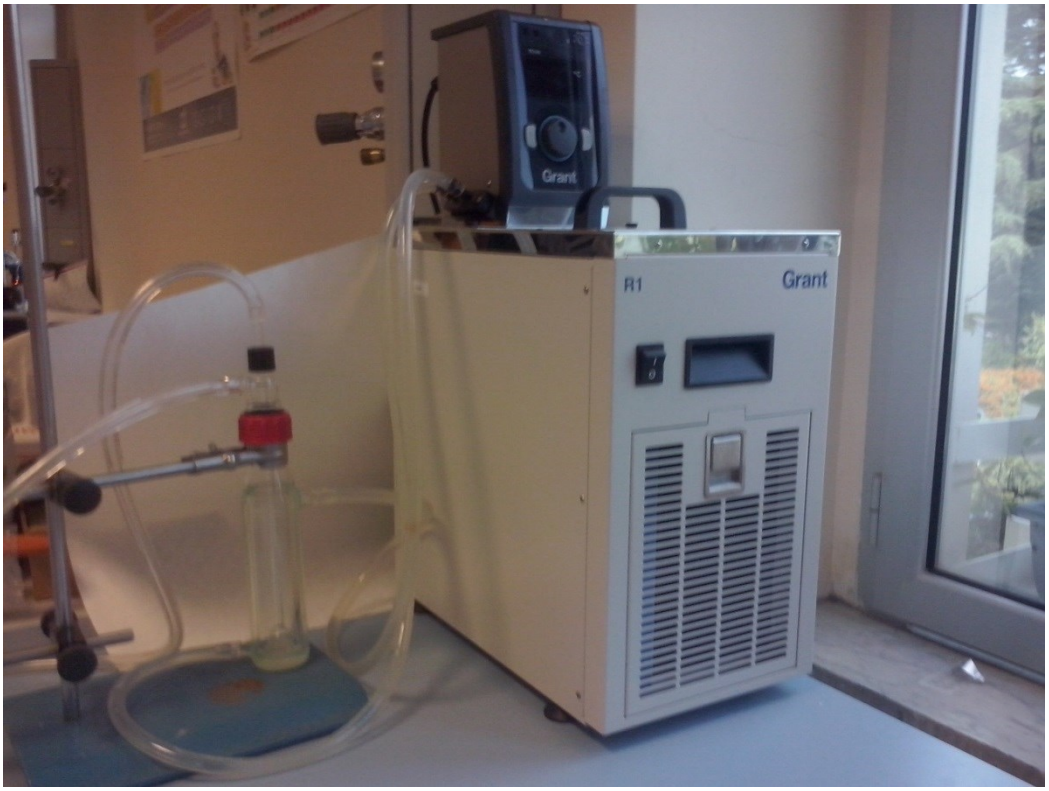

Fig. 2.9. System used for the plasticization of the microspheres.

2.5.2 Production process

The solvent mixture was composed by 4 ml of ethanol and 0.5 ml of DMC. The temperature and the pressure were set respectively at 25.3°C and 0.15 bar. This condition is the same for both processes, and it is good for the softening of PLGA microparticles which have been used.

In the first process the microspheres were disposed in the cavities of the PDMS mold, inserted whit the use of the microscope.

In the second phase, the mold with the microspheres was placed under the tube in which the plasticizing vapor flows. After a few minutes it can be observed the plasticization of the microspheres that assume the shape of microneedles, and then a hydrophilic matrix is deposited on the mold. This method does not allow the production of a very sharp tip because it is difficult to insert correctly the

microspheres at the deeper part of the stamp cavities. For this reason, it has been implemented the second process.

In this case the tip of microneedles is produced with a hydrophilic polymer inserted in the mold through degassing and then spinning. Then, in the second step similar to the process described previously microparticles were inserted in the cavities of the mold in order to produce the base of microneedles, and then the mold was placed under the tube in which the plasticizing vapor flows. The plasticization of microparticles occurs after few minutes. Finally, in order to extract the microneedles from the stamp a 3M medical tape with a hydrophobic layer was used.

2.6 Characterization of microneedles

The microneedles were characterized with several analyses to study morphological aspect, distribution of drug and mechanical resistance.

2.6.1 Multiphoton fluorescence microscopy

The multiphoton fluorescence microscope (MPM) uses pulsed long wavelength light to excite fluorophores within the specimen being observed. The fluorophore absorbs the energy from two long-wavelength photons which must arrive simultaneously in order to excite an electron into a higher energy state, from which it can decay, emitting a fluorescence signal (fig. 2.10). It differs from traditional fluorescence microscopy in which the excitation wavelength is shorter than the emission wavelength, as the summed energies of two long wavelength exciting photons will produce an emission wavelength shorter than the excitation wavelength.

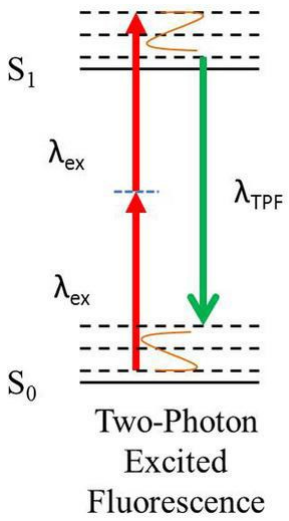

Fig. 2.10. Diagram indicating the absorption of two NIR photons to excite the fluorescent molecule to an excited state and the visible fluorescence emitted during relaxation.

Multiphoton fluorescence microscopy has similarities to confocal laser scanning microscopy. Both use focused laser beams that scan the sample to generate images, and both have an optical sectioning effect. Unlike confocal microscopes, multiphoton microscopes do not contain pinhole apertures, which give confocal microscopes their optical sectioning quality. The optical sectioning produced by multiphoton microscopes is a result of the point spread function of all elements included in optical path, namely depends on how the system blurs the image of a bright spot formed where the pulsed laser beams coincide.

Compared to similar optical imaging techniques, MPM holds inherent advantages for imaging living tissues by improving depth penetration and reducing photodamage. This is a direct result of employing near infrared (NIR) femtosecond lasers to generate observable nonlinear signals in the visible range. The NIR excitation enhances the ability to image deeper into a sample through a reduction light scattering proportional to the fourth power of the excitation wavelength.

2.6.2 Scanning Electron Microscope

For morphological analysis of microneedles sectioned a field emission SEM (Ultra plus Zeiss) was used. A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing signals that can be detected and that contain information about the sample's surface topography and composition. In a typical SEM, an electron beam is thermoionically emitted from an electron gun fitted with a tungsten filament cathode. The electron beam, which typically has an energy ranging from 0.2 keV to 40 keV, is focused by one or two condenser lenses to a spot about 0.4 nm to 5 nm in diameter. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the x and y axes so that it scans in a raster fashion over a rectangular area of the sample surface (fig. 2.11).

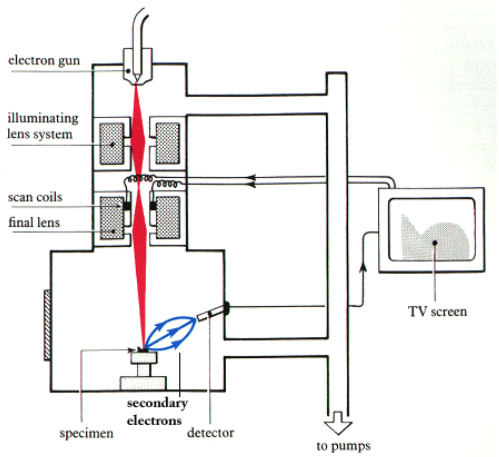

Fig. 2.11. Functioning scheme of a scanning electron microscope.

When the primary electron beam interacts with the sample, the electrons loose energy by repeated random scattering and absorption within a region of sample's volume, whose extension depends on the electron's landing energy, the atomic number of the specimen and the specimen's density. The interaction between

electron beam and surface sample produce a great number of particles, like secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays and transmitted electrons. In the standard detection mode, secondary electron imaging or SEI, the SEM can produce very high resolution images of a sample surface. Instead, back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. BSE are often used in analytical SEM along with the spectra made from the characteristic X-rays, because the intensity of the BSE signal is strongly related to the atomic number (Z) of the specimen.

2.6.3 Chromophore distribution

Different hydrophilic chromophores were used to produce microneedles, in order to study the distribution inside the microneedle. Microneedles were incorporated in PDMS, cured for 24 h at room temperature, and then frozen at -140 °C in Leica CryoUltra Microtome EM-FC7-UC7. Samples were sectioned at a thickness of 5 μm for confocal analysis in axial direction. Slices of samples were analyzed with a confocal Leica TCS at 543 nm using a 20X air microscope objective. While cutting, slices tend to bend so they are not perfectly outstretched on cover glasses. For this reason, since the surface is not included entirely in the same focal plane, in order to acquire an entire slice, stack acquisitions were performed on thick layers using 0.5 μm Z-steps. The acquired images were collected and analyzed using ImageJ (Java-based image processing program developed at the National Institutes of Health) studying fluorescence profile in axial direction and transverse direction at certain distances from the tip, as show in fig. 2.12.

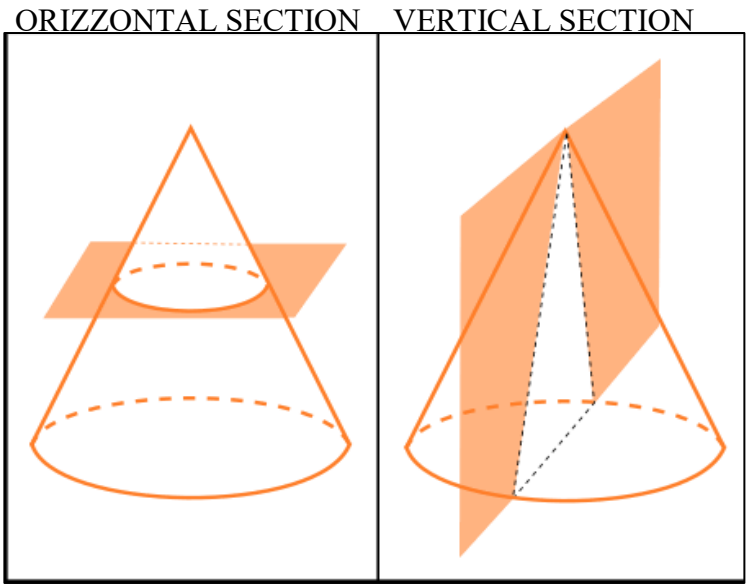

Fig. 2.12. Schematic direction illustration of section plane.

2.6.4 Porosity

The microneedles and microparticles were sectioned as described above at a thickness of 5 µm and a morphological analysis was performed using scanning electron microscope (SEM) (field emission Ultra plus Zeiss. This analysis allows understanding how the sintering process influences microneedles porosity. Samples were sputter coated with a 15 nm thick gold layer and to avoid any damage, a voltage (EHT) of 10 kV was imposed. Morphological analysis of porosity was carried out on the as produced microneedles.

2.7 Mechanical characterization

The fundamental requirement for microneedles is an adequate mechanical resistance, that allows to puncture the stratum corneum. For this reason, it is very important to study their capability to indent skin, in this way we demonstrate the mechanical properties of the microneedles.

2.7.1 Indentations in skin

First, indentation tests were performed in gelatin and paraffin wax.
Thin layers were prepared using these materials, the test was conducted under stereomicroscope. In order to confirm that the indentation had occurred the layer of paraffin was replicated with PDMS prepared as previously described.
Different arrays of microneedles with different dimensions were inserted in a full thickness cadaver pig skin without subcutaneous fat layer. The skin was shaved with depilatory cream and washed in a phosphate buffered saline (PBS) solution; finally, it was placed on absorbing paper for few minutes to eliminate the excess of water. Two different indentation tests were carried out with a SMICNA applicator system.

Fig. 2.13. SMICNA Applicator system.

In the first test, microneedles were removed after indentation and the skin was fixed in a solution of 10% neutral buffered formaline for 24 h, dehydrated in an incremental series of ethanol (75 %, 85 %, 95 % and 100 % and 100 % again, each step is performed for 30 min at room temperature) treated with two series of xylene for 30 min and then were embedded in paraffin.
Successively, samples were sectioned at thickness of 3μm, and stained with hematoxylin and eosin, and finally the sections were mounted with Histomount

Mounting Solution (INVITROGEN) on coverslips and the morphological features of constructs were observed with a light microscope (BX53; Olympus).

In the second test the skin was fixed in a solution of 10% neutral buffered formaline for 24 h. Samples were dehydrated by serial immersions, each of 30', in Methanol 50%, 70%, 80%, 90%, 100%, 100%. BABB solution was prepared containing Benzyl Alcohol (BA) and Benzyl Benzoate (BB) (SIGMA) in 1:2 ratio. Samples were moved into a glass bottle and treated with Methanol and BABB (1:1) for 4h and following with BABB for 24h at RT. Samples were investigated into fluorodish by Confocal Leica TCS SP5 II.

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Chapter III

Results and Discussion

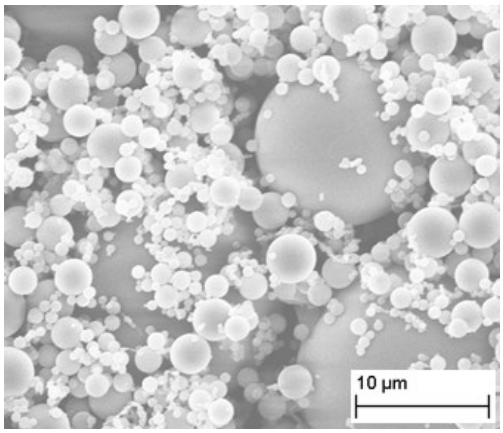
The main aim of this thesis was to produce polymeric microneedles embedding model drugs for dermal drug delivery. The method used in this work overcomes the limitations related to the known methods, in particular those related to the micro-casting and drawing lithography, since heating, multi-step processes and hazardous UV are not required.[1, 2] This study presents an approach for the realization of double-compartment microneedles for the delivery of two model drugs in sequence. In particular, one is a PVP compartment as a fast dissolvable matrix which was loaded with FITC and another compartment, based on porous PLGA microspheres assembled each other, which was loaded with Sulforhodamine B used to simulate the possible cargo. To fabricate the polymer microneedles needed to achieve these design goals, we employed a molding technique using (PDMS) molds. PDMS molds have been produced by replica molding from masters produced with different microfabrication techniques, such mold being provided with an array of cavities having the shape of a microneedle. Such microneedles have then been harvested from the mold by means of a water-soluble polymeric layer, morphological analysis was conducted and indentation tests *in vitro* have been carried out, to demonstrate the mechanical properties of microneedles product.

3.1 Microspheres production

Polymeric microspheres have been employed as starting material for the production of biodegradable polymeric microneedles. Such microspheres have been produced by means of a double emulsion process.

The choice of materials for the microparticles must satisfy multiple criteria that vary depending on the patch design and among the patch components. The microspheres were made of PLGA and Sulphorhodamine B, as a model drug. PLGA was selected since it is a very well-known biodegradable material, which is FDA approved, and has suitable mechanical properties for the production of microneedles for drug delivery.[3] In particular, a T 25 digital ULTRA-TURRAX® was used for the double emulsion according to the procedure reported in the Materials and Methods section, to produce porous microspheres in the range of 1-15 µm. The choice of using a double emulsion for microparticles is due to the possibility to use different cargos such as a hydrophobic one in the matrix of microparticles and a hydrophilic one in the pores of the microparticles.

a)



b)

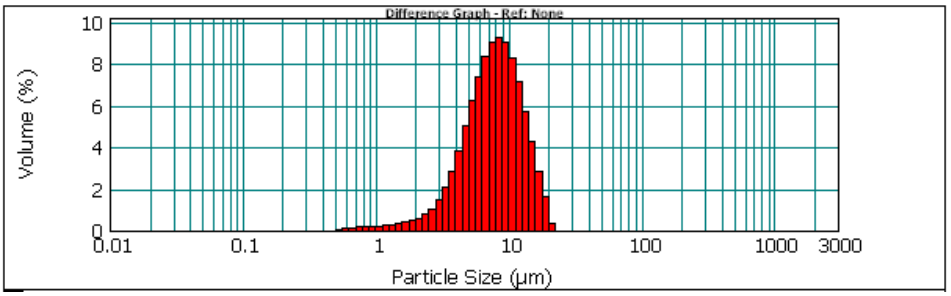


Fig. 3.1. a) SEM image of Porous Microspheres; b)Volumetrical analysis of microparticles with Mastersize.

3.2 PVP production

Drug is typically present in a water-soluble matrix that dissolves in the skin. This matrix must be not only biocompatible, but also suitable for delivery into and clearance from the body. This usually means, at a minimum, the matrix must be on the FDA's Generally Regarded As Safe list and, more ideally, has been safely used in parenteral (i.e., injectable) products. It also should be water-soluble and, when rapid onset of drug delivery is desirable, exhibit rapid dissolution in the skin. The material should also be compatible with the drug it encapsulates during manufacturing, storage, and delivery.

When this matrix is used as a coating on microneedles, it must form films on the microneedles that remain adherent during storage and skin insertion and should be amendable to coating processes. When this matrix forms the microneedle (i.e., dissolving microneedles), then it must also satisfy the mechanical strength requirement to make a strong microneedle. In this study, PVP was chosen as the structural material for the fabrication of the tips of microneedles , in an aqueous environment at room temperature. As it is well known, organic solvents and elevated temperatures may denature the activity of encapsulated drugs, this choice is also good for the encapsalution of thermolabile drugs. PVP is mechanically strong as MNs because of its chemical backbone that contains rigid rings. Additionally, PVP is water soluble, facilitating a rapid dissolution of MN arrays when inserted into skin. Once dissolved in the body, PVP can be safely excreted through the glomerulus within a few days [4]. The PVP used in this work is PVP at 55 kD. It was dissolved in 1 ml of water at a concentration of 25% w/v, with the addition of 0.5µl of FITC .200 µl of PVP at 25% was used to produce tip of microneedles .

3.3 Mold production

In order to prepare the microneedles, PDMS stamps, replicated onto masters made of different materials and techniques, were employed. The mold used to produce microneedles has conical shape and pyramid tip-shaped cylinders of different sizes, the first was entirely produced with a 2 Photon Laser, the second was produced by sequential combination of 2D Laser and ICP RIE techniques. With the first technique, the master was made of the negative photoresist IP-DIP, while with the second technique, the master was made of silicon. The first technique provided a master with high resolution, tunable different sizes and shapes. On the other hand, the photoresist can detach from the substrate after some uses; moreover, the serial production is very slow with a basic setup (piezo mode) but can be made faster with the new up-grade (galvo stage). In order to enhance the adhesion of the photoresist to the glass substrate, this was first treated with a plasma O_2 and then it was covered with the photoresist by the spin-coating technique, then was cured with UV lamp for 3 h. The cured photoresist was coated with another layer of uncured photoresist that is the ultimate layer exposed to the 2 Photon Laser. The microneedles layout was designed with selected conical hollows in order to reduce the production time.

With the second technique it was possible to obtain a master with excellent micromechanical properties and the process was quite fast after fine tuning of the process parameters. In this case, only limited dimensions and geometries can be realized and each time a long optimization procedure of the etching parameters during the ICP-RIE step is needed.

The dimension of the cone produced by 2 Photon Laser was 150 or 300 μm of base diameter and 300 or 600 μm of height, while the array of microneedles is extended onto an area of 1 cm^2. Microneedles are assembled into arrays that typically range from tens to tens of thousands of microneedles. Array area is often in the 1–10-cm^2 range. This can result in a wide range of microneedle densities. A large

microneedle density (i.e., requiring thinner and therefore shorter microneedles) can suffer from the “bed-of-nails” effect, where the insertion force is distributed among too many microneedles so that none penetrate the skin. This may be overcome by having sharper tips and stronger insertion forces.[5-7] Smaller microneedle density makes skin insertion easier and accommodates wider and therefore longer microneedles, but the smaller number of microneedles typically reduces the amount of drug that can be delivered. Increasing microneedle array area accommodates more microneedles but can increase pain.[8, 9]

The microneedles obtained by 2D laser and ICP-RIE have a pyramidal tip standing onto a cylindrical structure. In particular, the process was optimized to obtain a pyramidal tip with 60 µm of height and 112 µm of base diameter onto a cylindrical pillar with 150 µm of height and the same base diameter of the pyramidal structure.

a)

b)

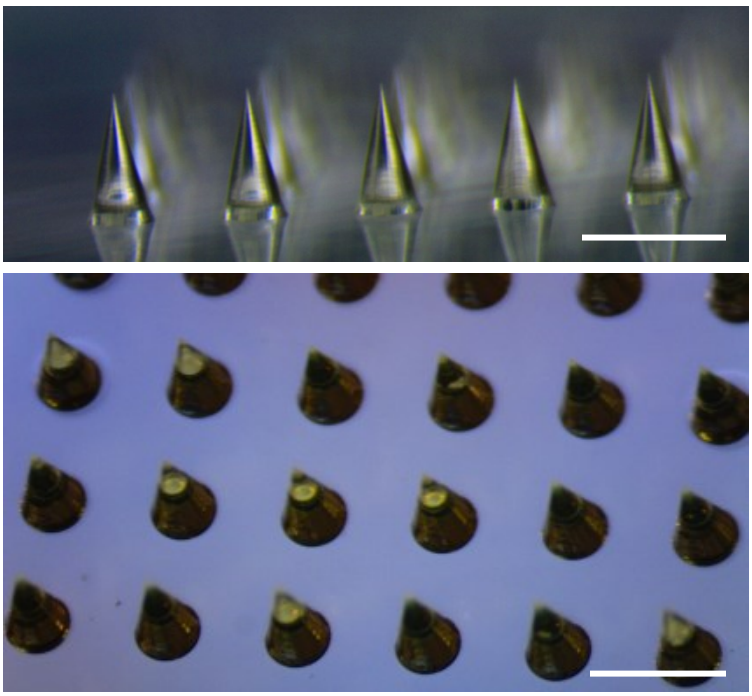


Fig. 3.2. a) Optical images of array of microneedles of 300 µm height and 150 µm diameter of base scale bar 600 µm; b) Array Microneedles of 600 µm height and 300 µm diameter of base scale bar 1.2 mm.

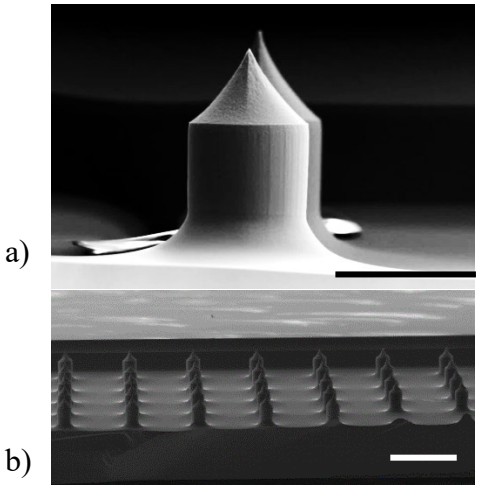


Fig. 3.3. SEM images of maicroneedles (scale bar 150 µm) (a) and array of microneedles (scale bar 600µm) (b) produced by 2D laser and ICP-RIE.

PDMS was used for the fabrication of the stamp. As previously described, PDMS prepolymer was mixed with the curing agent at the ratio of 10:1 and degassed under vacuum until complete disappearance of the air bubbles. Then, the PDMS was poured on the master, cured at 70 °C for 1 h and finally peeled off from the master. Fig. 3.3 shows the PDMS mold.

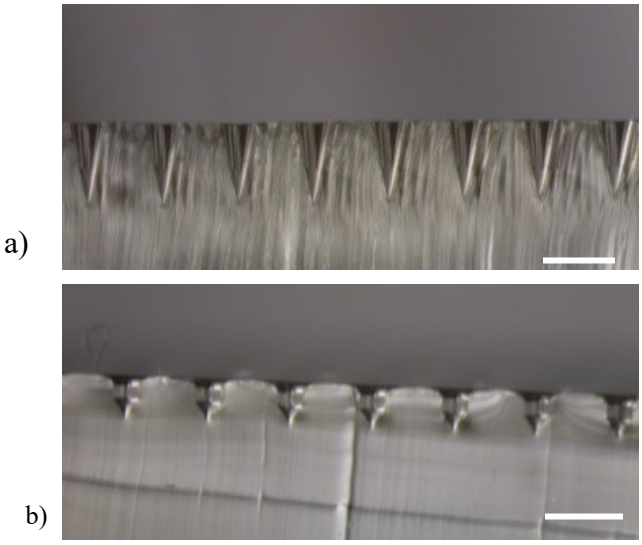


Fig. 3.4. Stereomicroscope images of PDMS mold used for microneedles fabrication taken after sectioning: a) conical shape (scale bar 600 µm) and b) cylinder with pyramidal tip (scale bar 300 µm).

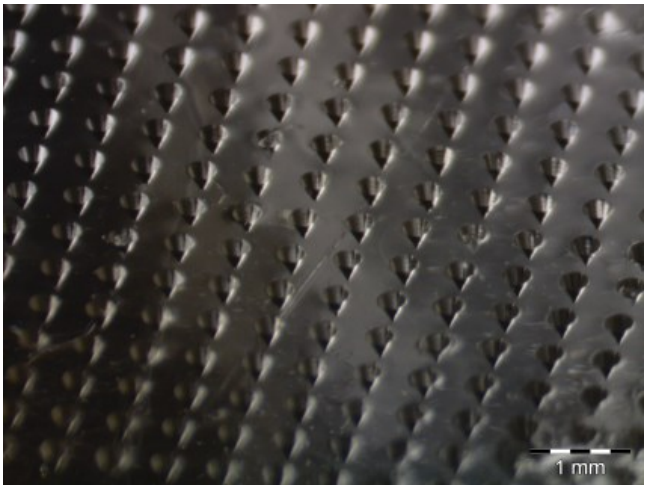


Fig.3.5. Optical microscope of the PDMS stamp with cone shape.

3.4 Production of microneedles

Polymer microneedles can be made using various methods most commonly involving casting liquid solutions onto an inverse mold of the MNP that is often made of polydimethyl siloxane to make dissolving microneedles, hydrogel microneedles, and, in some cases, coated microneedles.[10] After drying, the MNP is peeled out from the mold. MNPs made in this way have been studied for influenza vaccination in clinical trials.[11, 12] Two-photon polymerization has also been used to make polymer microneedles.[13] Casting solvent onto molds, where the solvent typically is water, is often used to make polymer microneedles.[10, 14] In some cases, two different formulations are casted sequentially onto the molds. The first cast contains the drug, in addition to other excipients, and is used to fill the mold cavities forming the microneedles. The second cast contains no drug and is used to fill any remaining space in the mold cavities and to form a film on the surface of the mold to create the MNP base substrate. In this way, drug is located only in the microneedles and not in the base substrate.

To make the microneedles, the formulation needs to meet certain requirements: it must be compatible with the drug, fillable into the mold cavities (e.g., sufficiently low viscosity, often with the aid of vacuum), and mechanically strong. To dissolve microneedles, it also needs to exhibit (rapid) dissolution in the skin. The formulation used to make the base substrate may need to have less drug solubility if the base substrate is designed to contain no drug. It could also have higher viscosity and be mechanically weaker after drying (and in some cases designed to be flexible). In addition, it may not need to be water-soluble.

After casting, the MNP needs to be dried before removal from the mold, assembled into a patch, and placed in packaging. When double casting is used, the first cast is often at least partially dried before the second cast is applied. Rapid drying is desirable (e.g., using heat and/or vacuum), but loss of drug activity (especially for biological molecules and vaccines) is often associated with drying, so drying conditions must be optimized to maintain drug stability.[15-17] Air drying is typical, but lyophilization has also been used.[17] With different techniques it is possible to obtain only a fast release of drug, after indentation in the skin. In this work I developed two approaches in parallel. In both cases, the aim was to develop microneedles able to combine bolus and long-term dosing of cargos released from cutaneous implanted PVP and PLGA depots, respectively.

First Procedure

The microspheres were placed inside the cavities of the mold under a stereomicroscope as shown in fig. 3.6.

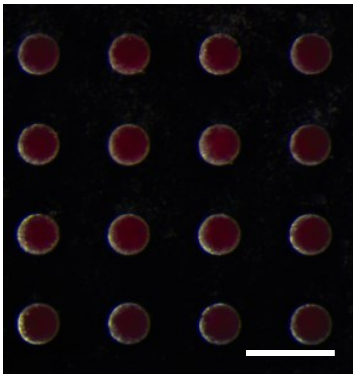


Fig. 3.6. Optical microscope of microspheres in the cavities of the mold scale bar 600 μm.

After filling up the mold with the microspheres, they were exposed to a vapor mixture of solvent non solvent in order to carry out a novel and mild plasticization able to assemble the microparticles in the shape of microneedles while keeping the microstructure of the starting microparticles.

This mild softening method was developed in a previous work for the mild deformation of single microparticles located in the cavities of a PDMS stamp.[5] In this work of thesis this patented method has been applied to multiple microparticles in order to promote their assembly by gentle sintering in the cavities of the stamp. In this way it has been possible to better reproduce more complex structures like microneedles with high aspect ratio with no need to use strong plasticization on single microparticles.[18] The advantage of inserting multiple microparticles consists on the formation of a sharper tip of the microneedles employing mild plasticization conditions that do not impair the internal structure and do not alter the properties of the encapsulated drug. The microneedles produced with the previous method are separated from each other; it's possible to extract single microneedles by the mold as possible to see in the figure 3.7.

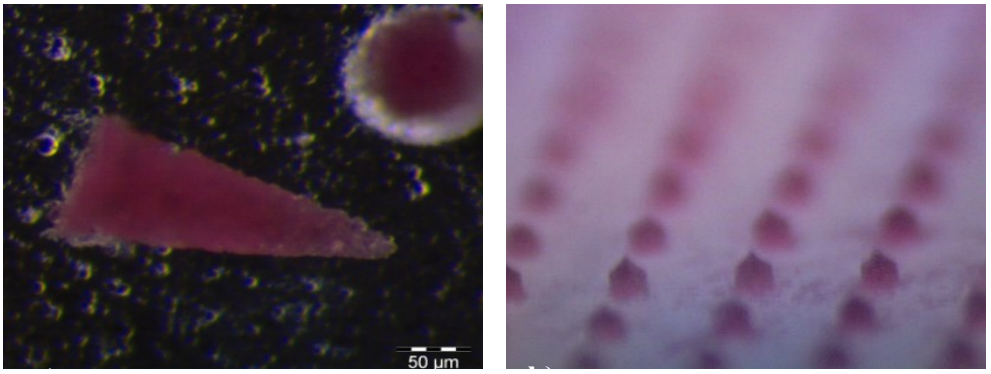


Fig. 3.7. Stereomicroscope images of (a) conical microneedles extracted from the mold and (b) array of cylindrical microneedles.

The as-produced microneedles show a tip with radius of curvature larger than corresponding mold. To overcame this limit, it has been used a hydrophilic matrix deposited on the mold cavities after the first step. The hydrophilic matrix was composed of PVP at 25% w/v in water. PVP was selected since it is biodegradable and biocompatible approved by FDA administration. Fig. 3.8 shown array of microneedles product with this process.

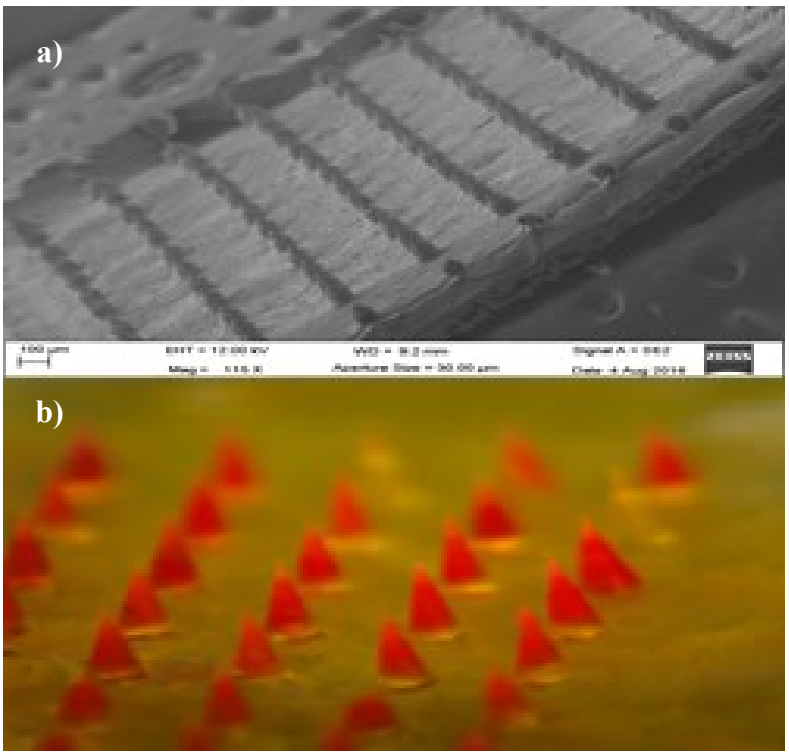


Fig. 3.8. a) SEM image of an array of cylindrical microneedles with pyramidal tip produced with the first process, and b) Stereomicroscope image of an array of conical microneedles produced with the first process.

Second procedure

Another procedure which is able to provide sharp tips has been developed during the work.

In this second procedure we started from a hydrophilic polymer solution with PVP at 25% (w/v), embedding a model drug, to produce the tip, by filling the cavities of the stamp, degassing and carrying out a spin coating to spread out the excess of solution from the stamp and allow a fast evaporation of the residual solvent.

Fig. 3.9. Production process of microneedles tip. a) Deposition of PVP with Gilson on the PDMS mold; b) Degassing process of solution with PUMP; c) spin coating technique to produce only PVP microneedles.

After filling with PVP the apical part of the cavities, next steps were the same. Stamp cavities were filled with the same microparticles in the range of 1-15 µm under microscope, as shown in fig. 3.4.

Then, it followed the same plasticization step by exposing microparticles to the vapor of solvent-non solvent mixture and the evaporation of the solvent. Then, it followed the same step of consolidation based on the evaporation of the liquid phase based on the solvent-non solvent mixture. To extract microneedles, it was used also in this case a 3M Medical tape but first a droplet of PVP solution was deposited on medical tape to promote more adhesion from the microneedles array and medical tape, as shown in fig. 3.10.

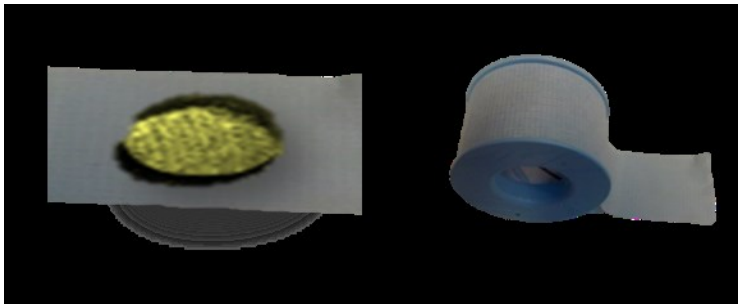


Fig.3.10. a) PVP droplets on the medical tape b) 3M Medical tape.

The microneedles obtained with this second procedure presented a different distribution of the two compartments of the microneedles since the fast dissolvable is localized in the tip.

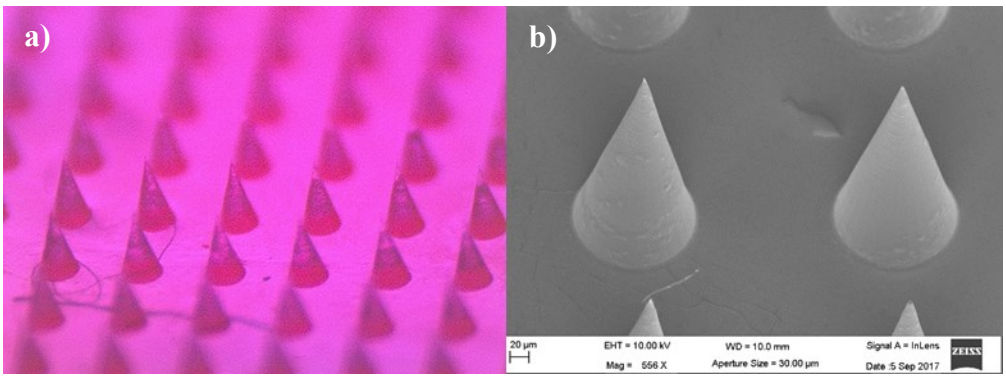


Fig. 3.11. a) Stereomicroscope image of microneedles array b) SEM Image microneedles array.

The most similar procedure reported so far is the one published by Irvine and his group, who manufactured arrays of microneedles composed of drug-loaded PLGA microparticles with a supporting and rapidly water-soluble PAA matrix.[19] In that work PLGA microparticles always obtained by double-emulsion-solvent-evaporation were applied to the surface of the mold in aqueous suspension and compacted into the mold cavities through centrifugation. Excess microparticles were then removed from the PDMS surface and the microparticle-loaded mold was allowed to dry. To form dissolving microneedles carrying dispersible PLGA microparticles, they next added a concentrated aqueous solution of PAA (35 wt%)

to the mold surface and infiltrated the PAA solution into the packed PLGA particle bed via centrifugation. The loaded molds were then dried at 25°C for 48 h before desiccation under vacuum to obtain solid PLGA-PAA microparticle matrices. The dry composite microneedles were finally removed from the PDMS mold.

That procedure is quite long and can lead to the loss of part of the hydrophilic drug from the microparticles which are dispersed in water during the process. Additionally, the centrifugation cannot guarantee a fine control over the homogeneous filling of mold cavities.

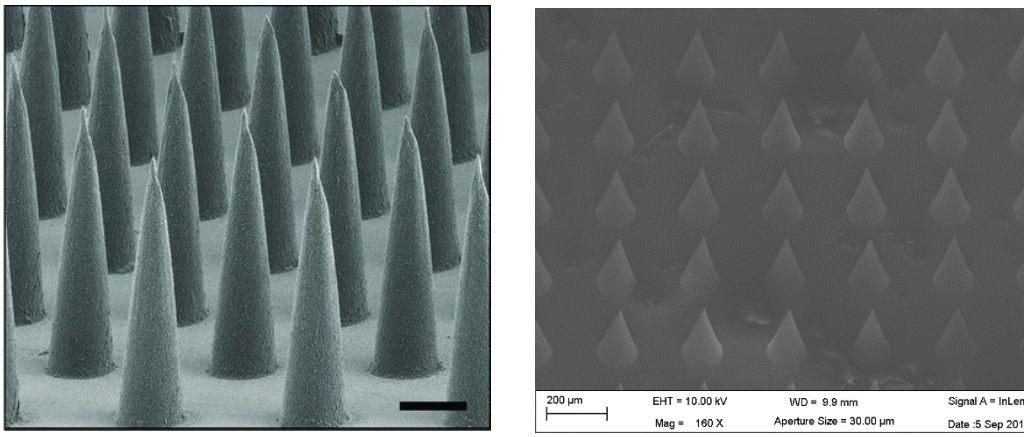


Fig.3.12. SEM images of (a) microparticles-encapsulating microneedle array (scale bar 200 µm)[19] and (b) of microneedles produced with the proposed protocol.

The MA microneedles proved thermostable and able to perforate the stratum corneum and implant the antigen/adjuvant-loaded PLGA microparticles in the epidermal compartment following patch removal due to rapid separation of microneedles from PVP layer used for the microneedles extraction.

3.5 Porosity analysis

The morphological analysis of microneedles was performed with a Scanning Electron Microscope (SEM), to understand if the process used for the production of microneedles could modified the morphology of the microspheres. For this reason, microneedles and microspheres were incorporated in PDMS, cured for 24 h at room temperature, and then frozen at -130 °C in Leica Cryo Ultra Microtome EM-FC7-UC7. Samples were sectioned at a thickness of 5 μm for analysis in axial direction.

Microneedles porosity is of fundamental importance for the drug release kinetics. Samples were sputter coated with a 15 nm thick gold layer and to avoid damage inside them, it was imposed a voltage (EHT) limited to 10-20 kV.

Morphological analysis of porosity was carried out both on microneedles and on microspheres.

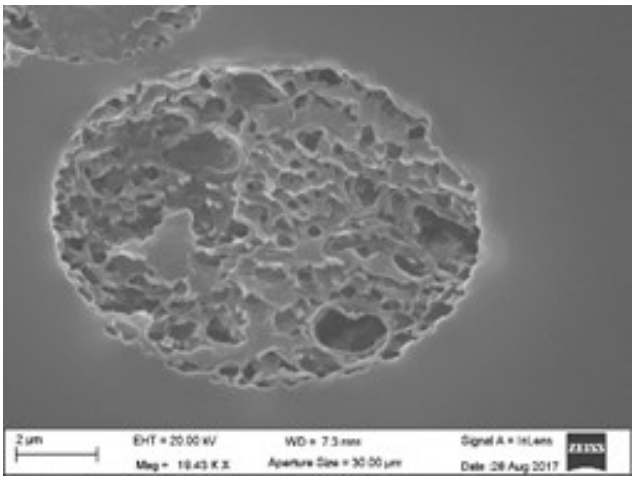


Fig. 3.13. SEM image of microspheres slice.

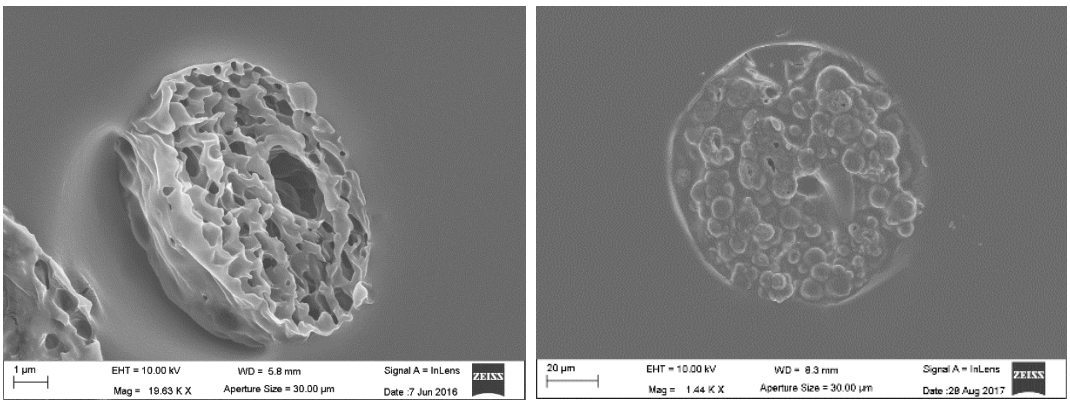


Fig 3.14. Representative SEM images of a) embedded PLGA microparticles and b) slice of microneedles product.

In both slices similar porosity can be observed, demonstrating that the treatment to which the microspheres are subjected for the production of microneedles do not involve any change in the morphological structure of microspheres. This is one of the most important aspects that this work demonstrated because in this way it is possible to exploit and apply the knowledge on the kinetic release of the cargo encapsulated in the PLGA microparticles to this novel device based on microneedles. Additionally, it is possible to combine a fast release from the fast dissolvable polymer matrix.

3.6 Hydrophilic drug loading

Two different hydrophilic chromophores have been used, to conduct the analysis of drug loading. Fitc used in the polymeric matrix, and Sulphorodamine B used in the pores of the microparticles. Confocal analysis was used to characterize both microneedles obtained with the two different procedures. Fig. 3.14 is referred to microneedles obtained with the first procedure. In fig. 3.14a it is depicted a microneedle extracted with no polymer matrix surrounding microparticles and despite the absence of such matrix microparticles are assembled each other and at the same time it is still possible to appreciate their individuality with their spherical

shape. In the fig. 3.14b it is reported a slice of such microneedles where the individuality of the microparticles is also evident inside the microneedle.

In fig. 3.14c it is depicted a microneedle with PVP matrix where it is possible to see the green color around microparticles. This is even more evident from fig 3.14d representing a slice of such microneedle. Green color is homogeneously distributed around the microparticles.

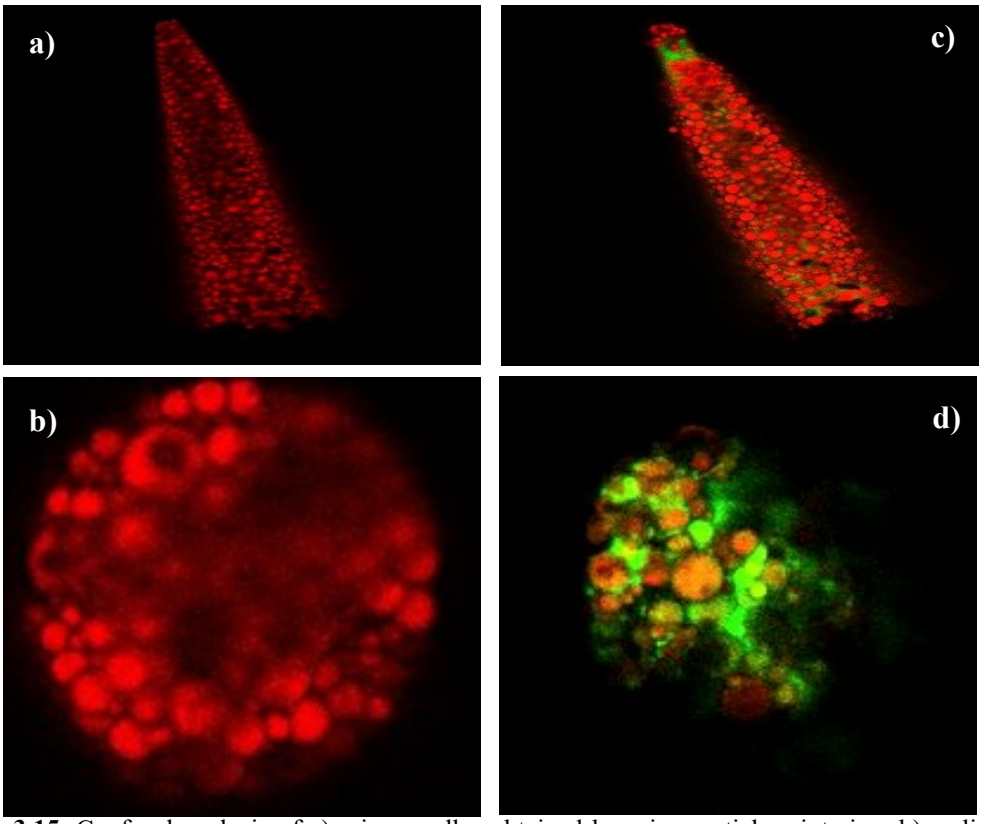


Fig. 3.15. Confocal analysis of a) microneedles obtained by microparticles sintering, b) a slice of microneedle obtained by microparticles sintering, c) microneedle obtained by microparticles sintering and hydrophilic matrix, d) slice of microneedle obtained by microparticles sintering and hydrophilic matrix.

Confocal analysis was also performed on microneedles obtained with the second procedure. In the mean time we optimized a reconstruction procedure that we used for the representation of the confocal results on the microneedles obtained with the second procedure. This is a reconstruction of z slice images which can be performed with LAS X software used for 3D reconstruction imaging. From the reconstructed image (fig. 3.15 and 3.16) it is clear that the fast dissolvable compartment made of PVP loaded with Fitc is located in the tip of the microneedle as expected from the process. Indeed, in this case the solution of PVP is dispensed in the cavities of the stamp before the insertion of the microparticles.

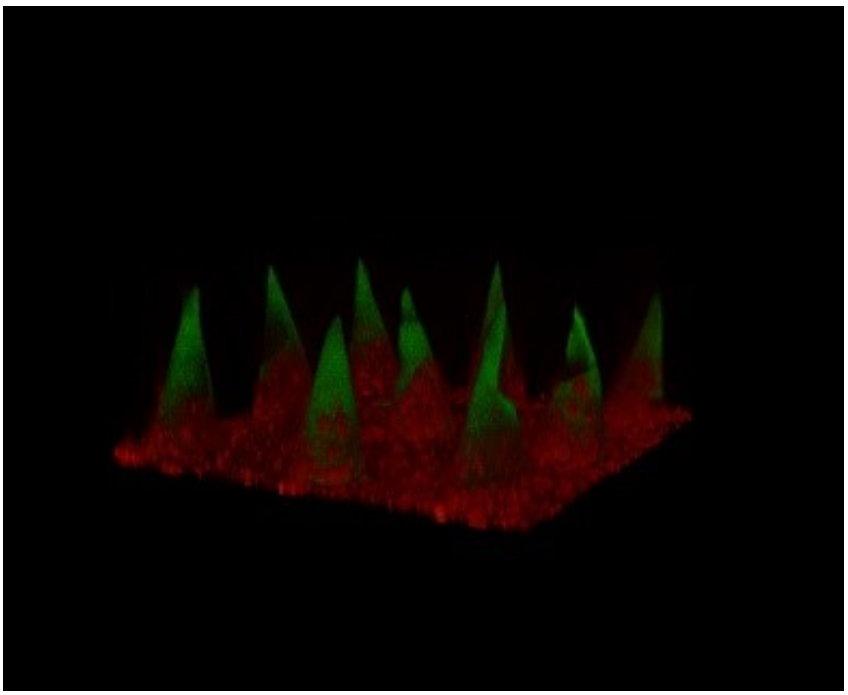


Fig. 3.16. 3D Confocal reconstruction of a microneedles array.

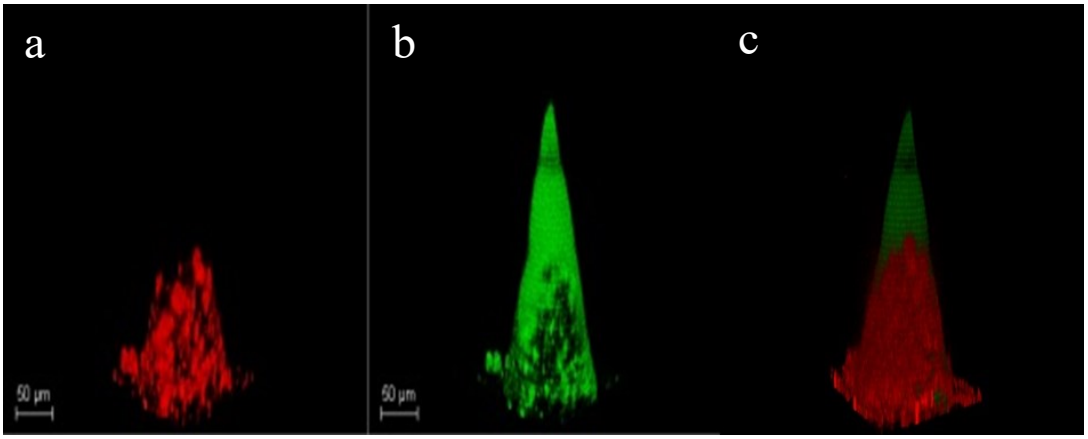


Fig. 3.17. 3D Confocal reconstruction of single compartments forming microneedles (a,b) and their matching (c).

3.7 Indentation tests

The fundamental requirement of the microneedles is that they are able to pierce the stratum corneum of the skin. For this reason, different indentation tests were carried out in this thesis.

3.7.1 Preliminary test

The first indentation test was performed in a photo-curable gelatin which is the simplest model of derma with the aim to check if thanks to the fast dissolvable base of the microneedles it was possible to release microneedles in the skin model. The gelatin used for this test was gelatin B with DS 64%. Briefly, 200 mg of gelatin and 2 ml of solution, in the ratio 10:1 were mixed and consolidated as described in the Materials and Methods section

Then the indentation tests were carried out by pressing the array of microneedles in the gelatin and after 1 h the array was removed. The microneedles stayed inside the gelatin showing the ability of our microneedles array to be released and therefore implanted upon indentation thanks to the fast dissolvable layer at the base of the microneedles themselves.

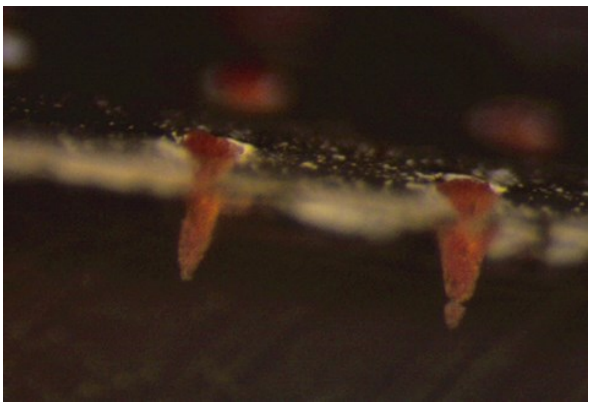


Fig. 3.18. Stereomicroscope image of microneedles in the gelatin.

The second indentation test was performed in a layer of paraffin wax prepared as described in Materials and Methods section. Paraffin is a more appropriate model if the aim is to assess the mechanical resistance of the microneedles and therefore their capability to survive to the applied pressure and promote the indentation. It is a fast model for a fast testing.

The indentation test was carried out by pressing the array of microneedles in the paraffin wax and after 15 min the array was removed.

To monitor the effective indentation of microneedles in the paraffin wax, a replica onto the paraffin wax with PDMS was carried out. The PDMS after 24 h was detached from the paraffin wax and it was observed at the SEM. Fig. 3.19, shows the replica of the indentations carried out with microneedles in the paraffin it is possible to clearly see the perfect replica of the microneedles used and the high resolution of the tip obtained.

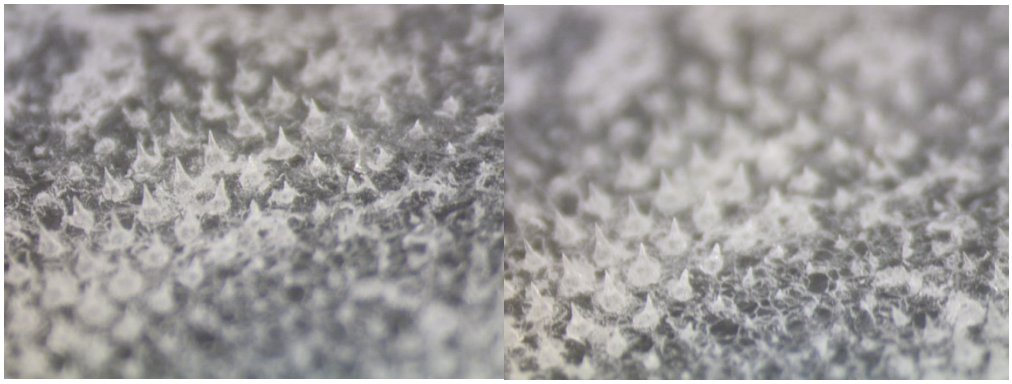


Fig 3.19. Stereomicroscope image of replica whit PDMS of paraffin wax

3.7.2 Skin indentation

Finally, indentation experiments were performed into real skin.

This analysis was carried out using the applicator system showed in Fig. 2.13. This applicator is suggested to perform reproducible indentations.

Pig cadaver skin was kept on an absorbing paper and microneedles were inserted into the skin and removed after 5 min.

The effectiveness of the indentation was confirmed by the cross sectional image of the stained skin at the site of microneedle penetration (fig. 3.20).

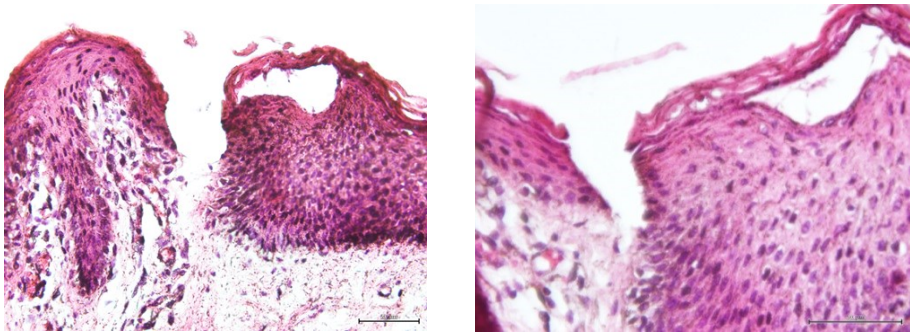


Fig. 3.20. Cross-sectional image of the skin after microneedle removal and skin fixing in formalin at 10% v/v.

The effectiveness of the indentation was confirmed also by the optical image, reported in fig.3.21, showing microneedles staying in the skin after indentation test and removal of the medical tape.

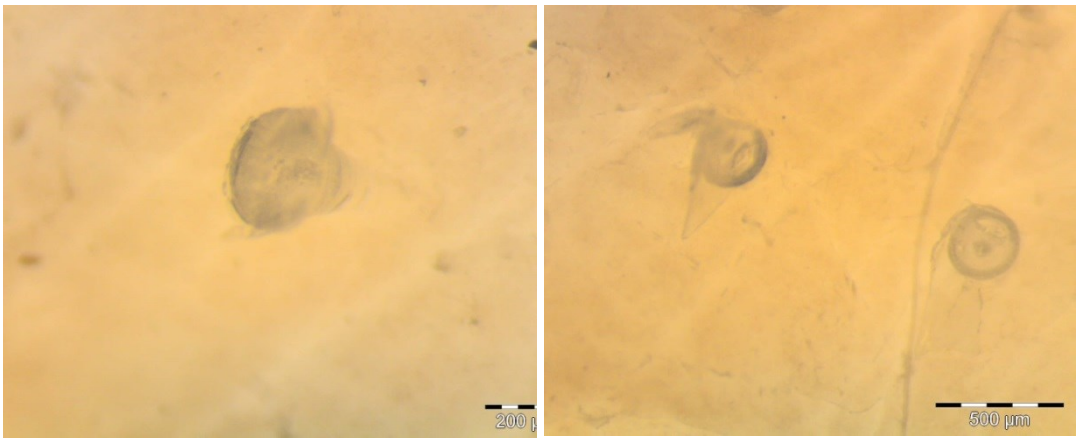


Fig. 3.21 Stereomicroscope image of microneedles into the skin.

The dermis was clearly reached by the microneedles, thus confirming the potential use for drug delivery. In particular, they were able to penetrate all the layers of the epithelium and part of the papillary dermis.

Moreover, the indentation was confirmed by the analysis of the cleared skin using a multiphoton microscope. Confocal images in Fig. 3.22 clearly show the formation of holes in the indented skin, visible thanks to the insertion of Sulforodhamine B into the microneedle tip that is released after skin indentation.

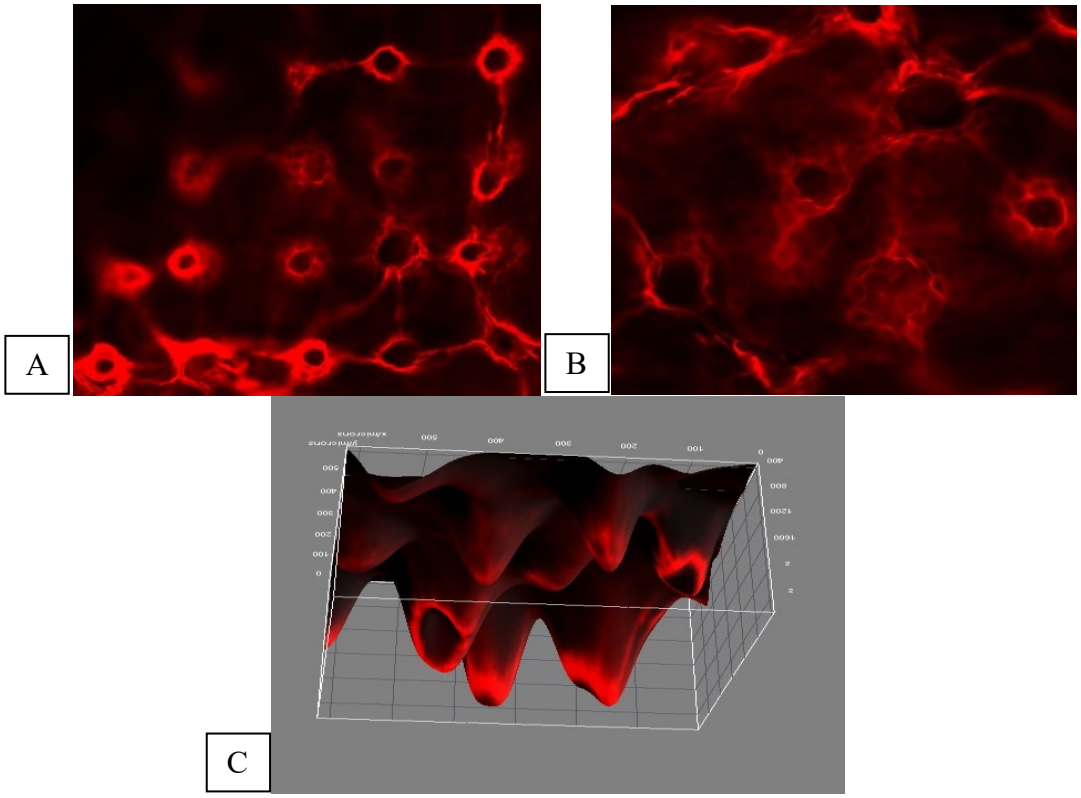


Fig. 3.22. Multiphoton image of indented skin (a and b), (c) 3D reconstruction of the holes.

Overall these results confirmed the effectiveness of the so obtained microneedles. The good indentation properties joined with the technological simplicity of the fabrication process might provide a significant breakthrough in the clinical development of the biodegradable microneedles.

3.8 Conclusions

Microneedles and in particular polymeric microneedles in the last years have been described as a great alternative to the traditional drug delivery systems. They have been mainly tested with different drugs and vaccines, and the main advantage is the possibility to encapsulate and deliver through the skin high molecular weight molecules being the tool able to indent and cross the stratum corneum. Main limitation is on the fabrication procedures which are typically time consuming and

therefore expensive. Additionally, there is still much room to improve the product by itself by improving the procedure and therefore the final performances of the product in terms of fine control of the release of both hydrophilic and lipophilic molecules.

Therefore, in this thesis, main focus was on the development of a novel procedures for the fabrication of microneedles remaining in the field of stamp based techniques. Additionally, the design of the procedure was such that we could also provide high performing microneedles and in this respect we produced biodegradable and multi-compartment microneedles with two different degradation times so to combine fast and prolonged release. This work started with the optimization of the processes to produce masters which are needed for the realization of stamps by replica. We developed two procedures which are both fast and carried out at room temperature to preserve thermolabile drugs, such as proteins. Depending on the procedure it is possible to change the distribution of the two compartments. For instance, in the second case the fast dissolvable compartment is localized in the tip of the microneedles while in the first procedure is homogeneously distributed all over the volume of the microneedle. In this way it is possible to confirm that the drug is encapsulated in the microneedles, so as to reduce the loss of medication.

Being stamp based procedures it is possible to obtain different shapes of microneedles which may be studied in future in terms of final performances.

The microneedles realized were separated each other which allow integration on flexible patches to improve the conformal contact with the skin. The multi-compartment feature allows a temporal and spatial control of the molecule to be released.

3.9 References

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CHAPTER IV

Microneedles for cosmeceutical applications

4.1 Introduction

Cosmeceuticals represent the future generation of skin care. They are based on the advances made within the world of dermatological products and the new backbone in skincare. Cosmeceuticals are typically cosmetic-pharmaceutical hybrids intended to enhance the health and beauty of skin. Some cosmeceuticals are naturally-derived while others are synthetic, but all contain functional ingredients with either therapeutic, disease-fighting or healing properties.

These are skin-care products that go beyond coloring and adorning the skin. In the last years, skin-lightening agents added to product formulations have become increasingly popular and such products are in demand.

4.1.1 Skin whitening

Skin whitening is the practice of using substances, mixtures, or physical treatments to lighten skin color. Skin whitening treatments work by reducing the content of melanin of the skin. Many agents have been shown to be effective in skin whitening; some have beneficial side effects (e.g.: are antioxidants,[1] nutrients, or decrease the risk of some types of cancer); some are of significant risk to health (for example, those containing mercury). Specific zones of abnormally high pigmentation such as lentigo spots, moles and birthmarks may be depigmented to match to the surrounding skin.

Cosmetics preparations for skin whitening are available in cream, soap, lotion, nasal spray and injectable form. When applied on the skin in the form of lotion the

product is not efficiently absorbed by the skin. When taken orally, it is hydrolyzed by enzymes in the gastrointestinal tract resulting in reduced bioavailability. On the contrary, intravenous administration delivers very high doses directly into the systemic circulation and is the preferred route. However, this method of administrating the antioxidant might flood the cells that may cause reductive stresses.

Both ablative and non-ablative lasers can have a profound effect on melasma. However, the results are not always consistent, and problems have been reported (such as hypo- or hyperpigmentation). Laser treatments of this kind are more likely to result in problems for those with darker skin tones.[2]

Another alternative to laser treatment is cryosurgery using liquid nitrogen. Controlled destruction of skin cells causes the skin to naturally regenerate itself. Excess melanin comes to the surface and peels off in a few days. This is particularly useful in sensitive areas like the genitals where laser treatment could leave a scar. Efficacy of the treatment depends on the depth of the pigment.

More recently, however, the use of MN in the cosmeceutical industry has been explored.

Two commercially available MN devices, namely Dermaroller® and Dermapen®, were developed based on these principles and Fernandes' percutaneous collagen induction (PCI) innovation.

PCI is achieved *via* multiple needle application.[3, 4] Roller devices with projecting needles have been designed to achieve this aim. The Food and Drug Administration (FDA)-approved Dermaroller® is described as a hand-held device equipped with medical grade solid steel MNs, projecting from a cylindrical roller fig. 4.1.a.[5] The rolling mechanism is applied directly over the skin, vertically, horizontally and diagonally. There are 24 circular arrays of eight needles each located on the roller (total 192 needles).[4] Fig. 4.1a, and the heights of the needles are specific to the nature of the treatment being employed.[6] Home Dermarollers® consist of the Home Roller C 8 (a single needle which is depicted in

Fig. 3b) and the Beauty Mouse®. The cosmetic C 8 is the name of model has needles ranging between 130 and 200 μm in height (Fig. 4.1b), with a penetration diameter of 70 μm. Another device intended for home use is the Beauty Mouse®, which incorporates 3 Dermarollers® with a total of 480 needles and has been developed to ensure coverage of larger skin surface areas, such as the arms, legs and buttocks for the treatment of stomach or thigh stretch marks and cellulite.[7]

A number of other companies, for example, Hansderma and White Lotus, have marketed products with similar functions.[8, 9] Despite the fact that MN devices are often categorized into home and medical use in product literature, they are available for purchase by any individual online from a multitude of commercial websites. With no restriction on purchasing of these products, it is clearly evident that there is potential for abuse and misuse of these devices.

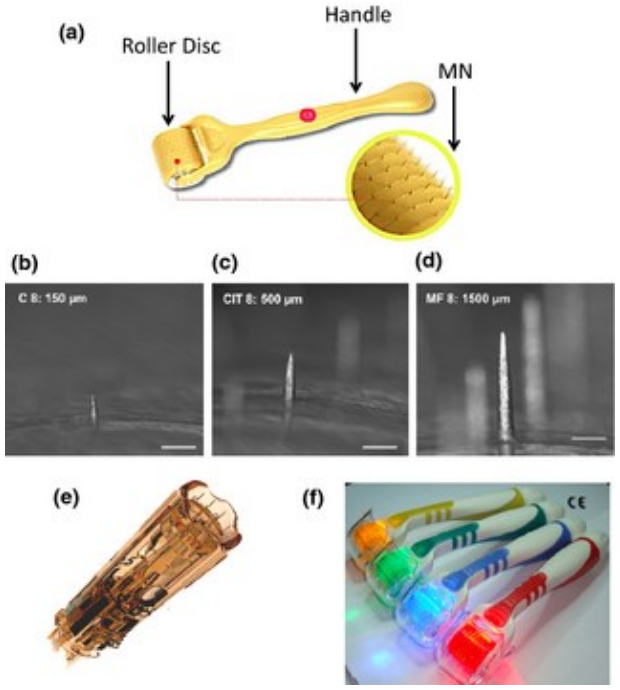

Fig. 4.1. FDA-approved MN devices. a) Image of a Dermaroller® MF 8 (35; (b, c and d) stereo microscopic images (Leica MZ 8, Switzerland) of the needles from different Dermaroller® models, namely b) cosmetic Dermaroller® C 8 model, medical models, c) Dermaroller®CIT 8 and d) Dermaroller®MF 8, scale = 500 μm.[4] e) Images of the MDerma FDS which will be ready for distribution in early 2015 (Images taken from a marketing PDF received from Dermapen®). f) An image of LED MicroNeedling Rollers.

PCI technology has subsequently evolved with the emergence of miniature versions of the Dermaroller®, termed Dermastamps™ onto the UK market.[10] These are sterile medical devices with needle heights of 200 μm, situated in a 5 mm diameter circular arrangement.[10] They are specifically designed to accommodate small confined areas where it is difficult for the Dermaroller® to achieve optimal stimulation, for example the upper lip. The device uses 'vertical penetration to create infusion channels' in the skin and is considered ideal for use on isolated scars and wrinkles.[10]

Designed to overcome the issues of varying pressure application by physicians/users and the subsequent MN depth penetration achieved, an advanced microneedling device has been launched, termed the Dermapen® (Fig. 4.1 e). It is described by manufacturers as a spring-loaded, fractional MN device, with an adjustment ring allowing for alteration of the heights of the MN, which carries out the function of 'fractional mechanical resurfacing'. It utilizes an electrically powered pen to deliver a vibrating stamp-like motion to the skin, creating a series of micro-channels in it. Treatments in acne scarring and photoageing are being investigated by the manufacturers, although no research studies focusing on this MN device have yet been published. Due to the substantial interest in these MN devices, more commercial products based on the same principles are currently under development.[6, 7, 11]

One of the most important advantages of these MN devices is in overcoming the use of traditional ablative methodologies. Ablative methods including dermabrasion, chemical peels, collagen injections, cortisone-like injections, cryosurgery and laser resurfacing are, by their nature, extremely physically disruptive to the targeted epidermis and superficial dermis.[12] In ablative methodologies such as these, undesired post-operative changes in the skin can result in significant healing times. As destruction, rather than disruption, of the epidermis initiates an inflammatory response that stimulates fibroblasts to produce thick branches of scar collagen,[13] skin has been shown to become more sensitive

to photodamage and one study has stated that a side effect of such treatment may be the development of dyschromias, a common skin disorder whereby there is a marked alteration in normal skin pigmentation, resulting in discolouration of the skin, hair and nails.[14] In contrast, microneedling, if limited to breacking the *SC* with needles of appropriate heights, would avoid the risks and negative side effects often observed with invasive ablative approaches. The main limit of this technique is related to the fast closure of the skin holes that prevents an effective injection of functional high molecular weight compounds.
In this scenario is set the present work aimed at the realization of biodegradable multi-compartment polymeric microneedles for cosmetic applications in particular for skin lightening application.

4.1.2 Laccase

Laccase is one of the few enzymes that have been the subject of study. In 1883, laccase was first described by Yoshida when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera.*[15] Their characteristic as a metal containing oxidase was discovered.[16] In 1896, both Bertrand and Laborde observed the presence of laccase in fungi for the first time.[17] Laccase has received lot of attention from researchers due to its ability to degrade a variety of recalcitrant pollutants. Compounds which are structurally similar to lignin can be oxidized [18] by fungal laccase (benzene diol: oxygen oxidoreductase, EC 1.10.3.2) along with ferroxidases (EC 1.16.3.1). Multicopper oxidases (MCO) typically contain two or four copper atoms per protein molecule and they catalyze oxidation reactions. In these reactions, electrons are removed from the reducing substrate molecules and transferred to oxygen in order to form water without the step of hydrogen peroxide formation.[19] Laccases have a wide substrate range, which can serve industrial purposes. The simple requirements of laccase catalysis (presence of substrate and O_2), as well as its apparent stability and lack of

inhibition (as has been observed with H_2O_2 for peroxidase), make this enzyme both suitable and attractive for industrial applications. In addition, laccase can oxidize a wide range of organic and inorganic substrates, including mono, di, polyphenols, aminophenols, methoxyphenols as well as metal complexes which are the major reason for their attractiveness for dozens of biotechnological applications.

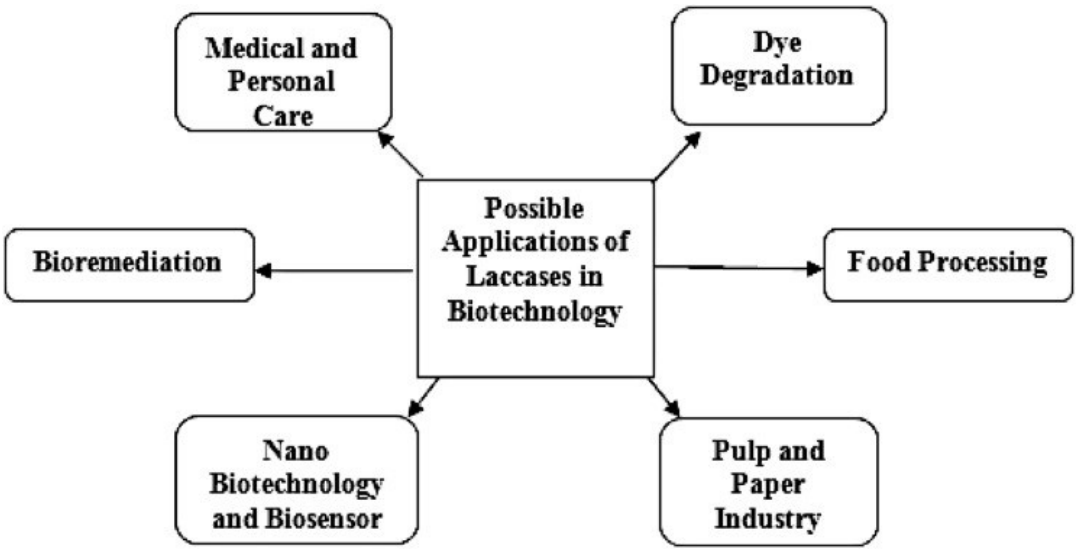

Fig.4.2. Application of laccase in biotechnology

4.1.3 Medical and personal care application

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are attracting active research efforts. Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc., including triazolo (benzo) cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline.

Poison ivy dermatitis is caused mainly by urushiol, which is a catechol derivative toxin. Laccase oxidizes, detoxifies and polymerizes urushiol, [20] which reduces the effect of poison ivy dermatitis (oxidized urushiol is non-toxic). Laccase can

oxidize iodide to produce iodine which is widely used as a disinfectant. It has several advantages over direct iodine application. In terms of handling, storage and transport, iodide salt is more stable and much safer as compared to iodine. Release of iodine from a laccase iodide system could be easily controlled. The system may be used in several medical, industrial, domestic, and other personal care applications. A novel application field for laccases is in cosmetics. For example, laccase-based hair dyes could be less irritant and easier to handle than current hair dyes. Laccase based systems may overcome drawbacks of chemical dyes by replacing hydrogen peroxide as the oxidizing agent in the dyeing formula.[21] More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed. Laccases may find use as deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers. Protein engineered laccase may also be used to reduce allergenicity.

4.2 Materials and methods

Poly (lactic-co-glycolic acid) 50:50 (PLGA RESOMER® RG 504H), 38000 - 54000 Dalton, was obtained by Boeringer Ingelheim and used as received. Polyvinylpyrrolidone (PVP 856568 Mw 55 KDa) Dimethyl Carbonate (DMC, D152927), as solvent of the PLGA, Poly(dimethyl-siloxane) (PDMS), used as flexible support, was provided by Sylgard® (184 Silicone Elastomer Kit, Dow Corning). Recombinant POXA1b laccase from *P. ostreatus* expressed in the eukaryotic host Pichia pastoris was kindly provided by Biopox srl (Italy). Pig cadaver skin was kindly provided by dott.ssa Antonelli Carmela of ASL Napoli 2 Nord, taken from the butchery implant ICS (Industria Carni Sud) of Caivano, Naples (IT). Pig cadaver skin was stained by synthetic blue pigment was kindly provided Biopox srl (Italy).

4.2.1 Assay of enzymatic activity with ABTS

Laccase activity was assayed at 25 °C by monitoring the oxidation of ABTS at 420 nm ($\varepsilon_{420} = 36 \times 10^3\ M^{-1}\ cm^{-1}$). The assay mixture contained $2 * 10^{-3}$ M ABTS in $1 * 10^{-1}$ M sodium citrate buffer, pH 3.0.

Enzymatic residual activity of enzyme-charged microneedles was assessed by dissolving 10 microneedles in 0.1 ml of DMC and, after a complete dissolution, the related solution was rapidly buffered in 50 µl of PBS buffer. Enzymatic activity of enzyme-charged microparticles was assessed by dissolving 5 mg of microparticles in 0.2 ml of DCM, and after a complete dissolution, the related solution was rapidly buffered in 895 µl of PBS buffer. The related enzymatic activity was compared with the one detected into the formulation solution by carrying out the same dilutions. Enzymatic activity of laccase was determined using ABTS as a substrate [22] by measuring UV absorbance at 420 nm. Maximum linear rate ΔA_{405nm}/minute was used to calculate the activity (Enzymatic assay of peroxidase from horseradish, EC 1.11.1.7, Sigma Prod. No. P-6782).

4.2.2 Microspheres production

For the preparation of the solution consisting of the discontinuous phase, 100 mg of polymer (83 mg of PLGA and 17 mg of Pluronic F68) were dissolved in 1 ml of solvent dichloromethane (DCM), then 70 U of laccase were added to the solution. The emulsification process was performed with the Ultra-turrax that is a homogenizer. The first emulsion has been prepared for 30 s at 15000 rpm and added to the continuous aqueous phase based on 10 ml of PVA solution (at 2% w/v). Second emulsion has been carried out always with the same instrument for 1 min at 20000 rpm. After that, the solution is kept under mechanical stirring for about 2 h and 30 min, at a speed of 450 rpm, to remove completely the polymeric solvent trough evaporation method. The whole process was carried out by refrigerating in ice, in order to avoid the enzyme denaturation. The microparticles

are rinsed with deionized water to remove the PVA from the solution with 3 centrifuges of 10 min at 15000 rpm. For their conservation, they are filtered from the water, frozen at – 80°C for 15 min and then subjected to freeze-drying over-night. Finally, the as produced microparticles were stocked at 4°C.

4.2.3 Polymeric microneedles

New method for to produce biocompatible multi-compartment microneedles for cosmeceutical application was discussed, it is possible divided the process in four steps:

1. production of microneedles tips with hydrophilic polymer through degassing and spinning;
2. microparticles inserted in the stamp;
3. PLGA microparticle sintered with a room temperature process, with a mixture of solvent not solvent;
4. deposition of hydrophilic polymer on the medical tape.

4.2.4 Production process

The solvent mixture was composed by 4 ml of ethanol and 0.5 ml of DMC. The temperature and the pressure were set respectively at 25.3°C and 0.15 bar. The tip of microneedles is produced with a hydrophilic polymer inserted in the cavities of the mold through degasing and then spinning. In the second step microparticles were inserted in the cavities of the mold in order to produce the base of microneedles, and then the mold was placed under the tube in which the plasticizing vapor flows. After 2 min it can be observed the plasticization of the microspheres that assume the shape of microneedles. Finally, in order to extract the microneedles from the stamp a 3M medical tape with a hydrophobic layer was used.

4.2.5 Characterization of microneedles

The microneedles were characterized with several analyses to study morphological aspect, distribution of drug and mechanical resistance.

4.2.5.1 Porosity

In order to check the final microstructures of the microneedles, they were sectioned as described above at a thickness of 5 μm. A morphological analysis was performed using scanning electron microscope (SEM) (field emission Ultra plus Zeiss). Microneedles microstructure was compared to the one of the basic microparticles to assess no significant variations consequent to the plasticizing process. Samples were sputter coated with a 15 nm thick gold layer and to avoid damage inside them, it was imposed a voltage (EHT) of maximum 10 kV.

4.3 Results and Discussion

The encapsulation of catalyzers in biodegradable polymer microneedles may represent a simple method to deliver enzymes in human skin for dermatology and cosmetics treatments.[23, 24] The cosmetics sector is interested in enzymes that enhance the beauty of the skin or defend itself against the ravages of the environment. One of the most interesting application of enzymes in this field is based on free radical scavengers that help to protect the skin by neutralizing dangerous substances generated by sun exposure and pollution.[25] Superoxide dismutase and peroxidase have been frequently used for their ability to reduce UV-induced erythema by working synergistically and they may help to slow the visible signs of aging and the damaging effects of the environment on the epidermis. The action of endogenous enzymatic system of human cells neutralizes the superoxide anions and can produce hydrogen peroxide having a detrimental effect on the lipid barriers of the skin. Peroxidase can work to neutralize hydrogen peroxide thus giving a full spectrum of free radical protection. However, since creams are not

effective in the delivery of high molecular weight molecules such as enzymes, invasive injection systems are typically used. In this scenario, painless biodegradable polymer microneedles represent a promising tool. In the present work we loaded polymeric and multi-compartmental microneedles with laccase as model enzyme.

The formation of a solid-state formulation in a microfabrication process involves recurring dipping-drying cycles, which can be potentially detrimental to sensitive biologically active agents.

The method used in this work to produce polymeric microneedles overcomes this limit. A new stamp based method has been used for the production of microneedles. The multi-compartmental feature of the proposed microneedles is obtained with a tip of hydrophilic matrix embedding enzyme and porous microspheres embedding enzyme everything processed at room temperature to preserve the enzyme activity. In particular, microparticles have been placed inside the cavities of the mold and then plasticized at room temperature by means of a suitable mixture of a solvent, DMC and a non-solvent, EtOH, with respect to PLGA to assemble microparticles in the shape of microneedles from one side and to keep their microstructures from the other side according to the patented procedure.[26] Such microneedles have then been harvested from the mold by means of a water-soluble polymeric layer, and indentation test *in vitro* have been carried out to demonstrate the mechanical properties of microneedles and the conservation of enzyme activity after the process of microneedles production.

4.3.1 Microspheres production

Polymeric microspheres have been employed as starting material for the production of biodegradable polymeric microneedles. Such microspheres have been produced by means of a double emulsion process to obtain micropores for the encapsulation of the enzyme. The double emulsion process allows to encapsulate hydrophilic and

hydrophobic molecular. The microspheres were made of PLGA and enzyme laccase. PLGA was selected since it is a very well- known biodegradable material, which is FDA approved, and has suitable mechanical properties for the production of microneedles for drug delivery,[27] In particular, a T25 digital ULTRA-TURRAX was used for the double emulsion which allowed to produce porous microspheres in the range of 1-15 μm.

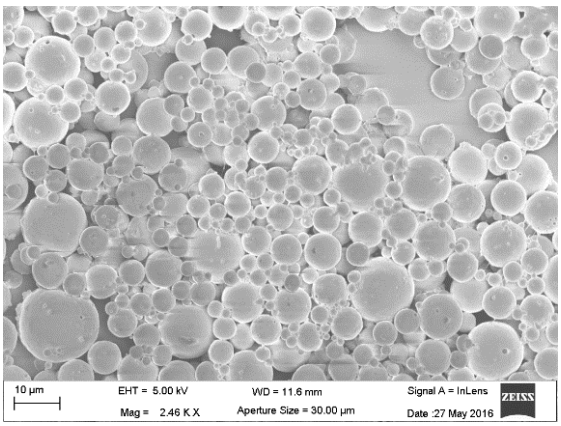

Fig. 4.3. SEM image of Porous Microspheres

4.3.2 Mold production

The techniques used for the production of microneedles is a stamp based method where the stamp is made of PDMS cavities of conical shape 300 μm of height and 150 μm of base diameter fig 4.4.

a)

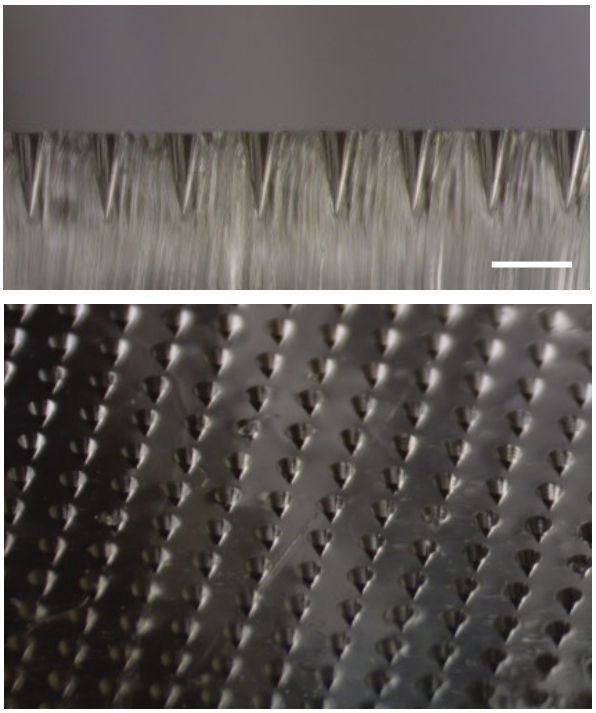

Fig. 4.4. Optical microscopy images of: a) section of the PDMS mold (scale bar 1.2 mm), b) view from the top of the PDMS mold showing conical shape (scale bar 900 μm).

4.3.3 Production of microneedles

As concern the production of the tip of microneedles from a PVP solution at 25% containing 70 U of laccase, all the steps of insertion in the stamp, degassing and spin coating are schematically reported in the fig. 4.5.

b)

Fig. 4.5 Production process of microneedles tip; a) deposition of PVP with Gilson on the PDMS mold; b) degassing process of solution with PUMP; c) spin coating technique to produce only PVP microneedles.

Then the PLGA microspheres containing the enzyme with a diameter in the range of 1-15 μm and previously described were placed inside the cavities of the mold under a stereomicroscope as schematically depicted in the fig. 4.6.

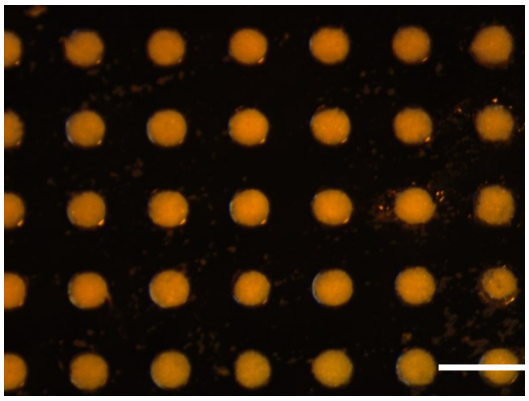

Fig. 4.6. Microspheres in the cavities of the mold (scale bar 300 μm).

After filling up the mold with the microspheres, it was placed under the tube in which the plasticizing vapour flows. This tube was connected to a jacketed

Dreschel Bottle, in which there is a mixture of solvent-non solvent. This set up has been developed ad hoc for this process and is reported in fig.2.7.

Fig. 4.7. System used for the plasticization of the microspheres.

The solvent mixture started to evaporate at room temperature 25.3°C, with a nitrogen pressure of 0.1 bar, and the plasticization of the microsphere started. After a time ranging from 2 min of this procedure the microspheres assembled assuming the shape of the mold cavities. In this process, to the extract the microneedles by the stamp a 3M Medical Tape was used after a deposition of a droplet of hydrophilic matrix.

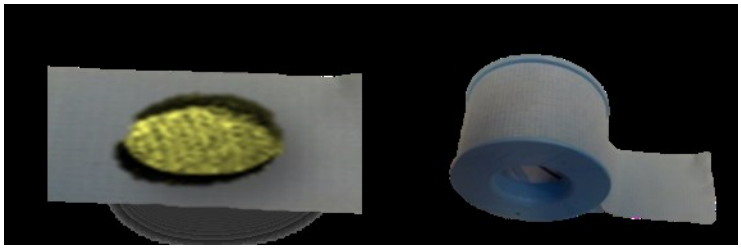

Fig. 4.8. a) deposition of hydrophilic polymeric matrix on medical tape; b) 3M medical tape.

The process here described is used to obtain multi-compartmental microneedles, as it is possible to observe from the optical image reported in the fig.4.8. Indeed, it is possible to observe the tip of microneedles obtained with only hydrophilic matrix, and the truncated cone in the base obtained with soft sintering of PLGA microparticles.

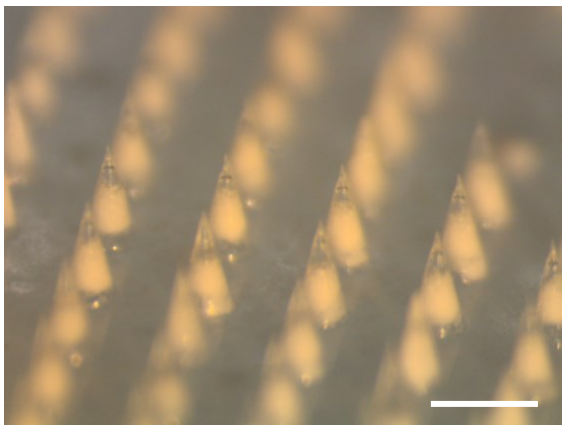

Fig. 4.8. Stereomicroscope image of microneedles array (scale bar 600 μm)

4.3.4 Porosity analysis

The morphological analysis of microneedles was performed with a Scanning Electron Microscope (SEM), to understand if the process used for the production of microneedles could alter the morphology of the microspheres. For this reason, microneedles and microspheres were incorporated in PDMS, cured for 48 h at room temperature, and then frozen at -130 °C in Leica Cryo Ultra Microtome EM-FC7-UC7. Samples were sectioned at a thickness of 5 μm for analysis in axial direction.

Microneedles porosity is of fundamental importance since it determines the drug release kinetics. Additionally, a possible loss of homogeneous distribution of the porosity may also affect mechanical properties of the final microneedles, compromising its indentation ability. Samples were sputter coated with a 15 nm

thick gold layer and to avoid damage inside them, it was imposed a voltage (EHT) 10-20 kV.

Morphological analysis of porosity was carried out both on microneedles and on microspheres sections fig. 4.9. The figure show that the microstructure is very similar. Our method allows to preserve both the microstructure and molecule distribution of the starting microspheres, providing shaped microparticles that can release enzyme over time. In particular, we demonstrate that the shaped microparticles keep a porous microstructure and enzyme distribution similar to that of the starting microspheres, whereby the release of enzyme embedded in the shaped microparticles and starting microspheres is equivalent.

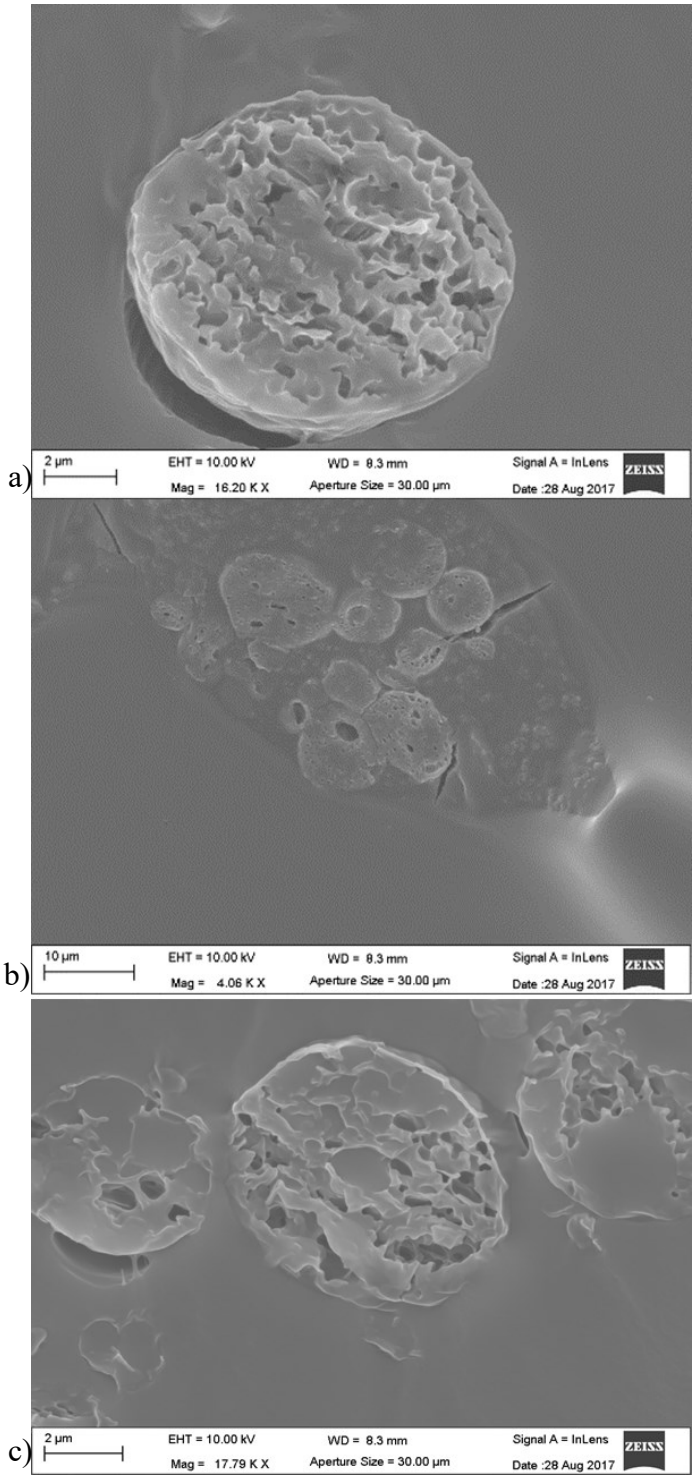

Fig. 4.9. a) SEM images of microparticles slice b) SEM image of micronedlees slice c) SEM image of microparticles performing microneedles

4.3.5 Efficiency Analysis

Microparticels were sectioned in slices to monitor the presence an the preservation of laccase encapsulated in their porous. In more detailes, a solution of ABTS and buffer was add on the slices in order to control the enzyme activites. In case of active enzymes, this contact produces an oxidation of the enzyme encapsulated in the porous of microparticles. In fact, from the fig. 4.10, the differnce between the slice without solution of ABTS in Buffer in the left an with solution in right. The presence and the activity of enzyme was confirmed to the green color in the pores of microparticles.

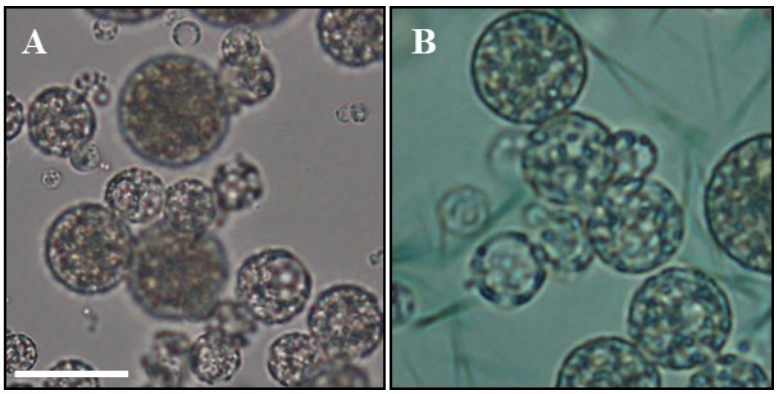

Fig. 4.10. a) microscope image of micoparticles slice; b) microscope image of microparticles slice with solution of ABTS and buffer. (Scale bar 10 μm)

By measuring the quantity of laccase encapsulated after the microparticles preparation and by comparing with the quantity of this enzyme used during the microparticles production, we obtained that the percentage ratio of encapsulated laccase, namely efficiency, was about 80 %. The amount of encapsulated laccase did not change in the day subsequent to the microparticle production as reported in fig. 4.11.

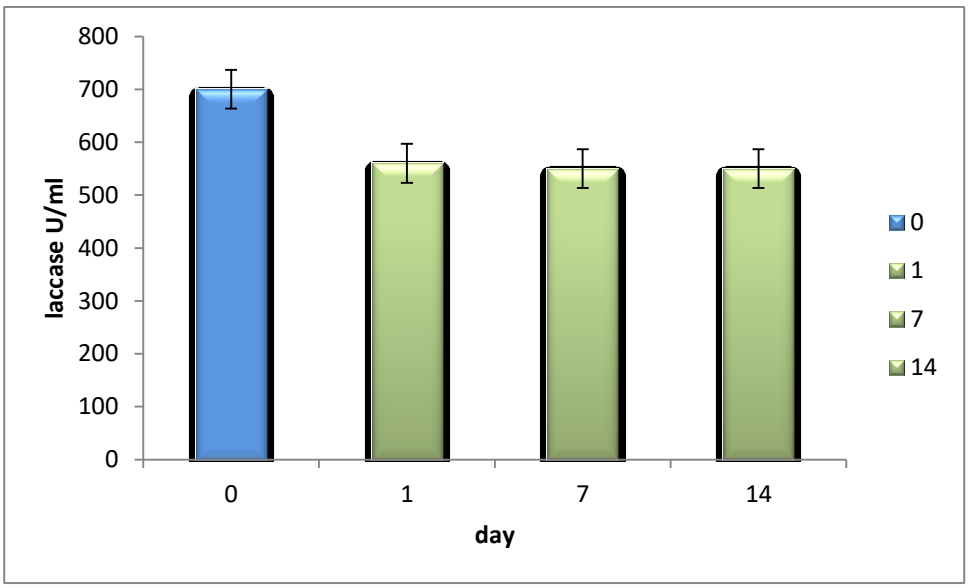

Fig 4.11 Amount of laccase encapsulation of microparticles after different days since the production. Blue column represents the initial enzyme used for the preparation of microparticles.

4.3.6 Skin decolorization

Indentation tests have been conducted to understand the mechanical properties of microneedles product with this now stamp based method, but at same time it is used for to understand if the encapsulated enzyme was still active after microneedles fabrication.

For this experiment the pig cadaver skin was stained by synthetic blue pigment kindly provided Biopox srl (Italy) and by using the applicator system of SMICNA for the application of the microneedles.

Two experiments were carried out in parallel, in fact two pieces of skin were stained with same pigment, and for the indentation two different arrays of microneedles were test fig. 4.12. The microneedles were prepared with same process, but one was loaded with the enzyme while the other one was without enzyme.

In parallel, control samples were maintained with heat inactivated partially purified laccase. After one day it was possible to see by the figure that there is a decoloration of the skin where was used the microneedles loaded with enzyme.

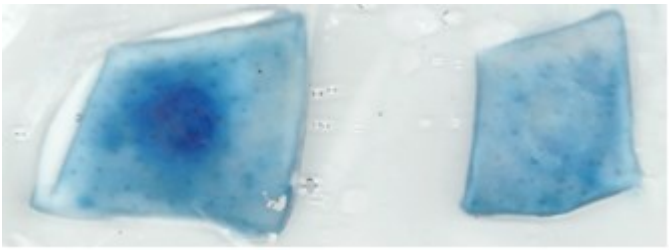

Fig. 4.12 Left) skin tested with microneedles without enzyme, and right) skin tested with microneedles embedding enzyme.

These preliminary results indicate that the conditions of the microneedles fabrication are mild enough to preserve laccase enzyme activity and that released enzyme from microneedles dissolution is functionally active.

4.4 Conclusions

Microneedles and in particular polymeric microneedles in the last years are considered as a great alternative to the traditional system for drug delivery.

They permit the encapsulation of high molecular weight, hydrophilic and hydrophobic molecules, since the microneedles pass through stratum corneum. In particular, this work was focused on the study of a new fabrication stamp based method of an array of microneedles for the encapsulation of an enzyme for skin lightening in the field of cosmeceutical applications. The results confirmed that the process do not produce a denaturation of the encapsulated enzyme.

Then, thanks to the multi-compartment approach for the realization of microneedles it is possible to promote a prolonged release combined to a fast release.

4.5 References

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