DELIVERY OF PROTEIN OR PEPTIDE INTO THE SKIN USING CUBOSOMES AND MICRONEEDLES

Diploma thesis

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Declaration

“I declare that the enclosed diploma thesis represents my own research work. I have clearly documented all of literal sources and material used. The diploma thesis was not used in the same or in a similar version to achieve an academic grading or is being published elsewhere.”

Hradec Králové, 25. 8. 2016                                      Simona Pekáčová
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Abstracts

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Transdermal transport of high-molecular-weight and hydrophilic substances is complicated by the protective skin layer, the stratum corneum. Physical breaching of this layer using microneedles combined with penetration-enhancing properties of cubosomes was previously reported as an effective approach to transdermal drug delivery and therefore employed in this project. (Rattanapak et al. 2012) Delivering abilities of solid (600 µm), coated (500, 600, 750 µm) and hollow (450, 600 µm) MNs were compared with intradermal injection by hypodermic needle. Cubosomes were prepared from phytantriol, poloxamer 407 and propylene glycol using liquid precursor method and loaded with fluorescent ovalbumin (FL-OVA) or SIINFEKL-TAMRA (ST) peptide. Several formulations for coating of solid microneedles were prepared using various solvents and excipients to investigate quality of coatings and their ability to deliver the drug into skin. Polyvinyl alcohol (PVA) turned out to be the most efficient coating excipient providing equally spread coatings that could be delivered into skin, allowing the drug to permeate to the deeper layers of dermis. As only limited amount of drug can be coated onto the surface of MNs tips, application using NanoPass MicronJet hollow MNs was investigated and resulted in massive fluorescence proving successful delivery by 450 µm long MNs. Solid MNs used to poke through the solution previously poured onto intact skin surface also showed successful delivery. Combination of cubosomes and microneedles is a promising approach to transdermal drug delivery and transcutaneous immunization, however, there is still a need to tackle several issues e.g. irritability of materials used for preparation of formulations, stability
of cubic phase, reproducible way of MNs application, preservation of protein or peptide structure during manufacture, storage and use or possible unwanted immunological effects.

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Transdermální přenos hydrofilních látek s vysokou molekulární hmotností je komplikován svrchní ochrannou vrstvou kůže, nazývanou stratum corneum. Fyzické rozrušení této vrstvy pomocí mikrojehel kombinované s penetrací usnadňujícími vlastnostmi kubozómů se již dříve ukázalo jako efektivní přístup k transportu léčiv přes kůži a bylo využito i v tomto projektu. (Rattanapak et al. 2012) Aplikační schopnosti pevných (600 µm), potahovaných (500, 600, 750 µm) a dutých mikrojehel byly srovnány s aplikací pomocí intradermální injekce. Kubozómy nesoucí fluorescenční ovalbumin (FL-OVA) či peptid SIINFEKL-TAMRA (ST) byly připraveny z phytantriolu, poloxameru 407 a propylenglykolu metodou tekutých prekurzorů. Za použití různých rozpouštědel bylo připraveno několik kombinací látek k potahování pevných mikrojehel a posouzena jejich kvalita a schopnost doručit látku do kůže. Polyvinyl alkohol (PVA) se projevil jako nejefektivnější z nich poskytující rovnoměrný potah uvolňující se do kůže a usnadňující permeaci látky do hlubších vrstev dermis. Jelikož pouze omezené množství léčiva je možné nanést na povrch pevných mikrojehel, aplikace pomocí NanoPass MicronJet dutých mikrojehel umožňujících transport větších objemů byla testována a ukázala masivní fluorescenci prokazující úspěšný transport látky pomocí mikrojehel dlouhých 450 µm. Metoda použití pevných mikrojehel k vytvoření mikrokanálů ve stratum corneum po nanesení disperze látky na intaktní povrch kůže se také ukázala jako úspěšná. Kombinace kubozómů a mikrojehel je slibným přístupem k transportu léčiv přes kůži a transkutální imunizaci, avšak, stále je potřeba vyřešit několik problémů,
týkajících se např. iritability použitých materiálů, stability kubické fáze, reprodukovatelnosti aplikace mikrojehel, zachování struktury peptidu či proteinu v průběhu výroby, skladování a používání nebo případných nechtěných efektů na imunitní systém.
# Table of Contents

1. List of abbreviations .................................................................................................................. 1

2. Introduction ............................................................................................................................... 2
   2.1. The human skin ...................................................................................................................... 2
       2.1.1. Stratum corneum .......................................................................................................... 3
       2.1.2. Viable epidermis .......................................................................................................... 4
       2.1.3. Dermis ......................................................................................................................... 4
   2.2. Dermal and transdermal drug delivery ................................................................................. 4
   2.3. Drug delivery using mechanical disruption of stratum corneum ......................................... 6
       2.3.1. Microneedles .............................................................................................................. 7
   2.4. Nanoparticles for transdermal drug delivery ....................................................................... 9
       2.4.1. Lipidic vesicles ........................................................................................................... 10
       2.4.2. Cubosomes ................................................................................................................ 10
   2.5. Combination of microneedles and cubosomes .................................................................... 12

3. Aims ........................................................................................................................................... 14

4. Materials and methods .............................................................................................................. 15
   4.1. Materials ............................................................................................................................ 15
   4.2. Preparation of cubosomes .................................................................................................. 15
   4.3. Cubosomes characterization .............................................................................................. 15
       4.3.1. Transmission Electron Microscopy (TEM) ................................................................ 16
       4.3.2. Particle size, polydispersity index and zeta potentials ............................................. 16
   4.4. Cubosomes stability testing ............................................................................................... 17
   4.5. Protein entrapment measuring ........................................................................................... 17
   4.6. Microneedles coating formulations .................................................................................... 17
   4.7. Coating technique ............................................................................................................. 18
   4.8. Microneedles Coating Assessment .................................................................................... 18
   4.9. Ways of Cubosomes Delivery into Skin ............................................................................ 19
   4.10. Acquisition of skin samples ............................................................................................. 19
   4.11. Skin Samples Processing ................................................................................................ 19
   4.12. Microneedles insertion into the skin .............................................................................. 20
   4.13. Skin Sections Imaging ..................................................................................................... 21

5. Results ...................................................................................................................................... 22
   5.1. Cubosomes Characterization ............................................................................................ 22
5.1.1. Trasmission Electron Microscopy (TEM) ................................................................. 22
5.1.2. Particle Size, PDI and Zeta Potentials................................................................. 22
5.2. Stability test ............................................................................................................ 23
5.3. Stability of cubosomes following passage through hollow microneedles ............. 25
5.4. Protein entrapment ............................................................................................... 26
5.5. Microneedles Coating ......................................................................................... 28
5.6. Delivery into skin using coated solid MNs ......................................................... 32
5.7. Other types of skin delivery .................................................................................. 34
  5.7.1. Solution poke-through by solid MNs ............................................................... 34
  5.7.2. Skin delivery using hollow MNs ..................................................................... 35
  5.7.3. Skin delivery using intradermal injection ...................................................... 36
6. Discussion ................................................................................................................. 37
7. Conclusion ............................................................................................................... 40
8. References ................................................................................................................. 41
1. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Cu0</td>
<td>Unloaded cubosomes</td>
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<tr>
<td>CuFL</td>
<td>Cubosomes with fluorescent ovalbumine</td>
</tr>
<tr>
<td>CuQN</td>
<td>Cubosomes with Quil-A and Nile red</td>
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<tr>
<td>CuQP</td>
<td>Cubosomes with Quil-A and SIINFEKL peptide</td>
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<tr>
<td>CuQPN</td>
<td>Cubosomes with Quil-A, SIINFEKL peptide and Nile red</td>
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<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Deionized water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDM</td>
<td>Electrical discharge machining</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FL-OVA</td>
<td>AlexaFluor-555-conjugated ovalbumin</td>
</tr>
<tr>
<td>GMO</td>
<td>Glyceryl monooleate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin</td>
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<tr>
<td>ID</td>
<td>Intradermal injection</td>
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<tr>
<td>LCs</td>
<td>Langerhans cells</td>
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<tr>
<td>MNs</td>
<td>Microneedles</td>
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<tr>
<td>NP450</td>
<td>450 µm long NanoPass MicronJet hollow MNs</td>
</tr>
<tr>
<td>NP600</td>
<td>600 µm long NanoPass MicronJet hollow MNs</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NR</td>
<td>Nile red</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature compound</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl pyrolidone</td>
</tr>
<tr>
<td>SC</td>
<td>Stratum corneum</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>ST</td>
<td>SIINFEKL-TAMRA peptide</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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2. Introduction

2.1. The human skin

The skin is the largest organ in the human body and acts as a protective barrier against influences from the outer environment. Skin provides temperature and hydration control, protects the body against ultraviolet radiation, chemicals, allergens and pathogens. (Alkilani, A.Z. et al. 2015) Furthermore, it works as a heat, cold, touch and pressure sensor organ connecting with the central nervous system. Skin has three distinct layers (Fig. 1): the epidermis, that further divides into stratum corneum and viable epidermis, the dermis and the subcutaneous fat tissue, also termed the hypodermis. (Larrañeta, E. et al. 2016) Other parts of the skin include hair follicles with sebaceous glands and eccrine sweat glands. (Chong, R. H. et al. 2013) Skin also contains a very important network of potent antigen-presenting cells – the epidermal Langerhans cells (LCs) and the dermal dendritic cells (DCs). These cells provide the first immunological defense barrier to outside pathogens. LCs and DCs are able to take up foreign antigens, migrate to the draining lymph node to present antigen fragments to naïve T lymphocytes and initiate antigen-specific immune responses. This makes skin an excellent target site for vaccine delivery, allowing the most effective immunization with small amount of antigen. (Widera, G. et al. 2006)
2.1.1. Stratum corneum

Stratum corneum (SC) is the outermost layer of the epidermis. The major protective barrier is formed by 10 – 25 rows of non-viable corneocytes. The SC composition is usually described as “the bricks and mortar” model, where flat, elongated, and polygonal corneocytes represent bricks that are embedded in lipophilic matrix representing mortar. Interstitial lipid bilayers prevent desiccation of the underlying tissues by inhibiting water loss and limit the penetration of substances from the external environment. This makes the SC the main barrier for penetration of molecules and nanoparticles. However, it also has hydrophilic regions that form aqueous pores facilitating the trans-epidermal polar route of skin absorption. The SC typically has a thickness of 10 – 20 µm, however, it is influenced by level of hydration and body location. For example, SC thickness on the palms and soles can reach 600 µm, whilst increased hydration can make the SC even 4 times thicker. (Chong, R. H. et al. 2013, Larrañeta, E. et al., 2016)
2.1.2. Viable epidermis

Viable epidermis lying beneath the SC is mainly composed of living keratinocytes. Besides, it contains melanocytes, Langerhans cells and Merkel cells. (Alkilani, A.Z. et al. 2015) Viable epidermis is usually 130 – 180 µm thick and can be divided into three distinct layers: stratum basale, stratum spinosum and stratum granulosum. (Chong, R. H. et al. 2013, Van den Maaden K. et al. 2012) Its basal layer lies directly above the dermo-epidermal junction and forms the most important structural and functional connection to the dermis below. (Ita, K. 2015) Stratum basale contains stem cells that actively divide and differentiate and therefore ensure tissue renewal and barrier function of the skin. (Chong, R. H. et al. 2013) In contrast to the SC, the epidermis has a hydrophilic nature that limits the permeation of lipophilic agents. Successful permeation of substances is also complicated by the activity of proteolytic enzymes that can degrade foreign substances and by the presence of tight junctions at the dermo-epidermal junction. (Larrañeta, E. et al., 2016)

2.1.3. Dermis

The dermis lies beneath the epidermis, with the dermo-epidermal junction connecting these two layers. Dermis is usually about 2 mm in depth. It mainly consists of collagen and elastin fibres and therefore gives strength and elasticity to the skin. (Alkilani, A.Z. et al. 2015) Another important function of the dermis is its ability of water retention thanks to polysaccharides–polypeptide complexes. (Chong, R. H. et al. 2013) Other dermal components include nerves, blood vessels, nociceptors, dendritic cells, lymph vessels, hair follicles and sweat glands. (Ita K. 2015)

2.2. Dermal and transdermal drug delivery

The biggest challenge of the transdermal delivery is to tackle the passage of the substance or particle through the SC layer. The mechanism of transepidermal transport can be passive or active (Fig. 2). Passive delivery methods do not disrupt the SC and the transport is mediated only by passive diffusion. Active delivery methods include physical penetration of top skin layers resulting in a
porous and permeable SC. (Larrañeta, E. et al., 2016, Watkinson, A.C. et al. 2016) After overcoming the SC, compounds can have different targets. When skin tissue is the final target, we talk about dermal drug delivery. When the aim is to produce quick adsorption through to blood capillaries to produce systemic effects, we talk about transdermal drug delivery.

There are three acknowledged routes of transdermal delivery: the intercellular route leading through the lipid matrix in between the cells in SC and viable epidermis, transcellular route leading through them, and transappendageal route via hair follicles and sweat glands. (Chong, R. H. et al. 2013) In general, transepidermal transport is limited to molecules with low melting point, maximum molecular weight of 500 Daltons and logP around 2. (Watkinson, A.C. et al. 2016) However, a huge amount of research has been carried out to study the possibility of transdermal delivery of hydrophilic and high-molecular-weight drugs, such as proteins, peptides, oligonucleotides and DNA and even larger entities such as vaccines, using various invasive and non-invasive penetration enhancers. (Van der Maaden, K. et al. 2012) Skin is the most accessible organ for drug delivery having a large surface area providing many placement options for drug application. (Alkilani, A.Z. et al. 2015) Besides, the compound bypasses the first-pass metabolism effect of the liver and produces steady blood concentrations, therefore decreases its toxicity and may increase efficacy. Furthermore, swallowing problems and drug absorption, stability or local toxicity problems in the gastrointestinal tract can be circumvented. (Van der Maaden, K. et al. 2012) Unconscious or vomiting patients can also benefit from this route of drug administration. (Alkilani, A.Z. et al. 2015)
Transdermal drug delivery methods can be divided into technologies using active methods of breaching the stratum corneum and passive methods changing physicochemical properties of compounds without physically disrupting the skin. Taken from Alkilani, A.Z. et al. (2015)

2.3. Drug delivery using mechanical disruption of stratum corneum

Intravenous, intramuscular and subcutaneous administrations are highly efficient and widely used ways of active transdermal delivery. However, the use of hypodermic needles brings along pain and is often associated with poor patient compliance. Application of injections usually requires skilled professional staff, clean environment and appropriate handling of sharp medical waste otherwise could cause harm. Especially in developing countries such conditions are not always accomplished and patients are often in danger of developing infections or disease transmission by re-use of the needles. An effective alternative to hypodermic needles is the use of transdermal delivery systems. (Larrañeta, E. et al. 2016)
2.3.1. Microneedles

A novel method of active SC perforation is the use of microneedles (MNs). They are minimally invasive devices that can be easily self-administered without causing pain and anxiety (Larrañeta, E. et al. 2016, Jeong, H.-R.R. et al. 2016). Very short needles (up to 1 mm) with or without a bore form micron sized channels in the skin that facilitate both local and systemic delivery of various substances including high-molecular-weight and hydrophilic drugs. (Van der Maaden, K. et al. 2012) Therefore, microneedle delivery system can be used for treatment of various genetic diseases related to skin, various types of malignancies and infectious diseases. (Bariya, S.H. et al. 2012) Also vaccine antigens were successfully delivered to target skin antigen presenting cells and to elicit potent antibody responses. (Widera, G. et al. 2006)

Massive progress in microfabrication manufacturing technologies over the past several decades enabled further development of MNs for both cosmetic and medical purposes. Nowadays, they can be divided into 4 groups (Fig. 3): solid, coated, dissolving and hollow MNs. Also, they can be formed into arrays with backing, which enables application and fixation on the skin surface like a bandage, such device is called Microneedle patch. (Ita, K. 2015)

Solid MNs can be fabricated from metal, silicon or polymer in various shapes and dimensions. They are used as a skin pretreatment before application of drug in a form of solution or patch or to poke through the solution applied to the skin surface.

Coated MNs are solid MNs with a drug-containing formulation coated on their tips. (Fig. 4) Preparation of coated MNs is a challenging procedure that requires formulations with appropriate qualities. Coating should be made of concentrated, ideally-viscous and fast-drying formulations that keep the drug stable during the whole process. Finally, they must be adherent enough to the MNs surface, but also must allow rapid separation in skin environment. The amount of coated drug is limited by the size of MNs surface. To load larger amount of drug onto MNs tips, thicker coating possibly can be made, but brings along reduced needle sharpness and penetration ability. Therefore, coated MNs
are only applicable for drugs effective in small doses, such as vaccines. (Van der Maaden, K. et al. 2012)

Dissolving MNs are made from drug-loaded polymers that can be dissolving, or hydrogel-forming. After insertion, they are kept in the skin and the drug is released from the polymer.

Hollow MNs (Fig. 4) resemble classic hypodermic needles, although much shorter. (Ita, K. 2015) Their advantage is a possibility of administration of larger amount of drug formulation. However, compression of the dermal tissue at the needle tip creates a pressure causing that tiny bores can get clogged during insertion, especially when dispersion of drug is used. Therefore, it is necessary to keep constant flow rate using adequate pressure. It could be beneficial to use MNs with wider bores or with the bore positioned on the side of the needle, but it is very important to keep their sharpness and strength. (Ita, K. 2015)

![Figure 3](image)

**Figure 3:** Application (A) of solid, coated, dissolving and hollow MNs into skin and diffusion (B) of delivered drug (yellow) through the skin layers. Taken from Kim, Y.-C.C. et al. (2012)
2.4. Nanoparticles for transdermal drug delivery

Incorporation of a drug into a nanoparticulate delivery system generally provides protection against proteolytic and chemical degradation, allows sustained drug release for a prolonged period of time and minimizes toxicity associated with systemic absorption or gastrointestinal problems. In transdermal drug delivery, beneficial physicochemical properties of nanoparticles (NPs) are employed to help the drug to penetrate the SC and reach its intended target sites in the deeper skin layers. (Larrañeta, E. et al., 2016, Desmet, E. et al., 2016)

NPs are very promising and useful tool for targeted drug delivery, however, we must be aware of possible toxicity or unwanted side effects of chemical enhancers used for their composition. Biodegradable and biocompatible components should be used to avoid skin irritation, inflammation or unwanted immune system activity. (Desmet, E. et al., 2016)
2.4.1. Lipidic vesicles

Liposomes are the most typical drug delivery systems from this group. They are formed by single or multiple lipid bilayers enclosing the aqueous phase inside. Hydrophobic drug can be incorporated into the lipid phase, whereas hydrophilic drug is dissolved in the aqueous phase inside the particle. Even though liposomes are the most popular nanocarriers, there are some limitations in their use, e.g. chemical and physical instability, variable purity, low drug load and solubility and side effects. This has led to the development of other lipid vesicle types with improved properties, e.g. niosomes with enhanced stability, transfersomes and ethosomes with improved ability to penetrate through skin or cubosomes. (Larrañeta, E. et al., 2016, Desmet, E. et al., 2016)

2.4.2. Cubosomes

Cubosomes are cubic liquid crystalline particles with unique structure of highly twisted, continuous lipid bilayer with two non-intersecting water channels (Fig. 5).

![Figure 5: Cubosomes imaged using cryo-transmission electron microscopy (A and B), 3D visualized using cryo-field emission scanning electron microscopy (C and D) and cubosomes models visualized using the differential geometry approach. Taken from Boyd, B.J. et al. (2007)
Such a complicated structure is spontaneously formed by variety of surfactant-water systems. The most commonly used cubic phase-forming lipids are Glyceryl monooleate (GMO) and phytantriol in combination with stabilizer (e.g. poloxamer 407) and hydrotrope (e.g. propylene glycol). Resulting viscous formulation is then dispersed in water forming cubosomes with internal cubic phase structure retained. (Rizwan, S. B. 2011) Several methods of preparation exists leading to cubosomes of particle size ranging from 50 to 250 nm. (Boyd, B.J. et al. 2007) As fatty acid-based structure of GMO (Fig. 6) is susceptible to esterase-catalyzed hydrolysis, phytantriol was proposed by Boyd et al. (2006) as a more stable alternative. Furthermore, it has been proven by Rizwan, S.B. et al. (2011) that GMO is likely to undergo conversion over time to hexagonal phase, whereas phytantriol is able to keep the desired cubic phase structure in the long term. Phytantriol was therefore chosen as a cubosomes-forming lipid for this project.

![Chemical structures of Phytantriol and GMO](image)

**Figure 6:** Chemical structure of Phytantriol and GMO. Taken from Rizwan, S.B. et al. 2011

The unique nanostructure with very large surface area makes cubosomes capable of loading lipophilic, hydrophilic and amphiphilic drugs simultaneously. (Bei, D. et al. 2009) However, compared to liposomes and transfersomes in study performed by Rattanapak et al. (2012), cubosomes had lower percentage of peptide entrapment. Interestingly, they showed the best ability of peptide permeation, suggesting that high peptide or protein entrapment might not be necessary. (Rattanapak, T. et al. 2012) Cubosomes also play important role in drug release as they are capable of controlled and sustained release of drugs of various molecular weights and polarities. (Karami, Z. and Hamidi, M. 2016)
Rattanapak, T. et al. (2012) also reported that cubosomes significantly enhanced skin penetration compared to liposomes and transfersomes. Phytantriol has penetration enhancing properties itself and propylene glycol acts as a humectant and therefore improves hydration of SC. Their final cubic structure is compatible with SC lipids, having fluidizing effect on them. Moreover, addition of adjuvants can have a positive effect on skin penetration. For instance, Quil-A used in the later stage of this project is a surfactant that can disrupt the SC lipids and was reported to increase peptide permeation in vitro. (Rattanapak, T. et al. 2012) Cubosomes also have bioadhesive properties and form depositions on the skin surface or mucous membranes (Garg, G. et al. 2007, Karami, Z. and Hamidi, M. 2016). Successful transdermal delivery of several drugs including indomethacin (Esposito, E et al. 2005) and hydrophilic plant extracts from Berberis koreana. (Kwon, TK et al. 2010) has been already performed. (Rattanapak, T. et al 2014) Other potential ways for cubosomes delivery includes oral, intravenous, ophthalmic or intranasal routes. (Karami, Z. and Hamidi, M. 2016)

2.5. Combination of microneedles and cubosomes

Even though cubosomes themselves are penetration enhancers, transcutaneous delivery was found to be more successful in previously physically penetrated skin using solid microneedles. (Rattanapak T. et al. 2014) Cubosomes were chosen as the delivery system for vaccines thanks to their biphasic structure, which enables entrapment of both adjuvants and the antigen. This turned out to be crucial as the application of aqueous mixture onto the penetrated skin surface was ineffective. When the skin is pretreated with MNs, cubosomes retain in microchannels and act as a depot. Rattanapak T. et al (2014) suggests, that peptide antigens entrapped into lipid nanocarrier can potentiate immune responses by facilitating uptake of vaccine by dermal dendritic cells. Furthermore, cubosomes can facilitate the movement of unentrapped peptide antigen and adjuvants into the deeper layers of dermis as well. When vaccine-loaded cubosomes were used in MNs pretreated skin, recruitment of dermal dendritic cells occurred much faster than when applied to
an intact skin. (Rattanapak T. et al. 2014) The combined approach to transcutaneous immunization has been shown to be more dose-effective than conventional intramuscular and subcutaneous immunization in both human and animal studies. (Van der Maaden, K. et al. 2012)
3. Aims

Microneedle-based transdermal drug delivery seems to be very promising and numerous studies have been carried out regarding this matter. As more dose-effective one-step process could provide easier application and higher efficiency, this project was focused on other ways of transdermal delivery of cubosomes using solid, coated and hollow MNs. Zhu et al. (2009) reported, that MNs coated with influenza virus antigen were able to elicit stronger immune response then conventional intramuscular immunization. Therefore, the main interest of this project was producing and evaluating the coatings of cubosomes formulations onto solid MNs in order to deliver macromolecular substances including vaccines.
4. Materials and methods

4.1. Materials

All reagents were obtained from Fisher Scientific, UK unless otherwise stated. Solid microneedle arrays were fabricated from stainless steel sheets using wire electrical discharge machining (EDM). They were made by colleagues in Cardiff University.

4.2. Preparation of cubosomes

Cubosomes were formulated using the liquid precursor method. Cubosomes with no protein (Cu0) were prepared using 268.3 mg of propylene glycol (Sigma Aldrich, UK), 15 mg of poloxamer 407 (Sigma Aldrich, UK) and 100 mg of phytantriol (Tokyo Chemical Industry UK Ltd, UK) were weighed into a 25 ml round bottom flask. Components were dissolved in 10 ml of chloroform and final solution was mixed well. Round bottom flask was attached to the rotary evaporator (Rotavapor R110, Büchi, Switzerland) with a water bath heated to 45°C. Once all chloroform evaporated round bottom flask was removed and 2.5 ml of deionized water were added to the solution, vortexed gently and transferred to the 50 ml falcon tube. Another 2.5 ml of deionized water were added to the empty round bottom flask, mixed to remove any residual components and added to the previous 2.5 ml in the falcon tube. Final 5 ml of the formulation were vortexed at high speed for 10 minutes and stored protected from light under nitrogen at room temperature. (Rizwan, S. B. et al, 2011)

In certain batches ovalbumin protein with Alexa Fluor™ 555 Conjugate (Invitrogen, UK) was used as a model protein (FL-OVA). These fluorescent cubosomes (CuFL) were prepared by adding 10 µl of FL-OVA solution (100 mg/ml) to the resulting lipid mix and vortexed shortly until visually homogenous. The water was added afterwards and vortexed as mentioned above. During the
project 2 batches of cubosomes loaded with FL-OVA were prepared (CuFL1, CuFL2).

At the final stage of the project, SIINFEKL-TAMRA (ST) peptide (peptides&elephants GmbH, Germany) with adjuvant Quil-A (Brenntag Biosector, Denmark) was used. Nile red (Thermo Fisher Scientific, UK) was used to stain the lipid phase of cubosomes (NR). Cubosomes were formed as stated above, lipid soluble NR (38.325 µg/ml) was added in the chloroform, water soluble Quil-A (0.4 mg/ml) and ST peptide (0.2 mg/ml) were added to the resulting lipid mix and vortexed. Water was added afterwards as stated above and vortexed at high speed for 10 mins.

To assess influence of NR and SIINFEKL-TAMRA (ST) peptide on cubosomes characteristics, 3 types of cubosomes formulations were made:

1. Cubosomes with Quil-A and NR (CuQN)
2. Cubosomes with Quil-A and ST peptide (CuQP)
3. Cubosomes with Quil-A, ST peptide and NR (CuQPN)

### 4.3. Cubosomes characterization

#### 4.3.1. Transmission Electron Microscopy (TEM)

Aliquots of 20µl each of the CuFL1 and Cu0a formulations were submitted to a collaborator in the School of Optometry & Vision Sciences, Cardiff University for analysis by cryogenic TEM using a JEM-2100 LaB6 Transmission Electron Microscope (JEOL, USA) with a high-resolution digital camera (Gatan, UK) utilised for image capture.

#### 4.3.2. Particle size, polydispersity index and zeta potentials

Size, polydispersity index (PDI) and zeta potentials were measured using Zetasizer Nano-ZS (Malvern Instruments Ltd, UK) to characterize cubosomes. For size and PDI measuring 20 µl of cubosomes formulation were taken and diluted in 980 µl of deionized water. Three samples of each formulation before and after 10 minutes vortexing were measured at 25°C with refractive index
1.45. Vortexed samples were removed into a disposable folded capillary cell (Malvern Instruments Ltd, UK) and used to measure zeta potentials.

4.4. Cubosomes stability testing

Three batches of cubosomes were used in 4 weeks long stability test. Size, PDI and zeta potential were measured as mentioned above to characterize cubosomes stability.

4.5. Protein entrapment measuring

For protein entrapment measuring cubosomes formulations were centrifuged (Thermo Fisher Scientific, UK) at 20,800 × g for 30 minutes at room temperature. Aqueous phase was isolated and diluted with deionized water in ratio 1:1. Then 200 µl of this solution were taken out and its fluorescent intensity was measured by fluorescent spectrophotometer (Cary Eclipse, Agilent Technologies, UK) using precision cell made of Quartz SUPRASIL® (Hellma, USA). Percentage of peptide entrapment was calculated against standards made of FL-OVA and deionized water. Concentrations of FL-OVA used for the calibration curve were 50, 100 and 150 µg/ml.

4.6. Microneedles coating formulations

Formulations for MNs coating were prepared using lipid phase isolated from centrifuged fluorescent cubosomes formulation (CuFL). Following types of coating formulations were used:

- Formulation F0a: FL-OVA (10 mg/ml)
- Formulation F0b: FL-OVA (200 µg/ml)
- Formulation F1: CuFL1 + ethanol (EtOH)
- Formulation F2: CuFL2 resuspended in deionized water (ddH2O)
- Formulation F3: 20 µl CuFL2 + 2 µl polyvinyl alcohol (PVA)
- Formulation F4: 60 µl CuFL2 + 3 mg polyvinylpyrrolidone (PVP)
- Formulation F5: 50 µl CuFL2 + 7.5 mg Trehalose
- Formulation F6: SIINFEKL-TAMRA (5.25 mg/ml)
4.7. Coating technique

Formulations F0 – F5 were coated onto solid MNs using 0.2 μl of solution per 1 MN array. Usually, 0.4 μl of coating formulation were taken into a pipette tip, the tip was carefully removed and 2 arrays of MNs were coated under Leica Zoom 2000 stereomicroscope (Leica Microsystems Ltd, UK). The coating formulation held in a pipette tip was applied onto MNs by touching their tips and pushing the formulation out by index finger (Fig. 7). This procedure was repeated several times at both sides of the MN until the whole content of the pipette tip was used. Afterwards, MNs were kept in the fridge overnight with desiccant to enable proper drying.

Formulation F6 was coated onto short (500 μm) and long (750 μm) MNs using 1.5 μl and 3.4 μl in both per 1 MN array. Method of coating was the same as mentioned above.

![Microneedle array with 600 μm long tips held in a metal holder coated using transparent formulation.](image)

**Figure 7:** Microneedle array with 600 μm long tips held in a metal holder coated using transparent formulation.

4.8. Microneedles coating assessment

Coated MNs were imaged before and after insertion to the skin using Leica DM IRB epifluorescence microscope (Leica Microsystems (UK) Ltd, UK) with Openlab imaging software (Perkin Elmer, UK) using fluorescence and bright fields.
4.9. **Ways of cubosomes delivery into skin**

Following types of needles and deliveries were used. Fresh skin was used when available due to limited supply, defrosted skin sometimes had to be used.

1. Solid MNs coated with formulations F0 – F5 were inserted into the human skin for 2.5 minutes. Defrosted skin was used for F0 – F1, fresh skin was used for F2 – F5.
2. 50 µl of vortexed formulations CuFL1 and CuFL2 were poured onto human skin surface and poked through several times by solid MN. Defrosted skin was used for F1, fresh skin for F2.
3. 50 µl of vortexed formulation CuFL1 were injected into the defrosted human skin via NanoPass MicronJet hollow MNs 450 (NP450) and 600 µm (NP600) long (NanoPass technologies Ltd, Israel). Same amount of vortexed formulation CuFL2 were then injected into fresh human skin via NP450.
4. 50 µl of vortexed formulation CuFL2 were injected into the fresh human skin via intradermal injection (ID)

4.10. **Acquisition of skin samples**

Fresh human breast skin samples were obtained from subjects of breast reduction and mastectomy following informed consent and with full ethical approval from South East Wales Local Research Ethics Committee (reference 08/WSE03/55).

4.11. **Skin samples processing**

After surgery, breast skin tissue was placed into the plastic box containing Dulbecco’s modified eagle medium (DMEM) with penicillin and streptomycin and transported to the laboratory on ice. Upon arrival at the laboratory, the skin tissue was stored for a short time at 2 to 8 °C until use. Skin samples were processed in the laminar flow cabinet. First of all, the subcutaneous adipose tissue was cut-off with surgical scissors. The excised skin was briefly rinsed in media and was then stretched and pinned using 1 cm push pins (Staples, UK) on a planar corkboard covered in tin foil with the epidermal side facing up.
(Chong, R. H. et al. 2013) Unused skin samples were covered in tin foil and frozen upon arrival at the laboratory or after removing the adipose tissue. Frozen skin samples used for MN insertion were allowed to thaw for 30 minutes at room temperature in the laminar flow cabinet and washed in sterile Phosphate buffered saline (PBS).

4.12. Microneedles insertion into the skin

2 arrays of coated MNs were placed into a plastic holder, inserted into the skin and kept there for 2.5 mins (Fig. 8). During this time MNs were constantly pushed down and gently moved from side to side to enable the coating to get into skin. After their removal, channels were stained by methylene blue (Sigma Aldrich, USA). After few seconds, redundant methylene blue was wiped of the skin surface. The places of insertion of each MN array were cut out separately, placed on a piece of cork with the epidermis facing to the side, mounted with Optimal Cutting Temperature compound (OCT), snap frozen in chilled hexane, placed into chilled vials and kept on dry ice until stored in -80 °C.

![Figure 8: Two solid MN arrays coated with fluorescent formulation placed into plastic holders.](image)

After skin injections by NP MicronJet hollow MNs or intradermal skin injection (ID) round skin samples were cut out using 8 mm diameter biopsy punch (Kai Medical, Germany). To maintain the viability of skin samples the air-liquid interface of a Trowell-type organ culture was used to simulate in vivo conditions. (Trowell, 1954)
Skin samples were placed epidermal side up on the metal gauze covered in lens cleaning tissue paper soaked with 5 ml of organ culture media (DMEM with 100 mg/ml penicillin and 100 unit/ml streptomycin) and placed in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ in air for 24 hours. After 24 hours rounded samples were cut in half, placed on a piece of cork epidermal side facing to the side, mounted with OCT, snap frozen in chilled hexane, placed into chilled vials and kept on dry ice until stored in -80°C. After at least overnight stay in -80 °C, 10 µm thick sections were cut using cryostat (Cryotome FSE, Thermo Scientific, USA) with chamber set to temperature -20 °C, cryobar to -60 °C and specimen to -20 °C. Sections were captured on Superfrost Plus® microscope slides and stored in -80 °C.

4.13. Skin Sections Imaging

Histological skin sections were kept in chilled (-20 °C) acetone for 15 minutes to fix them. After fixation, sections were washed in PBS and stored for a short time immersed in it. Sections were observed using the Leica DM IRB epifluorescence microscope (Leica Microsystems (UK) Ltd, UK) with Openlab imaging software (Perkin Elmer, UK) using fluorescence and bright fields. After fluorescent microscopy selected slides were subjected to Haematoxylin & Eosin (H&E) staining. The slides were rinsed in running tap water, immersed in Haematoxylin for 2 minutes, washed again in tap water, differentiated in acid alcohol for 10 s, rinsed under running tap water and kept there for 1 minute, immersed in eosin for 3 s and rinsed under running tap water again. Redundant water was carefully wiped off the slides and they were air-dried in a room temperature for a few minutes. Dry sections were mounted with ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, UK) and cover-slipped. Slides were kept in the dark at room temperature for at least 24 hours, then imaged using the Olympus BX-50 microscope (Olympus (UK) Ltd, UK) and captured by DP-10 digital camera (Olympus (UK) Ltd, UK).
5. Results

5.1. Cubosomes Characterization

5.1.1. Transmission Electron Microscopy (TEM)

Cubosomes with no peptide (Cu0) were visualized using Transmission Electron Microscopy (TEM) to ensure that the approximate conformation of cubosomes was reached. Even though the quality of images obtained from TEM was not ideal, cubic-shaped particles of correspondent size were recognizable as shown in Figure 9.

![TEM visualization of cubosomes](image)

**Figure 9**: Transmission Electron Microscopy (TEM) visualization of cubosomes, three examples are marked with red arrows. White arrows are indicating unwanted bubbles, an artifact of the TEM imaging process.

5.1.2. Particle Size, PDI and Zeta Potentials

Cubosomes of appropriate sizes were obtained in formulations Cu0 (171.4±1.9 nm), CuFL (167.7±1.3 nm) and CuQN (152.9±2.2 nm). Addition of SIINFEKL peptide caused formation of bigger particles (340.6±30.0 nm), when cubosomes
were loaded with the peptide, Nile red and Quil-A the size was almost 10 times bigger (1649.0±329.4 nm) then the size of unloaded cubosomes. In almost all formulations except CuQP (0.66±0.09) PDI was lower then 0.3 proving relatively homogenous particle distribution. Cubosomes have a negative zeta potential due to the stabilising effect of poloxamer 407 adsorbed on the surface acting as a coating layer to prevent aggregation. (Rattanapak, T. et al. 2012) In formulations Cu0, CuFL and CuQPN the values of zeta potentials were higher then indicated by Rattanapak, T. et al. (2013) and dispersions formed aggregates, however, it was possible to redisperse them by vortexing even after long-term storage (See chapter 5.2. Stability test). Results summary is shown in Table 1.

Table 1: Mean values of particle size, PDI and zeta potentials of prepared cubosomes formulations compared to target values reported by Rattanapak, T. et al. 2013

<table>
<thead>
<tr>
<th></th>
<th>Target values</th>
<th>Cu0</th>
<th>CuFL</th>
<th>CuQN</th>
<th>CuQP</th>
<th>CuQPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size</td>
<td>158 - 182 (± SD)</td>
<td>171.4</td>
<td>167.7</td>
<td>152.9</td>
<td>340.6</td>
<td>1649.0</td>
</tr>
<tr>
<td>PDI</td>
<td>&lt; 0.3 (± SD)</td>
<td>0.17</td>
<td>0.16</td>
<td>0.20</td>
<td>0.66</td>
<td>0.20</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>-21 - -25 (± SD)</td>
<td>-16.7</td>
<td>-15.9</td>
<td>-27.3</td>
<td>-21.1</td>
<td>-1.9</td>
</tr>
</tbody>
</table>

5.2. Stability test

A four week cubosome stability test was performed with three batches of Cu0, where size of particles, PDI and zeta potentials were measured. Stability test results are depicted in Figure 10.
Figure 10: Particle size (A), PDI (B) and Zeta potential (C) changes in 4 weeks long stability test.
From the graphs above it is apparent that major differences following storage were in size of particles. Formulations tended to aggregate due to zeta potentials values were not low enough to keep the formulations stable. However, after 10 mins vortexing required size of particles was reached again. PDI values were slightly affected by storage and vortexing, but stayed in stated levels.

One batch of FL-OVA loaded formulation CuFL1 was also subjected to the four week cubosome stability test to find out if protein influence the stability of the carrier. Results were comparable to Cu0, therefore FL-OVA does not influence the long-term stability of cubosomes. Stability test with loaded SIINFEKL peptide could not have been performed due to time constraints.

5.3. Stability of cubosomes following passage through hollow microneedles

The influence of passage through bores of 450 µm (NP450) and 600 µm (NP600) long NanoPass MicronJet hollow MNs was tested in both unloaded (Cu0) and loaded (CuFL1) cubosomes formulations to find out any possible changes in structure following exposure to mechanical pressure during application process. Surprisingly, there were no significant differences in particle size and PDI, however, zeta potentials dropped down significantly. As lower zeta potentials means higher stability of dispersion without forming aggregates, this could be considered as a benefit, especially in case of this project, where desired values of zeta potentials (-21 - -25 mV) were not reached during cubosomes formation. The length of MNs did not influence measured values. Results are depicted in figure 11.
Figure 11: Graphs showing the influence of passage through 450 µm (NP450) and 600 µm (NP600) long hollow MNs on particle size, PDI and zeta potentials of unloaded (Cu0, blue) and loaded (CuFL1, red) cubosome formulations.

5.4. Protein entrapment

To examine cubosome delivery of testing protein excitation and emission spectra of FL-OVA were measured as shown in Figure 12. FL-OVA excitation spectrum peak wavelength of 552.94 nm and emission spectrum peak wavelength of 571.91 nm were used in further measurements.
**Figure 12:** Excitation and emission spectra of FL-OVA

Concentration-calibration curve was made out of FL-OVA standards using concentrations 0, 50, 100 and 150 µg/ml as shown in Figure 13.

**Figure 13:** Concentration-calibration curve of FL-OVA standards

Three samples of CuFL1 prepared as mentioned above were used to measure protein entrapment in cubosomes. Results are depicted in Table 2.
Table 2: Percentage of entrapment of FL-OVA in cubosomes

<table>
<thead>
<tr>
<th></th>
<th>Intensity (a.u.)</th>
<th>Concentration (µg/ml)</th>
<th>Protein entrapment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>164.223</td>
<td>72.7</td>
<td>30.76</td>
</tr>
<tr>
<td>Sample 2</td>
<td>152.825</td>
<td>67.0</td>
<td>27.96</td>
</tr>
<tr>
<td>Sample 3</td>
<td>150.520</td>
<td>65.8</td>
<td>26.89</td>
</tr>
</tbody>
</table>

Average protein entrapment measured at the day of cubosomes preparation was 28.54 ± 1.63 %. Another 3 samples from the same batch of CuFL were measured 7 days later with the result of 89.69 ± 8.05 %. High percentage was most probably not caused by entrapping more protein into the cubosomes, but rather by destruction of free protein, which was not entrapped in the cubosomes and therefore could not have been measured. Cu0 formulation without any protein was measured as a control with the result of 98.59 %.

Unfortunately, SIINFEKL peptide entrapment study could not have been performed due to time constraints. However, previous work reported by Rattanapak, T. et al. (2013) shows that entrapment is possible and comparable to FL-OVA.

5.5. Microneedles Coating

Various formulations were used for coating of solid metal MNs of various lengths. Formulations F0 – F5 were coated onto 600 µm long MNs. FL-OVA in two concentrations was used as a standard. Formulations F0a and F0b had lower viscosity that made coating slightly more difficult and sometimes caused unintended losses of formulation caused by bubbles and excessive leakage of formulation that did not stay on the tips but also got to the further parts of the MN array. As only MNs tips can get through the skin, appropriate coating should reach up to 2/3 of their length to deliver as much drug as possible.

Coated formulations of FL-OVA easily dissolved from the MNs after contact with the inner skin environment.

For the first coating of cubosomes formulation ethanol was used as a solvent (F1). Coating was easy to do and formulation dried fast on the surface of MNs, but unfortunately did not stick to them properly and almost whole content got
wiped off after contact with the skin. When lipid phase of cubosomes was resuspended in deionized water (F2) instead of ethanol, certain amount of formulation was wiped off as well, but part of it was still able to get into the skin. Three different coating excipients were used to improve equality and stability of coating and dissolution off the MNs. PVA turned out to be the best option, as the most of the formulation (F3) was able to get into the skin. Too high surface tension of formulation F4 containing PVP caused that it did not spread equally on the surface of the MNs, got wiped off after insertion and no amount of formulation got into the skin. Trehalose formed aggregates of formulation F5 on the MNs surface and did not get into the skin at all either.

Two different amounts of ST peptide were coated onto two different types of the MNs to find out what amount is possible to fit onto needles 500 µm and 750 µm long and how much is possible to deliver from them. Coating was equally spread and even onto the shorter MNs it was possible to coat the higher amount of 3.4 µl of F6. However, the coating thickness decreased the needles sharpness and therefore could complicate their insertion into skin. Furthermore, thick coatings might be unstable on the tips and prone to breakage. Unfortunately, due to time constraints the delivery into skin could not have been examined. Results are depicted in Figure 14.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Coated MNs</th>
<th>MNs after skin insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0a</td>
<td><img src="image1" alt="Coated MNs" /></td>
<td><img src="image2" alt="MN after skin insertion" /></td>
</tr>
<tr>
<td>FL-OVA (10 mg/ml)</td>
<td><img src="image3" alt="Coated MNs" /></td>
<td><img src="image4" alt="MN after skin insertion" /></td>
</tr>
</tbody>
</table>
F0b
FL-OVA (200 µg/ml)

F1
CuFL + EtOH

F2
CuFL in ddH2O

F3
CuFL + PVA

F4
CuFL + PVP
Figure 14: Images of coated MNs and MNs after insertion into skin in combined bright and fluorescence fields. Scale is 200 µm.
5.6. Delivery into skin using coated solid MNs

Delivery of coated formulations was detected in formulations F0a – F3 as a channel with broken stratum corneum and red fluorescence as shown in figure 15. H&E staining was used for better visualization of the tissue to determine whether stratum corneum was disrupted and how deep the needles penetrated. Formulations F4 and F5 were not delivered into skin at all and therefore showed no fluorescence. Formulation F6 was not examined. Formulations containing FL-OVA itself separated from MNs surface and were delivered to the deeper layers of dermis. Thanks to higher concentration of FL-OVA in formulation F0a the fluorescence was stronger and coloured protein was visible in the H&E stained sections as well. As mentioned above, formulation F1 did not stick to the MNs properly and therefore could not have been delivered into the skin. Fluorescence was visible only on the skin surface. Formulation F2 was showing more successful delivery as certain amount of formulation apparently got into the skin, on the other hand, in comparison with F0b the fluorescence is weaker which could have been caused by residual of formulation that stayed on the MNs surface. The most promising was formulation F3 containing PVA, where bright fluorescence was shown even in the deeper layers of dermis.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Bright + fluorescence field</th>
<th>H&amp;E stained channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0a</td>
<td><img src="image" alt="Bright + fluorescence field" /></td>
<td><img src="image" alt="H&amp;E stained channels" /></td>
</tr>
<tr>
<td>FL-OVA (10 mg/ml)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


**F0b**
FL-OVA (200 µg/ml)

**F1**
CuFL + EtOH

**F2**
CuFL in ddH2O

**F3**
CuFL + PVA
Figure 15: Images of skin delivery of formulations F0a, F0b, F1, F2 and F3 using coated solid MNs visualized in combined bright and fluorescence fields and H&E stained sections with more visible channels in stratum corneum.

5.7. Other types of skin delivery

5.7.1. Solution poke-through by solid MNs

The same MNs (600 µm) as used for coating were used uncoated to poke through the cubosomes formulation poured onto the skin. This type of delivery seemed to be very effective as a big amount of formulation was delivered into deeper skin layers as shown in figure 16.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Bright + fluorescence field</th>
<th>H&amp;E stained channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuFL1</td>
<td><img src="image" alt="CuFL1 bright fluorescence" /></td>
<td><img src="image" alt="CuFL1 H&amp;E stained" /></td>
</tr>
<tr>
<td>CuFL2</td>
<td><img src="image" alt="CuFL2 bright fluorescence" /></td>
<td><img src="image" alt="CuFL2 H&amp;E stained" /></td>
</tr>
</tbody>
</table>

Figure 16: Images of skin delivery of formulations CuFL1 and CuFL2 using formulation poke-through method visualized in combined bright and fluorescence fields and H&E stained sections with more visible channels in stratum corneum.
5.7.2. Skin delivery using hollow MNs

NanoPass MicronJet hollow MNs 450 µm (NP450) and 600 µm (NP600) long were used for this type of delivery. NP450 showed as a very promising way of skin delivery with very intense fluorescence as shown in figure 17. Surprisingly, delivery via NP600 was not successful with almost no fluorescence.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Bright + fluorescence field</th>
<th>H&amp;E stained channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuFL1</td>
<td><img src="image1" alt="CuFL1 NP450" /></td>
<td><img src="image2" alt="H&amp;E stained CuFL1" /></td>
</tr>
<tr>
<td>NP450</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuFL2</td>
<td><img src="image3" alt="CuFL2 NP450" /></td>
<td><img src="image4" alt="H&amp;E stained CuFL2" /></td>
</tr>
<tr>
<td>NP450</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 17:** Images of skin delivery using 450 µm NanoPass hollow MNs (NP450) visualized in combined bright and fluorescent fields and H&E stained sections with more visible channels in stratum corneum. On the picture showing H&E stained section after insertion of formulation CuFL1 the channel in SC was not properly caught on the slide, however, as shown on the previous image, the fluorescence was clearly emerging from the place of needle application. Therefore, inability to find proper channel in H&E stained sections was most
probably caused by wrong position of the sample during cryosectioning, not by inability of needle to break the SC.

5.7.3. Skin delivery using intradermal injection

Method using intradermal injection (ID) has already been proven effective and is used in clinical practice, so it was used as comparison to methods using MNs. Intradermal injection goes deeper into the skin and makes longer channel, which was hard to capture in one section. Massive fluorescence was shown in deeper layers of dermis but was impossible to capture in one shot having the channel included (Fig. 18).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Bright + fluorescence field</th>
<th>H&amp;E stained channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuFL2</td>
<td><img src="image" alt="Bright + fluorescence field" /></td>
<td><img src="image" alt="H&amp;E stained channels" /></td>
</tr>
<tr>
<td>ID</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 18**: Images of skin delivery of formulation CuFL2 using intradermal injection (ID) visualized in combined bright and fluorescent fields and H&E stained section with more visible channel in stratum corneum.
6. Discussion

When working with cubosomes, it is important to characterize them to make sure that stable particles of right dimensions were formed. Preparation of unloaded cubosomes and cubosomes loaded with FL-OVA and Nile red and Quil-A resulted in particles with satisfactory parameters, however, addition of ST peptide caused significant growth in size. In previous study performed by Rattanapak et al. (2013) cubosomes loaded with ST peptide were prepared using the same method and resulted in particles of appropriate size. This suggests that the problem is most probably not connected with the peptide itself, but even small differences in preparation process or quality of chemicals might affect the formation of particles. Due to time constraints only one batch of each formulation containing ST peptide was prepared so the source of difference could not has been indicated.

Stability tests showed that both loaded and unloaded cubosomes are stable following long-term storage or mechanical pressure caused by passage through bores of NanoPass Micronjet hollow MNs. Positive effect of passage through hollow MNs on zeta potential values suggests that a kind of homogenizing device could be useful in final stages of cubosomes manufacturing and could lead to less aggregating dispersion if low enough zeta potentials are not reached during preparation. These measurements give us very important information about outer surface and spread of mass in cubosomes dispersion, however, additional examination of inner nanostructure would be useful as there is a risk of undergoing conversion from cubic phase to another and therefore losing their delivering abilities. Cubosomes structure should have stabilizing effect on protein or peptide loaded inside, however, it’s necessary to focus on each loaded drug individually and make sure that macromolecular structure has not been altered during manufacture, storage and application. Such structural changes or degradation could lead to inefficiency of therapy or unwanted adverse reactions.
Another question is how much of drug is possible to load and release from cubosomes and deliver into skin. With method used in this project we were only able to measure the protein entrapment of FL-OVA at the day of preparation as later on the fluorescence was not detectable by fluorescent spectrophotometer. To quantify the non-fluorescent protein in the long term, a different analysis method, such as HPLC, could be employed. Also, coated MNs provide only limited space for drug loading. Coating of too many layers is not the best solution due to thickness of the coating, which decreases the sharpness of needles and prolongs the dissolution. This could be overcome by using multiple MN arrays rather than using longer needles as Gupta, J. et al. (2011) reported, that increasing the length of needles resulted in more painful application than increasing their number.

Coating of cubosomes formulations onto the MNs tips and their delivery into skin is technically possible. PVA turned out to be the most efficient excipient allowing the coating and enhancing penetration of substance. Even though there was a bit of formulation left on the base of MNs, deep delivery of significant amount was reached and appeared comparable to the delivery of protein formulation F0b. Lowering the concentration of PVA might help to prevent the wiping of the formulation. Again, attention must be given to cubosomes structure as addition of solvents improving coating properties of formulations could change physicochemical properties of particles and cause coagulation or lost of cubic phase. Furthermore, solvents can harm the loaded drug or cause irritation of the skin. Formulation poke-through is an effective method circumventing the problems related to addition of another solvent to the formulation. Even though this method is capable of efficient delivery of significant amount of drug, amount of formulation poured onto the skin surface is never completely absorbed which makes it difficult to determine the real dose of drug that effectively got into the skin. Delivery of whole content is assured by use of hollow MNs. NanoPass MicronJet hollow MNs 450 µm long (NP450) were able to deliver large amount of formulation into dermis with no residua and no need for additional solvents. Bores provide fast and efficient delivery, however, they got clogged pretty often during application and needed to be changed. Also, mechanical strength of hollow MNs is lowered and they are
more likely to break then solid ones. Application via hollow MNs is generally more complicated but more consistent. Needles must go deep enough into skin under the right angle and formulation must be pushed out using consistent pressure to form a bleb. When the bleb was formed and the whole content of syringe was used, the application could have been considered as successful. Application of solid MNs is easier, however, significant pressure must be used to break the SC. The pressure often varies when applying them manually and has major impact on the depth of needles penetration. This is even more important for coated MNs as they need to be applied precisely deep enough to allow the dissolution of coatings. Ideally, the same pressure should be kept for the whole duration of application allowing gentle movement from side to side. Also, distribution of drug in the skin environment was influenced by skin properties. Both frozen and fresh skin samples were used from different individuals, therefore the level of hydration and thickness of particular skin layers differed and together with manual application could have been a cause of differences in amounts and depths of delivered formulations or could have caused unsuccessful deliveries.
7. Conclusion

Delivery of protein or peptide into skin using cubosomes and microneedles was successfully performed. Application via NP450 hollow MNs can be considered as most efficient method, delivering the largest amount of drug. Solid MNs coated with right formulations also showed promising results, which make them a good candidate for delivery of drugs effective in small doses, such as vaccines. This combined approach definitely holds promise for transdermal delivery of hydrophilic, macromolecular substances that are not able to penetrate the stratum corneum without enhancers. Furthermore, painless and safe application is beneficial for patients supporting their compliance. However, there are several challenges remaining to be overcome and lot of work to be done before proceeding to a clinical trial.
8. References


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