Revolutionizing Vaccines: Soluble Microneedles and the Future of Painless Transdermal Delivery

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Abstract. Trypanophobia, an intense fear of needles in a medical setting, affects 33% to 66% of children and up to 10% of the total population, hundreds of millions of people. Additionally, many side effects result from traditional needle injections, such as a high risk of infection and contamination. Finding an alternative to traditional hypodermic vaccine delivery is vital for patients worldwide. Microneedles are small arrays of needles that penetrate a small area with restricted depth. They cause minor inflammation and damage to the dermal layers compared to traditional needles and were engineered to penetrate the epidermis to convey a drug directly into the subepidermal vesicular without reaching nerve endings and blood vessels which cause the pain associated with needles. This study aimed to develop water-soluble microneedles from hyaluronic acid that include both proteins OVA and BSA. The microneedle we developed demonstrated significant protein delivery in human umbilical vein endothelial cells and is expected to be a new method of vaccine delivery in the future.

Keywords: Microneedles, Hyaluronic Acid, Vaccine Delivery, Bovine Serum Albumin, Ovalbumin.

1. Introduction

The skin, the largest organ in the body, is a beneficial site for vaccination as it contains a rich network of immune cells and is accessible to penetration [1]. It protects the body from outside environments by acting as a barrier to prevent fluid and loss of salts and regulates body temperature [2]. The skin has three layers—the epidermis, dermis, and hypodermis—with an average thickness of 1.5mm and a surface area of 1.75m² [3]. However, the stratum corneum, the top layer of the epidermis, prevents vaccine delivery into the skin, which calls for a method of piercing that layer to allow for successful vaccination [4].

The conventional needles used are hypodermic needles; however, the pain associated with them presents major problems in transdermal drug delivery. Trypanophobia, an intense fear of needles in medical settings, is present among 10% of the adult population and is even more common among adolescents aged 5-16 years. Additionally, hypodermic injections are not medically safe as there is a high risk of infection and contamination which can lead to hypersensitivity, swelling, and bleeding in the patient [5].

Among several developed devices for intradermal vaccine administration, the Transdermal Drug Delivery System (TDDS), or specifically microneedles (MN) is currently one of the most promising technologies. They are small, needle-like structures with micron-scale lengths measured in micrometers, typically assembled as arrays. MNs are versatile and very efficient, engineered to penetrate the epidermis and convey a drug directly into the subepidermal vasculature. They allow for painless delivery of large substances, eliminate first-pass metabolism, and lower inflammation of the dermal layers and damage compared to transdermal delivery routes with traditional needles [6].

There are many types of MNs, each having its own benefits. Dissolvable MNs were chosen for their lack of risk of injury, being prepared from biodegradable and safe materials that do not produce sharp waste in comparison to other types of MNs [7, 8]. It's able to encapsulate drug or water-soluble and biodegradable biomaterials, and completely disintegrate after being inserted to release the substance into the skin [9, 10].

Hyaluronic acid (HA) is a highly versatile polymer with significant benefits and diverse applications in drug delivery systems, particularly in dissolvable MNs. Its biocompatibility and

biodegradability, being a natural component of the skin and connective tissues, make it ideal for safe and efficient drug delivery without triggering immune responses. HA's unique ability to encapsulate both hydrophilic and lipophilic molecules enhances drug stability and bioavailability, while its enzyme-sensitive nature enables controlled, targeted release. It is widely used in transdermal drug delivery, including insulin and anticancer therapies, by promoting high drug loading and precise release. HA's water-binding capacity supports skin hydration and regeneration, making it valuable in cosmetic applications like anti-aging and dermal repair. Additionally, HA MNs offer sufficient mechanical strength to penetrate the skin without fracturing, while their dissolvable nature eliminates biohazardous waste. These properties have made HA a key material in advancing immunization, gene therapy, regenerative medicine, and dermo-cosmetic innovations, highlighting its critical role in modern drug and cosmetic formulations [11].

This study aimed to develop soluble MNs loaded with OVA and BSA that are able to successfully dissolve and deliver the proteins while not harming cells within the body. Human umbilical vein endothelial cells (HUVEC) cultured in a DMEM medium were selected. Soluble MNs were prepared from HA that contained OVA and BSA. The solubility and durability of the MNs were evaluated through piercing tests in pig skin. A flow cytometer was used to test the ability of MNs to perform intracellular delivery of proteins. The effects of MNs on cell proliferation were tested by a CCK-8 assay.

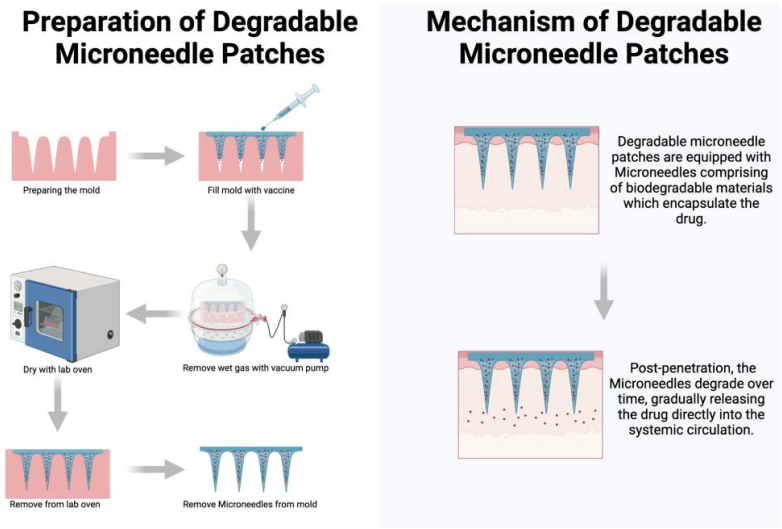

Figure 1. Schematic flow of soluble microneedle preparation

2. Materials and Methods

2.1. Materials

1.5-1.6 million Da Hyaluronic Acid (HA), 200k-400k Da HA, 5k-10k Da HA, and Bovine Serum Albumin (BSA) were purchased from Beyotime. Other reagents and instruments used were as follows: ins ovalbumin (OVA), phosphate-buffered saline (PBS), Trypan Blue dye, human umbilical vein endothelial cells (HUVEC), DMEM medium containing 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, CCK-8 solution, a CO_2 incubator, an ultrasonic cleaner, yellow FITC-NHS (fluorescein isothiocyanate), blue Cy5-NHS (cyanine dye), PDMS molds, centrifuge tubes, a vacuum pump, a drying oven, ethanol C_2H_6O, pig skin, EMITECH K550X Gold coating machine, Hitachi S-4800 Scanning Electron Microscope (SEM), Supernatant, VICTOR X4 2030 Multilabel reader, and CytoFLEX 5 Flow Cytometry.

2.2. Synthesis of Microneedles

3.0 grams of 1.5-1.6 million Da HA, 1.0 gram of 200,000-400,000 Da HA, and 1.0 grams of 50,000-100,000 Da HA were weighed by using an electronic analytical balance. The three HA

samples were mixed with 100mL of distilled water to create a 5% HA solution. The solution was stirred and set overnight to mix the HA and water completely. Afterward, the PDMS mold was placed in a petri dish and filled with the HA solution. The petri dish was then placed in a vacuum pump for 3 minutes to remove all air. The mold was then taken out from the vacuum pump and placed in a drying oven overnight.

2.3. Microneedle Protein Loading

0.1 grams of BSA and OVA were measured using an electric analytical balance. Then, each was placed in a separate centrifuge tube. 5 mL of distilled water was added to each tube and mixed until dissolved completely. Afterward, 20 mL of the 5% HA solution was put into two centrifuge tubes, each holding 10 mL using a pipette. 500 µL of the BSA solution was added to one centrifuge tube, and 500 µL of the OVA solution was added to the other tube. 100 mL of each yellow FITC-NHS and blue Fy5-NHS solution was added into one HA solution. Subsequently, 4 molds were placed in a petri dish; 2 were filled with the colored HA solution, while 2 were filled with the clear HA solution and all four were placed in the vacuum pump. The vacuum pump was used to remove all air from the HA solutions, and the four molds were then placed in the drying oven overnight for 24 hours. Then, the microneedles were taken out of the mold.

2.4. Preparation For Skin Penetration Test

75% Ethanol (C_2H_6O) was obtained by mixing 10 mL of H_2O and 30 mL of Ethanol. Then, pig skin was soaked in this solution for 5 minutes. Afterward, the pig skin was rinsed three times with PBS (Phosphate-buffered saline) for 1 minute for each rinse. Then, the pig skin was cut into 1cm x 1cm pieces and dyed blue using 0.4% Trypan blue.

2.5. SEM Observations

One colored HA microneedle containing BSA and OVA and one clear HA microneedle were taped onto a metal plate and put into a gold spraying machine for 4-5 minutes to improve images produced by the scanning electron microscope (SEM). Then, they were placed into an SEM and various photos were taken from different depths.

2.6. CCK-8 and Flow Cytometry Trials

First, frozen HUVEC cells were thawed quickly in a 37°C water bath. Then, the cells were separated by centrifugation, and the liquid was removed. After that, 10 mL DMEM was used to resuspend the HUVEC cells. The cell solution was put into 500 µL increments in a three-by-six grid of wells in a 24-well culture plate (refer to the image below). The culture plate was then incubated at 37 degrees Celsius and 5.0% CO_2 for 24 hours. The next day, a clear and colored microneedle was cut up into 6 pieces, put into the corresponding wells in the cell culture plate, and put back into the incubator for 24 hours. After that, the cell culture plate was taken out of the incubator and 1 µL of CCK-8 solution was added to 9 wells on the left marked “experiment 1”, and were left to sit for 50 minutes for the CCK-8 solution to activate. Then, the solution was taken out of the right 9 wells marked “experiment 2” and transferred to individual little tubes. The tubes were placed in the centrifuge to separate the cells from the liquid. After it was separated, the liquid was taken out of each tube and filled with 500 µL of PBS. Following this, 500 µL of solution in each tube was moved to a separate tube after mixing to prepare for the flow cytometer. Going back to experiment 1, the CCK-8 solution, after 50 minutes had passed, 100 µL of solution in each well was transferred into a smaller 96-well cell culture plate. Subsequently, a CCK-8 Multilabel Reader and Flow cytometer were used to observe the corresponding effects of the microneedles on cells.

2.7. Fluorescent Microneedle Testing

A fluorescent microscope was used to take pictures of the two different types of microneedles created: clear and colored, with clear being one without protein and purely HA while colored being

microneedles with protein and HA. Many different angles were used in taking pictures, including facing the front, sideways, and diagonally.

3. Results and Discussion

3.1. Constructing Microneedles from Hyaluronic Acid

Hyaluronic acid (HA) solutions of varying molecular weights were prepared to construct soluble microneedles with tailored mechanical and dissolution properties. Specifically, 3.0106 g of 1.5–1.6 MDa HA, 1.0011 g of 200–400 kDa HA, and 1.0021 g of 50–100 kDa HA were weighed using an electronic analytical balance and dissolved in 100 mL of distilled water. The solution was sonicated and stirred until fully dissolved, forming a viscous gel. To remove air bubbles, the mixture was allowed to rest at room temperature for 24 hours. Three formulations of microneedles were then prepared: one loaded with ovalbumin (OVA) labeled with fluorescent dye FITC-NHS and bovine serum albumin (BSA) labeled with fluorescent dye Cy5-NHS, and another without proteins (MNs). These dyes enabled visualization and tracking of protein delivery. Separate solutions of OVA (0.1018 g) and BSA (0.1016 g) were prepared in 5 mL of distilled water. Subsequently, 10 mL of the HA solution was combined with 500 µL of each protein solution and 100 µL of the respective fluorescent dye. The HA-protein solutions and protein-free HA solution were poured into PDMS molds and placed in a vacuum pump to eliminate air bubbles and ensure uniform filling. Finally, the molds were dried in an oven overnight to solidify the microneedles. The microneedle array obtained from the mold of 0.9 cm x 0.9 cm is composed of 121 individualized microneedles in an 11 x 11 matrix.

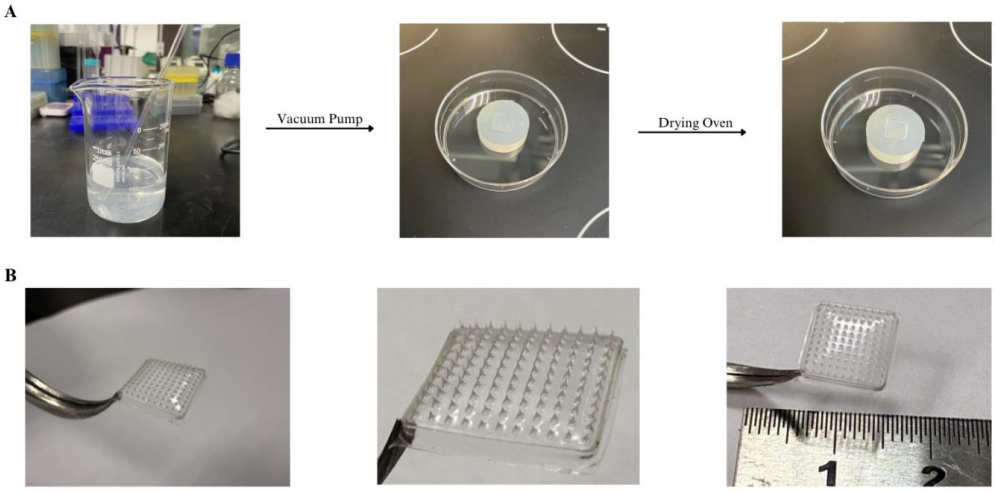

Figure 2. (A) Process of creating microneedles from Hyaluronic acid. (B) Pictures of the microneedle from different angles and against a ruler

3.2. Examinations Through SEM

Through the images captured by the scanning electron microscope (SEM) at increasing magnifications, the uniformity of the microneedle array and the sharpness of the needle tips highlight the high-quality fabrication process. The well-defined pyramidal geometry ensures consistent and reliable skin penetration, a key feature of microneedle-based drug delivery systems. Such precision is essential to facilitate painless insertion while minimizing tissue damage. At higher magnifications, the surface roughness becomes more apparent, likely resulting from the incorporation of OVA and BSA within the microneedle. However, observed irregularities, such as cracks, may indicate challenges with material stability or brittleness, potentially affecting mechanical integrity during insertion.

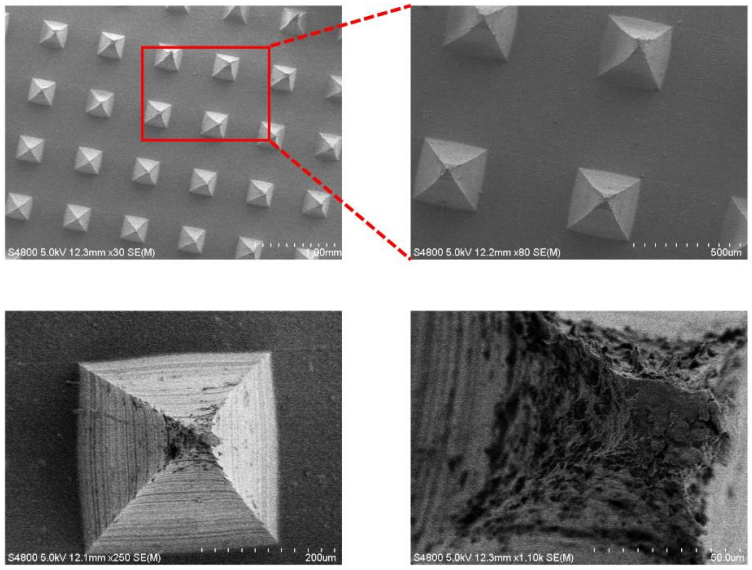

Figure 3. Image of microneedles containing OVA and BSA under an SEM microscope

3.3. Characteristics of OVA and BSA's spacial distribution within MNs

Three different types of images were captured through the fluorescent microscope, including brightfield, green fluorescence, and red fluorescence. The brightfield images reveal the geometric precision and arrangement of the microneedle array, highlighting their uniformity and sharp tips, which are essential for effective penetration. Under fluorescent microscopy, the green and red fluorescence images illustrate the successful incorporation of fluorescently labeled materials into the microneedles, which is OVA for the green fluorescence (FITC-NHS) and BSA for the red fluorescence (Cy5-NHS). The fluorescence distribution, visible from both bird's-eye and sideways perspectives, demonstrates uniform labeling and material integration throughout the microneedle structure. Additionally, the images emphasize that the two proteins can coexist in one microneedle as OVA is seen to be more concentrated towards the tip while BSA is concentrated towards the base and center of the microneedle. These images emphasize the microneedles' potential for delivery of bioactive compounds while offering visual confirmation of the loading and spatial distribution of OVA and BSA within the array.

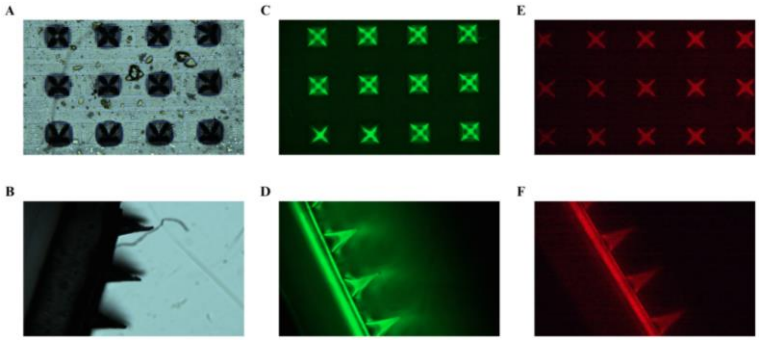

Figure 4. (A) Image of microneedles under a brightfield microscope, birds-eye view. (B) Image of microneedles under a brightfield microscope, sideways view. (C) Fluorescent microscopy showcases the same array of microneedles under green fluorescence, birds-eye view. (D) Same array of microneedles under green fluorescence, sideways view. (E) Same array of microneedles under red fluorescence, birds-eye view. (F) Same array of microneedles under red fluorescence, sideways view

3.4. Pig Skin Penetration

A microneedle was employed to penetrate a 1 cm x 1 cm section of pig skin, mimicking a scenario commonly used in biomedical and pharmaceutical research to study drug delivery systems. The

microneedle was held in place and pressed firmly against the skin for approximately one minute to ensure adequate penetration and tissue interaction. Afterward, the pig skin was treated with a 0.4% Trypan Blue Dye solution, a staining agent often used in biological studies to highlight structural changes or areas of tissue disruption. The dye served to enhance the marks left by the microneedle penetration, providing a clear visual representation of the needle's effect on the skin. As depicted in Figure 5, the microneedle not only punctured the skin but also began to dissolve within the tissue. This dissolvability is characteristic of soluble microneedles, which are designed to deliver vaccines, drugs, or other therapeutic agents directly into the skin layers while gradually breaking down. The process left the microneedle tip noticeably less sharp, indicating successful interaction with the skin and a potential release of its contents. The alteration in the needle's structure highlights its functionality and supports its intended use for transdermal delivery applications.

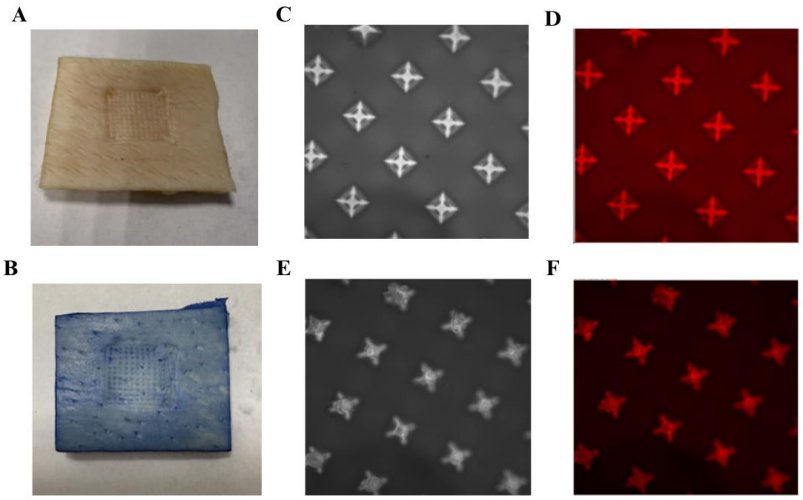

Figure 5. (A) Image of pig skin after microneedle penetration. (B) Image of pig skin after microneedle penetration and soaked with 0.4% Trypan blue dye. (C) Image of microneedles before penetration of pig skin under normal lighting. (D) The same array of microneedles before penetration under red fluorescence. (E) Image of microneedles after penetration of pig skin under normal lighting. (F) The same array of microneedles after penetration under red fluorescence

3.5. Cell Viability Assessment Using CCK-8 Assay

After confirming that MNs were capable of successfully penetrating the skin, further testing was conducted to evaluate its biocompatibility and potential cytotoxic effects. Specifically, the experiment aimed to determine whether the microneedle contained any substances that could negatively impact cell viability. Human Umbilical Vein Endothelial Cells (HUVECs) were chosen for this test due to their relevance in vascular biology and their sensitivity to toxins, making them a suitable model for assessing cellular responses. The HUVEC cells were thawed from cryopreservation and prepared according to standard cell culture protocols. After defrosting, the cells were put in a 24-well plate containing DMEM and allowed to stabilize under optimal growth conditions in a CO_2 incubator. To assess cell viability, the cells were treated with the Cell Counting Kit-8 (CCK-8) solution, a commonly used reagent that provides a colorimetric readout correlating with cell metabolic activity. Nine wells of the plate were designated for the experiment. The wells were divided into three groups: the MN group with MN in the solution along with the HUVEC and CCK-8, the P-MN group with P-MN in the solution along with the HUVEC and CCK-8, and the control group containing only the HUVEC and the CCK-8 solution to serve as a baseline for comparison. The plate was analyzed using the VICTOR X4 2030 Multilabel Reader. The results indicated that the cell viability in the MN and P-MN groups was similar, demonstrating no significant differences between the two types of microneedles. However, both groups showed statistically significantly reduced viability compared to the control group, which was free from any microneedle treatment. This minor decrease could be attributed to the physical interaction or minor stress caused by the microneedle material, but it was

not substantial enough to suggest overt toxicity. However, the cell viability for all groups was over 85%, which signifies that the microneedle would be safe for medical use.

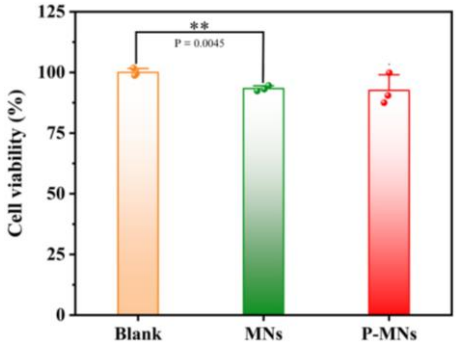

Figure 6. Microneedles without proteins and microneedles with proteins soaked in an HUVEC in comparison to a control HUVEC to test for cell viability

3.6. Cell Toxicity Assessment Using Flow Cytometry

Since the microneedles (MNs) were able to successfully penetrate the skin without exhibiting any hazardous effects, a subsequent experiment was conducted to verify whether the protein-loaded microneedles (P-MNs) could effectively deliver their proteins OVA and BSA into Human Umbilical Vein Endothelial Cells (HUVECs). For this purpose, flow cytometry was used, a sophisticated technique that allows for the detailed analysis of cells' physical and chemical properties as they flow in a single-cell suspension through a laser beam. In this experiment, flow cytometry was specifically used to quantify the uptake of two fluorescently labeled molecules, FITC-NHS and Cy5-NHS, within the HUVECs. These fluorescent labels were put into the P-MNs, enabling precise detection and measurement of protein delivery into the cells. HUVECs were exposed to either MNs, P-MNs, or nothing in a DMEM culture, followed by incubation to allow for protein release and cellular uptake. The treated cells were then analyzed using flow cytometry, where fluorescence intensity served as an indicator of the amount of FITC- or Cy5-labeled protein present in the cells. As depicted in Figure 7, the results demonstrated that the P-MNs were effective in transferring proteins into the HUVECs. Approximately 40% of the cells showed fluorescence corresponding to FITC-NHS, and about 34% exhibited fluorescence for Cy5-NHS, confirming successful protein delivery. In contrast, the untreated control group and cells exposed to regular MNs exhibited no detectable fluorescence, as expected, since no protein transfer occurred in these groups. There was a very significant difference between the P-MNs and the control and MNs. These findings illustrate the capability of P-MNs to deliver two therapeutic proteins, BSA and OVA, into target cells with measurable efficiency.

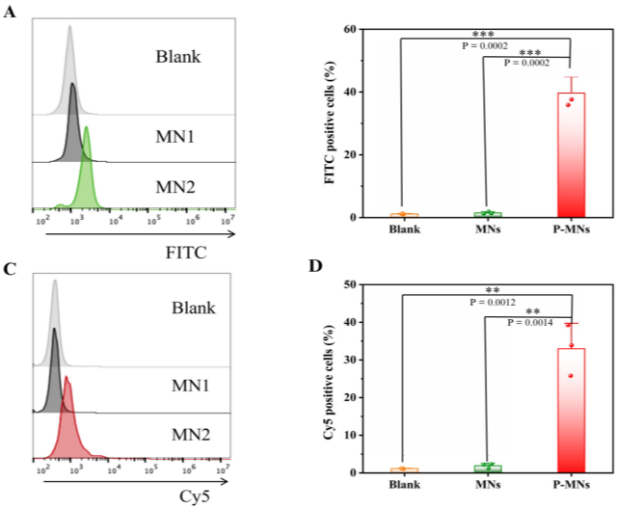

Figure 7. Flow cytometry images of HUVEC with FITC and Cy5 dyes in a control group of cells, microneedles without proteins, and microneedles with proteins (BSA and OVA)

4. Conclusion

In summary, soluble MNs were successfully synthesized using HA with different molecular weights and were discovered to be optimal for transdermal drug delivery. The fabrication process demonstrated precise control over the geometry pyramid shape of MNs, ensuring uniformity and sharpness which is essential for efficient skin penetration. SEM imaging confirmed the structural integrity and sharpness of the MNs, while fluorescence microscopy verified the successful incorporation and distribution of proteins within the MNs. The MN's ability to penetrate the pig skin and deliver proteins as seen by its' dissolving inside the pig skin demonstrates that MNs are soluble, biodegradable, and promising to non-invasive drug delivery systems. Additionally, the incorporation of both proteins BSA and OVA into the MNs, labeled with fluorescent dyes FITC-NHS and Cy5-NHS, facilitated visualization of protein delivery. P-MNs exhibited effective protein delivery to HUVEC, as confirmed by the flow cytometry analysis, highlighting its potential for targeted therapeutic applications. Furthermore, biocompatibility testing using the CCK-8 assay revealed no significant cytotoxic effects, indicating that MNs and P-MNs are safe for potential clinical use.

These findings pave the way for the development of microneedle-based delivery platforms for a wide range of applications, including vaccines and therapeutic proteins. The ability to incorporate multiple agents and control their spatial distribution within the microneedles further enhances the versatility of MNs for drug delivery. Future studies should focus on optimizing the stability, release, and mechanical properties of MNs to improve their efficacy and practicality. Moreover, scaling up production methods and conducting many preclinical and clinical trials will be crucial to ensure their realistic translation into human clinical applications. As research progresses, microneedles have the potential to revolutionize non-invasive drug delivery, providing a safer, more efficient, and patient-friendly alternative to traditional methods.

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