Proceedings of ICEGEE 2026 Symposium: Drugs Analysis and Biomedicine Applications
DOI: 10.54254/2753-8818/2026.KA33838

Soluble Hyaluronic Acid Microneedles Loaded with Methotrexate for Inflammatory Responses Modulation

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Abstract. In the treatment of inflammatory diseases, systemic administration has drawbacks such as severe side effects and poor targeting. Microneedle-based local drug delivery is an ideal alternative, but existing materials have deficiencies in biocompatibility and degradability. In this study, biodegradable microneedles were prepared using hyaluronic acid (HA) with different molecular weights, loaded with methotrexate (HA-MTX microneedles). HA-MTX microneedles has a regular pyramidal structure, which can effectively penetrate pig skin and degrade to release drugs. Fluorescence imaging showed uniform drug distribution. CCK-8 assay confirmed no toxicity to HUVECs. Flow cytometry and RT-qPCR indicated that HA-MTX microneedles can promote the polarization of macrophages from M1 to M2 and reduce the expression of pro-inflammatory factors, providing a safe and efficient new tool for inflammatory treatment.

Keywords: microneedle, inflammatory response, hyaluronic acid, drug delivery, soluble polymer

1. Introduction

Inflammatory diseases are a group of inflammatory response syndromes caused by immune imbalance, infection, or tissue damage, including rheumatoid arthritis, psoriasis, ankylosing spondylitis, and other types [1]. Their core pathological mechanism lies in the overactivation of the immune system, leading to the massive secretion of pro-inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which cause local tissue redness, swelling, pain, and dysfunction. Long-term progression can result in irreversible damage such as joint deformity and organ fibrosis, seriously reducing patients' quality of life [2]. Statistics show that there are over 23 million rheumatoid arthritis patients worldwide, and such diseases have become a significant burden on public health.

Current clinical treatments mainly rely on systemic administration, including oral non-steroidal anti-inflammatory drugs and intravenous injection of immunosuppressants. However, this model has significant drawbacks: first, large doses of drugs are required to achieve effective local concentrations, which easily cause systemic side effects such as gastrointestinal ulcers and liver and kidney function damage; second, when drugs reach the inflammatory site through the bloodstream, their bioavailability is low due to metabolic loss, resulting in delayed onset, making it difficult to quickly control acute inflammation; third, about 10% of adults and 33%-66% of children suffer from

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trypanophobia, which causes strong resistance to injection therapy, leading to decreased treatment compliance. These issues highlight the urgency of developing local targeted drug delivery systems.

Methotrexate (MTX) is a commonly used anti-inflammatory drug in clinical practice [3]. It blocks purine synthesis by inhibiting dihydrofolate reductase, thereby inhibiting T lymphocyte activation and pro-inflammatory factor secretion, with clear efficacy in the treatment of autoimmune diseases such as rheumatoid arthritis. Compared with other anti-inflammatory drugs, MTX has both immunosuppressive and anti-inflammatory effects, and its low cost makes it suitable for local drug delivery scenarios [4]. However, its systemic administration can easily cause side effects such as bone marrow suppression and mucosal damage, which can be avoided by local delivery [5].

As a new transdermal drug delivery tool, microneedles consist of micron-scale needle arrays, which can penetrate the stratum corneum (the outermost barrier of the epidermis) and deliver drugs directly to the superficial dermis, combining the advantages of "efficient drug delivery" and "painless safety" [6]. Compared with traditional injections, they avoid stimulation of nerve endings and blood vessels, significantly reducing the risk of pain and infection; at the same time, the local drug concentration is high, reducing systemic exposure and side effects [7]. Microneedles can achieve "precision drug delivery", and soluble microneedles have no sharp waste residues, with better safety [8]. However, existing microneedles mostly use synthetic polymers, which have problems such as mismatched degradation rate and drug release, and strong irritation of degradation products. Some non-degradable microneedles may also cause secondary skin damage, limiting their clinical application [9, 10].

Based on the above background, this study integrates the advantages of hyaluronic acid (HA) and microneedle technology to design biodegradable microneedles loaded with MTX (HA-MTX microneedles). As a natural glycosaminoglycan, HA is a natural component of the skin and connective tissues, with excellent biocompatibility, biodegradability, and hydration capacity. HA is suitable for preparing soluble microneedles and can avoid damage to drugs caused by high-temperature processing. In this study, HA with different molecular weights was mixed to regulate the mechanical properties and degradation rate of microneedles, making them match the skin characteristics; at the same time, MTX was loaded to achieve local controlled release, aiming to solve the side effects and compliance problems of existing treatments and provide a new therapeutic strategy for inflammatory diseases.

2. Experiment

2.1. Materials

Sodium Hyaluronate 1.5 - 1.6 million Da, Sodium Hyaluronate 10k Da, Sodium Hyaluronate 50k-100k Da, Sodium Hyaluronate 200k-400k Da, were purchased from Sigma-Aldrich.

Taipan Blue dye, Cy5-NHS, DMEM medium, Fetal bovine serum (FBS), streptomycin and penicillin, CCK-8, were bought from Gibco.

PDMS mold (Polydimethylsiloxane),methotrexate, human umbilical vein endothelial cells (HUVEC) were purchased from Beyotime.

2.2. Synthesis of HA microneedles

An electronic analytical balance weighted 2.0g of sodium hyaluronate 1.5-1.6million Da, 1.0g of sodium hyaluronate 10k Da, 1.0g of sodium hyaluronate 50k-100k Da, 1.0g of sodium hyaluronate 200k-400k Da. The four weighted sodium hyaluronate were combined with 100ml of distilled water

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to create a 5% sodium hyaluronate solution. This solution was stirred by a small megaton put inside the solution, and set overnight on the magnetic stirrer machine to mix the sodium hyaluronate and water completely.

Afterward, the PDMS mold was placed in a Petri dish and filled with the mixed solution. Then, the Petri dish was placed in a vacuum pump for 3 minutes to ensure all air was pulled out. The mold was then removed and placed in a drying oven overnight.

2.3. HA microneedles drug loading

50mg of methotrexate was measured using an electric analytical balance. Then, 10ml of 5% HA solution was put into a tuber, and 50mg of methotrexate was added into the tuber. Afterwards, methotrexate mixture solution was put into the ultrasonic reactor. Take another tuber and fill it with 10ml of 5% HA solution and mix it up with 2mg of Cy5-NHS. After taking out the methotrexate mixture solution out of the ultrasonic reactor, put 8 PDMS molds in 2 Petri dishes. Fill 4 PDMS molds with methotrexate mixture solution, and the other 4 with 5% Ha + 2mg Cy5-NHS mixture. Use the vacuum pump to suck out the air bubbles of all eight molds and put them into the drying oven at 37 Celsius for 24 hours.

2.4. Soluble HA-MTX microneedles testing

10 mL of water and 30 mL of Ethanol was mixed to create a 75% ethanol. Soak the pig skin into the 75% ethanol for 5 minutes and rinse the pig skin afterwards. Cut the pig skin into 1 cm by 1 cm pieces and pierce it with the micro-needle. Use the pipette to transfer taipan blue dye into a tube filled with distilled water to make a 4% taipan blue staining solution. Then, soak the pierced pig skin into the 4% taipan blue staining solution in a platelet.

2.5. SEM observation

To prepare the micro-needle for scanning electron microscopy (SEM) analysis, a clear micro-needle was removed from the Petri dish and placed onto a metal plate. This setup was then inserted into the EMITECH K550X gold coating machine for 4-5 minutes and 30 no mA, where the surface of the micro-needle was evenly coated with a thin layer of gold to enhance conductivity. Once coated, the micro-needle was transferred into the Hitachi S-4800 scanning electron microscope. This advanced machine allowed for a better imaging, enabling detailed observation of the micro-needle's tip.

2.6. CCK-8

Use the pipette to transfer raw 264.7 cell into the centrifuge tube and centrifuge the cells at 1000 rpm for 4 minutes. Then, pour out the liquid and only preserve the sediments. Transfer culture media into the tube that contain the cells using a pipette, and use the pipette to suspend the cells. Afterwards, put 100 μL of cell suspensions into the 96-well plates for 3 groups. Incubate the cells overnight, and add micro-needle pieces into the 96-well plate after 24 hours. Finally, conduct the Cck-8 assay by using the Microplant reader.

2.7. Flow cytometry trials

Use the pipette to transfer macrophage cell into the centrifuge tube and centrifuge the cells at 1000 rpm for 4 minutes. After 4 minutes, pour out the liquid and only preserve the sedimented cells.

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Transfer culture media into the tube that contain the cells using a pipette, and use the pipette to suspend the cells. Afterwards, put cell suspensions into the centrifuge tube. Make sure that each tube has over 10 thousand cells. Finally, analyze the cells using Flowcytometer.

2.8. Fluorescent HA-MTX microneedles testing

Activate the fluorescent microscope and place a clear micro-needle in it to take pictures without fluorescent light. Take pictures of the clear micro-needle on a side way view. Then, place the colored micro-needle in the microscope to take pictures on a side way view. After that, open the green fluorescent light and take pictures of the colored micro-needle on a front view and a side view. Repeat the same procedure for the colored micro-needle using red fluorescent light.

3. Results and discussion

3.1. Synthesis of soluble MN-MTX

The successful synthesis of soluble HA-MTX microneedle depends on the reasonable ratio and molding process of hyaluronic acid (HA). Four molecular weights of HA were mixed and prepared a 5% HA solution, which was magnetically stirred overnight to ensure complete dissolution. The design of this ratio is based on the influence of HA molecular weight on microneedle properties. High molecular weight (1.5-1.6 mDa) HA can provide mechanical strength to ensure sharp needle tips for skin penetration,while low molecular weight HA (10 kDa) promotes degradation, facilitating drug release, which is consistent with the design to regulate microneedle mechanical and dissolution properties [11].

After injecting the HA solution into the PDMS mold, vacuum defoaming was performed for 3 minutes to remove air bubbles (to avoid pores in the microneedles after molding), followed by overnight drying and solidification in a drying oven. The demolded HA-MTX microneedle was transparent with a complete array structure (Figure 1A). Optical images showed in Figure 1B, ensuring uniform drug delivery area. This synthesis process is stable and controllable.

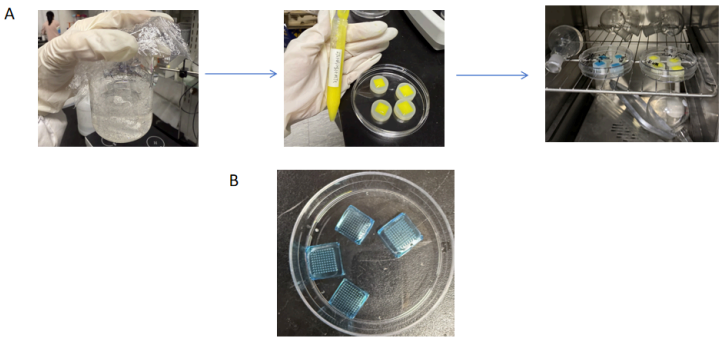

Figure 1. Synthesis of microneedle. A) Process of creating microneedles from hyaluronic acid. B) pictures of the microneedle

3.2. Characterization of soluble HA-MTX microneedle

The structural characterization of HA-MTX microneedle is crucial for evaluating its drug delivery potential. Scanning electron microscopy (SEM) observation showed that under 40×, 60×, and 220× magnifications, the microneedles had a regular pyramidal structure with sharp tips and neatly

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arranged arrays (Figures 2A-C). The advantages of this structural can disperse the puncture force reducing skin damage and ensures the efficiency of penetrating the stratum corneum. Under high magnification, the microneedle surface was smooth with no obvious cracks, indicating that the HA solution was uniformly mixed, and no structural defects were generated due to stress during the drying process, ensuring mechanical integrity.

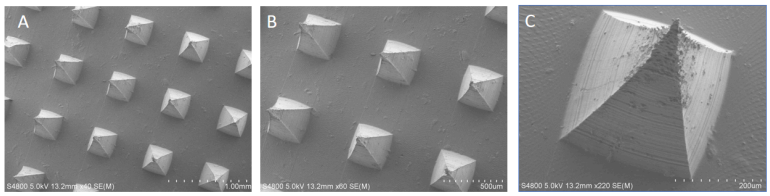

Figure 2. SEM images of micro-needles containing methotrexate microneedles at A) 40x, B) 60x and C) 220x different magnifications

Figure 3A is a micro-needle under a bright field microscope at a birds-eye view. Fluorescence microscopy analysis further verified the drug loading effect. Using Cy5-NHS to label MTX, fluorescence images showed that green fluorescence was uniformly distributed in the microneedles (Figures 3B, D), indicating good compatibility between MTX and HA matrix without aggregation. Side observation showed that the microneedles had triangular lateral faces (Figures 3C, D), which increased the drug loading space and facilitated force conduction during puncture.

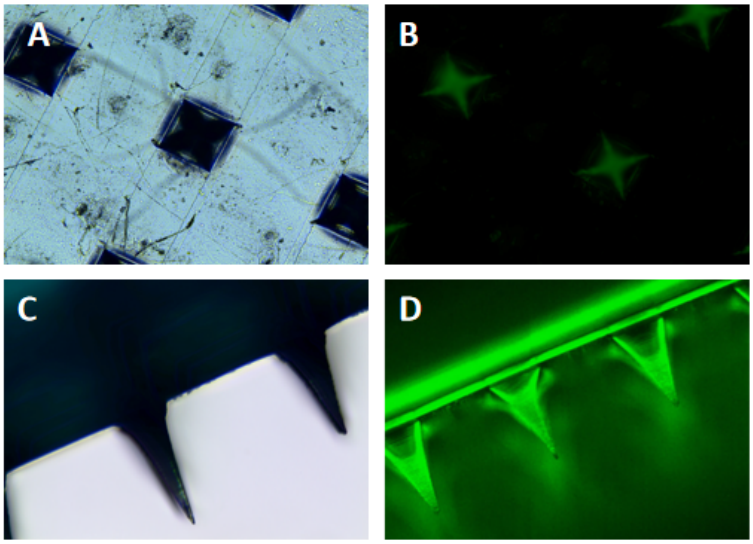

Figure 3. A) Image of microneedles under a brightfield microscope, birds-eye view. B) Fluorescent microscope showcases the same array of microneedles under green fluorescence. C) Image of microneedles under a brightfield microscope, sideways view. D) Sideways view of microneedles under green fluorescence

3.3. Pig skin penetration of HA-MTX microneedle

Pig skin is often used as a model for skin penetration experiments because its structure is similar to human skin (both containing stratum corneum and dermis). Figure 4A and 4B shows 2 images of micro-needle before penetration,having sharp and intense needle tips. After HA-MTX microneedle punctured pig skin for 1 minute, clear needle tip marks were visible (Figure 4C). After staining with

DOI: 10.54254/2753-8818/2026.KA33838

0.4% Taipan blue, the punctured area showed blue spots (Figure 4D), indicating that the microneedles successfully penetrated the stratum corneum, confirming that MN-MTX has effective skin penetration ability. The morphological changes of microneedles after penetration revealed their degradation characteristics. Figure 4E showed blunted needle tips, and the green fluorescence intensity weakened in Figure 4F, indicating that the microneedles began to dissolve in the moist skin environment, and MTX was released with matrix degradation.

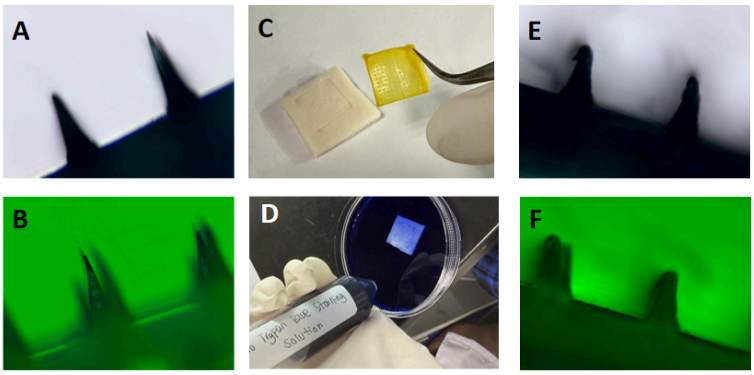

Figure 4. A) Image of MN before penetration of pig skin under normal lighting. B) Same array of MN before penetration under red fluorescence. C) Image of pig skin after MN penetration. D) Image of pig skin after MN penetration and soaked with 0.4% Taipan blue dye. E) Image of MN after penetration of pig skin under normal lighting. F) Same array of MN after penetration under red fluorescence

3.4. Cell viability assessment of HA-MTX microneedle

Cell viability assessment is the core of verifying the biocompatibility of microneedles. The CCK-8 method was used to detect the viability of HUVECs co-cultured with HA-MTX. The results showed that the cell viability in the MN-MTX group was >90%, with no significant difference from the control group (no treatment) ($P>0.05$), and the viability of the MN group without MTX (only HA microneedles) was similar (Figure 5). This indicates that neither HA matrix nor MTX loading caused obvious cytotoxicity.

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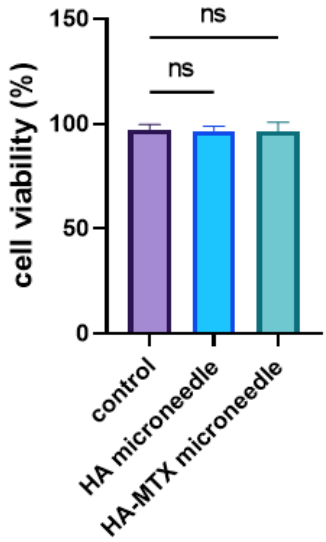

Figure 5. Microneedles without methotrexate and mirconeedles with methotrexate soaked in an HUVEC in comparison to PBS to test for HUVEC cell viability

3.5. Anti-inflammation assessment of HA-MTX microneedle

The anti-inflammatory efficacy of HA-MTX microneedle was systematically evaluated through a combination of cellular phenotypic analysis and molecular level detection, with a focus on macrophage polarization and pro-inflammatory factor expression, which are key indicators of inflammatory response regulation. Macrophages, as core immune cells in the inflammatory microenvironment, exhibit functional plasticity. M1-type macrophages secrete a large number of pro-inflammatory factors (such as TNF-α, iNOS) to promote inflammatory responses, while M2-type macrophages are mainly involved in anti-inflammatory and tissue repair processes. In this study, lipopolysaccharide was used to induce macrophages into M1 pro-inflammatory phenotype, simulating the pathological state of inflammatory diseases, and then the regulatory effect of HA-MTX microneedles on macrophage polarization was observed. Flow cytometry was used to detect the expression of surface markers. CD86 was selected as the specific marker of M1 type, and CD206 as the specific marker of M2 type, which is consistent with the common phenotypic identification method in inflammatory research. The results showed that in the LPS-stimulated control group, the proportion of M1-type macrophages was as high as 85.1%, while the proportion of M2-type was only 8.37%, indicating that the inflammatory model was successfully constructed (Figure 6).

After treatment with HA microneedles without MTX, the proportion of M2-type macrophages increased slightly to 14.5%, which may be related to the inherent anti-inflammatory properties of HA, as a natural component of the extracellular matrix, can weakly regulate immune responses. The HA-MTX microneedles treatment group showed a significant shift in macrophage polarization. The proportion of M1-type macrophages decreased from 85.1% to 67.5%, while the proportion of M2-type increased significantly to 41.3%. This significant change indicates that MTX loaded in microneedles plays a key role in regulating macrophage phenotype. This is consistent with the known anti-inflammatory mechanism of MTX, and the local delivery mode of microneedles ensures that MTX reaches the inflammatory site in high concentration, enhancing its regulatory effect on macrophages.

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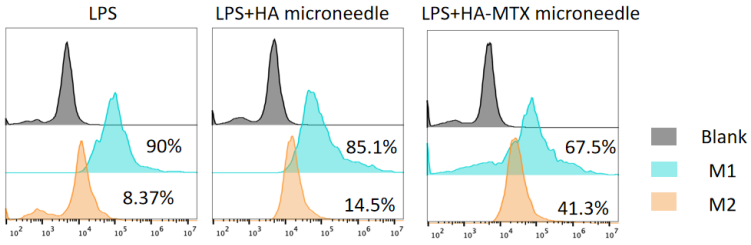

Figure 6. Flowcytometry images of macrophage with CD86 and CD206 dyes in a control group of cells, microneedles without methotrexate, and microneedles with methotrexate

To further verify the anti-inflammatory effect at the molecular level, RT-qPCR was used to detect the mRNA expression levels of key pro-inflammatory factors iNOS and TNF-α. The results showed that in the LPS group, the expression levels of iNOS and TNF-α were significantly up-regulated (Figure 7). After treatment with HA-MTX microneedles, the expression of iNOS and TNF-α were reduced compared with the LPS group. This result is consistent with the phenotypic changes of macrophages, confirming that HA-MTX microneedles can inhibit the expression of pro-inflammatory factors at the transcriptional level, thereby alleviating the inflammatory response. In addition, the comparison between the HA microneedles group and the HA-MTX microneedles group further confirmed the synergistic effect of MTX and HA. HA provides a safe and effective delivery platform for MTX, ensuring that MTX is released locally and stably, while MTX exerts specific anti-inflammatory effects.

In summary, the combination of flow cytometry and RT-qPCR results clearly demonstrates that HA-MTX microneedle can effectively regulate macrophage polarization and inhibit the expression of pro-inflammatory factors, exerting significant anti-inflammatory effects. This provides a solid experimental basis for the application of HA-MTX microneedles in the local treatment of inflammatory diseases.

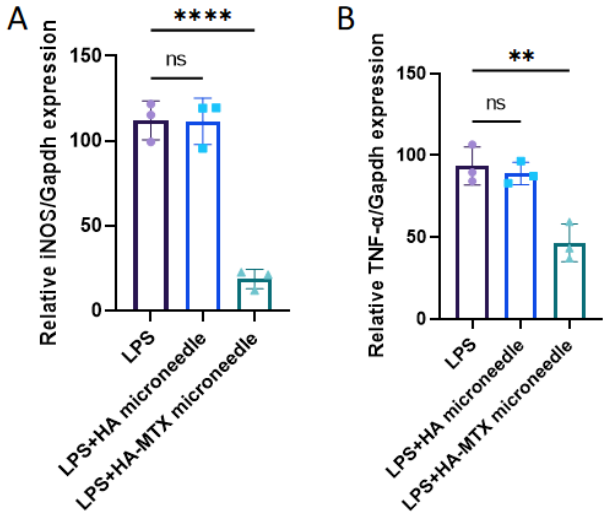

Figure 7. RT-qPCR images of macrophages with A) iNOS and B) TNF-α dyes in a control group of cells, microneedles without methotrexate, and microneedles with methotrexate

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4. Conclusion

This study successfully prepared biodegradable hyaluronic acid microneedles loaded with methotrexate (HA-MTX microneedles). The microneedles were synthesized using mixed molecular weight HA, with a regular pyramidal structure and sharp tips, which can effectively penetrate pig skin and gradually degrade to achieve uniform drug release. Fluorescent labeling confirmed uniform distribution of MTX. CCK-8 assay showed no significant toxicity to HUVECs, with good biocompatibility. Importantly, HA-MTX microneedles can significantly promote the polarization of macrophages from M1 pro-inflammatory phenotype to M2 anti-inflammatory phenotype and reduce the expression of pro-inflammatory factors, with clear anti-inflammatory efficacy. In conclusion, HA-MTX microneedles has both safety and effectiveness, providing a highly potential new tool for local targeted treatment of inflammatory diseases and is expected to promote the clinical transformation of microneedle drug delivery technology.

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