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Summary of Ph.D. Thesis

FORMULATION AND CHARACTERIZATION OF INNOVATIVE DERMAL FOAM SYSTEMS

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ABBREVIATIONS

AD-MSC	Human adipose-derived mesenchymal stem cell
ANOVA	Analysis of variance
CAM	Chorioallantoic membrane assay
CMA	Critical Material Attributes
CPP	Critical Process Parameters
CQA	Critical Quality Attributes
DEXP	Dexpanthenol
DMEM-HG	Dulbecco's Modified Eagle's Medium with high D-Glucose concentration
DS	Diclofenac sodium
FE	Foam expansion
FVS	Foam volume stability
HA	Hyaluronic acid
HA_{CL}	Cross-linked Hyaluronic acid
HA_{HMW}	High molecular weight Hyaluronic acid
HA_{LMW}	Low molecular weight Hyaluronic acid
HEC	Hydroxyethylcellulose
HET-CAM	Hen's Egg Chorioallantoic Membrane Test
HPMC	Hydroxypropyl methylcellulose
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IPA	Isopropanol
IS	Irritation score
IVPT	*In vitro* permeation test
IVRT	*In vitro* release test
logP	Logarithmic octanol-water partition coefficient
LVE	Viscoelastic range
NIAC	Niacinamide
PEG 200	Polyethylene glycol 200
QbD	Quality by Design
QTPP	Quality Target Product Profile
REM	Risk Estimation Matrix

RI	Relative intensity
RFD	Relative foam density
SDS	Sodium dodecyl sulfate
SLES	Sodium laureth sulfate
TPP	Target Product Profile
UHPLC	Ultra-high performance liquid chromatography
XANT	Xanthan gum

1. INTRODUCTION

A wide range of dermal formulations is available during the development of preparations. According to the classification of the 8th edition of the European Pharmacopoeia, within traditional forms, solid, semisolid, and liquid preparations are distinguished. In addition to traditional forms, the pharmaceutical industry has seen an increasing number of new, innovative forms, such as nanoemulsions, niosomes, film-forming systems, foams, etc. In the dermal field, foams have gained focus in recent years, especially in the treatment of burns and wound healing. They are being applied in several new areas, and environmentally friendly designs have become increasingly important. Consequently, propellant-containing systems are gradually being replaced by propellant-free systems.

Foam-forming systems offer significant potential, providing advantages to both the pharmaceutical and cosmetic industries. They provide easy and convenient application with precise dosage, allowing complete removal from the skin surface. Despite their numerous advantages, formulating these systems poses considerable challenges. The key consideration in composition design is ensuring the product to remain on the skin for a sufficient duration. Meeting user needs, the product should spread quickly and provide a pleasant skin feel.

There are limited studies on medicated foams, therefore understanding, developing, and investigating these systems can result in a formulation with significantly improved properties.

2. EXPERIMENTAL AIMS

This Ph.D. work aimed to design stable foam formulations and to determine the proper methods to investigate their physicochemical, biopharmaceutical and biocompatibility properties. Another goal was to examine the permeation of foams through the skin and gain a deeper understanding of the mechanism of their action.

The research was carried out in accordance with the following steps:

I. In the initial part of my Ph.D. work, stable foam compositions were formulated based on the Quality by Design (QbD) approach, and appropriate methods were developed to analyze their physicochemical, structural characteristics, and stability. In addition, the effect of different polymers on foam stability as well as on foam structure were investigated.

II. During the second part of my work, emphasis was placed on the formulation of a foam drug delivery system with diclofenac sodium and investigation of its biopharmaceutical properties. Furthermore, a comparative assessment of the research results was carried out with the foam bulk liquid and a conventional hydrogel, which falls within the category of traditional carrier systems.

III. In the subsequent part of my research, the biocompatibility of foam formulations was examined with *in vitro* cytotoxicity tests and an *in ovo* model.

The cytotoxicity of the components was assessed using human adipose-derived mesenchymal stem cells (AD-MSCs) and keratinocytes. Additionally, the formulations were evaluated for their effects on wound healing using two types of wound healing models involving AD-MSCs.

The *in ovo* model was employed to assess the irritation potential of the formulations.

3. MATERIALS AND METHODS

3.1. Materials

Polyoxyl castor oil was purchased from BASF SE Chemtrade GmbH (Ludwigshafen, Germany). Caprylocaproyl Polyoxyl-8 glycerides, obtained from Gattefossé (Saint-Priest Cedex, France), were provided by Azelis Hungary Ltd. (Budapest, Hungary). Xanthan gum (XANT) from CP Kelco A Huber Company (Atlanta, GA, USA) and the blend of Phenoxyethanol and Caprylyl Glycol were both provided as gifts by Biesterfeld Speciális Kemikáliák Magyarország Ltd. (Budapest, Hungary). Hydroxyethylcellulose (HEC) was acquired from Molar Chemicals Ltd. (Budapest, Hungary), and purified, deionized water from the Milli-Q system by Millipore (Milford, MA, USA). Additionally, HyaCare50 (HA_{LMW}), HyaCare Filler CL (HA_{CL}), and HyaCare Tremella (HA_{HMW}) were product samples from Finecon s.r.o. (Bratislava, Slovakia). Diclofenac sodium (DS) and fluorescein sodium were obtained from Sigma-Aldrich (Budapest, Hungary), Isopropanol (IPA) from Avantor (Radnor, PA, USA), Hydroxypropyl methylcellulose (HPMC) from Colorcon (Budapest, Hungary), Polyethylene glycol 200 (PEG 200) from Merck KGaA (Darmstadt, Germany), and the cellulose acetate filter from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Additionally, 70% Sodium laureth sulfate (SLES) and Niacinamide (NIAC) was provided by Biesterfeld Speciális Kemikáliák Magyarország Ltd. (Budapest, Hungary), and Dexpanthenol (DEXP) by DSM Nutritional Products Ltd. (Basel, Switzerland). For the chorioallantoic membrane assay, Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (Steinheim, Germany).

3.2. Methods of experiment part 1 – Formulation and characterization of the physicochemical properties of foams

3.2.1. The Quality by Design approach

The QbD methodology starts by establishing objectives and prioritizes understanding both the product and the process, focusing on process control through quality risk management.

The QbD approach includes identifying the target product profile (TPP), the quality target product profile (QTPP), critical material attributes (CMAs), critical process parameters (CPPs), and critical quality attributes (CQAs) of the product at the outset of development.

3.2.2. Preparation of foams

Firstly, various foam compositions, detailed in Table 1, were prepared. To extend the lifetime of foams, viscosity can be increased by incorporating polymers into the composition, which were present in varying concentrations (Phase B). Additionally, surfactants served as the main foaming agents (Phase A), with consistent amounts across all formulations, while Phase C included the microbiological preservative.

Table 1. Composition of different formulations ('+' indicates that the formulation contains the excipient, '−' indicates that the formulation does not contain the excipient).

	F-0polymer	F-XANT_0.1	F-XANT_0.2	F-HEC_0.2	F-HEC_0.4	F-HA_{LMW}_0.1	F-HA_{LMW}_0.2	F-HA_{HMW}_0.1	F-HA_{HMW}_0.2	F-HA_{CL}_0.1	F-HA_{CL}_0.2
Phase A											
Caprylocaproyl Polyoxyl-8 glycerides /surfactant/	+	+	+	+	+	+	+	+	+	+	+
Polyoxyl castor oil /surfactant/	+	+	+	+	+	+	+	+	+	+	+
Phase B											
Xanthan gum /polymer/	−	0.1%	0.2%	−	−	−	−	−	−	−	−
HEC /polymer/	−	−	−	0.2%	0.4%	−	−	−	−	−	−
HA_{LMW} /polymer/	−	−	−	−	−	0.1%	0.2%	−	−	−	−
HA_{HMW} /polymer/	−	−	−	−	−	−	−	0.1%	0.2%	−	−
HA_{CL} /polymer/	−	−	−	−	−	−	−	−	−	0.1%	0.2%
Purified water /solvent/	+	+	+	+	+	+	+	+	+	+	+
Phase C											
Blend of Phenoxyethanol and Caprylyl Glycol /preservative/	+	+	+	+	+	+	+	+	+	+	+

3.2.3. Characterization of the critical parameters of the foams

3.2.3.1. Macroscopic characterization of foams

The macroscopic properties of the foams were determined using the cylinder method.

After stirring the bulk liquid for 5 minutes, the foam was poured into a glass measuring cylinder, and the initial and aged volumes of the foam after 30 minutes were recorded. The parameters assessed include relative foam density (RFD), foam expansion (FE), and foam volume stability (FVS), calculated using specific formulas.

3.2.3.2. Microscopic characterization of foam kinetics and bubble morphology

Microscopic measurements were conducted using the Leica DM6 B Fully Automated Upright Microscope System (Leica Biosystems GmbH, Wetzlar, Germany). This method allows for the determination of foam structure and bubble size, providing valuable insights into foam kinetics. The kinetics of the samples can be observed through bubble size analysis. The number of bubbles was recorded at time points 0, 10, 20, and 30 minutes. Foam uniformity can also be determined with this method as the homogeneity of air bubbles.

The size, roundness, and the aspect ratio of incorporated air bubbles as well as bubble amount in a predetermined area are the parameters of interest in foam characterization.

3.2.3.3. Rheological investigations

The rheological properties of the foams were investigated using an Anton Paar Physica MCR302 Rheometer (Anton Paar, Graz, Austria) employing a parallel plate type measuring device (diameter: 50 mm, gap height: 2 mm). Flow curves of the bulk liquids were recorded with a cone-plate type measuring device (diameter: 25 mm, gap height: 0.1 mm), over the shear rate range from 0.1 to 100 1/s and from 100 to 0.1 1/s at 25°C. The analysis of the foams involved amplitude sweeps, wherein the strain value increased from 0.1% to 100%, with an angular frequency of 10 rad/s.

3.2.3.4. Assessment of spreadability

The spreadability of foams was evaluated using a TA.XT plus Texture Analyzer (Stable Micro Systems Ltd., UK) equipped with a TTC Spreadability Rig, consisting of a male 90° cone probe and a precisely matched female perspex cone-shaped product holder.

The spreadability was determined by measuring the force required for the product to flow outward at 45° between the male and female cone surfaces, with the maximum force (firmness) recorded in the force-distance curve, modeling the application of semisolid dermal dosage forms.

3.3. Methods of experiment part 2 - Biopharmaceutical analysis of a diclofenac sodium-containing foam drug delivery system compared to foam bulk liquid and hydrogel

3.3.1. Preparation of the formulations

In this part, properties of the foam formula were compared to the foam bulk liquid (which is a polymer solution) and to a conventional hydrogel. Regarding the formulations, both the foam/bulk liquid and hydrogel shared identical concentrations of non-ionic emulsifiers and preservatives. The variations were observed in solvent types, polymer types, and concentrations.

The foams used in the experiments were generated using a propellant-free foam pump. The detailed compositions are provided in Table 2.

Table 2. The composition of foam, hydrogel, and bulk liquid ('–' indicates that the formulation does not contain the excipient).

	Hydrogel	Foam/Foam bulk liquid
DS (%) /active ingredient/	1	1
PEG 200 (%) /solvent/	3.5	–
IPA (%) /solvent/	15	–
HPMC (%) /polymer/	3	–
Xanthan gum (%) /polymer/	–	0.2
Caprylocaproyl Polyoxyl-8 glycerides (%) /surfactant/	2	2
Polyoxyl castor oil (%) /surfactant/	2	2
Blend of Phenoxyethanol and Caprylyl Glycol (%) /preservative/	0.5	0.5
Purified water (%) /solvent/	up to 100	up to 100

3.3.2. Preformulation test of foam formula: Investigation of *ex vivo* permeation through the skin using fluorescent microscope

Fluorescent microscopy was utilized to model the permeation of the foam formulation through the *stratum corneum*, including the assessment of permeation capacity of the blank formulation (without the active ingredient).

Experiments employed *ex vivo* human skin samples obtained from a Caucasian female patient after routine plastic surgery (Ethical Permission: BMEÜ/2339-3/2022/EKU).

After plastic surgery, the skin underwent a gentle cleansing process and was stored at −20 °C for a maximum of 6 months before use.

Fluorescein sodium dye visualized the permeation process on full-thickness abdominal skin samples, with observation times of 10- and 30-minutes following application of each formulation. Skin sections were analyzed using a Leica light microscope (LEICA DM6 B, Leica Microsystems GmbH, Wetzlar, Germany) with a red fluorescence filter, capturing images for comparison with untreated and SLES-treated skin controls. Image analysis with ImageJ software determined relative intensity, indicating the extent of color intensity increase compared to untreated skin.

3.3.3. Rheological properties of the foam formula compared to bulk liquid and hydrogel

The viscosity of the bulk liquid, foam, and hydrogel was evaluated using an Anton Paar Physica MCR302 Rheometer (Anton Paar, Graz, Austria) equipped with a cone-plate type measuring device. Measurements were performed at 25°C, and the RheoCompass software facilitated viscosity calculations at a shear rate of 50 1/s, with flow curves generated over a shear rate range from 0.1 to 100 1/s based on three simultaneous measurements.

3.3.4. Biopharmaceutical investigation of the foam formula compared to bulk liquid and hydrogel

3.3.4.1. *In vitro* drug release and permeation tests (IVRT and IVPT) using Franz Diffusion Cell System

Drug release from the bulk liquid, foam, and hydrogel through a synthetic membrane, as well as its permeation through human heat-separated epidermis, were studied using the Vertical Franz diffusion cell system (Hanson Microette TM Topical & Trans-dermal Diffusion Cell System, Hanson Research Corporation, Chatsworth, CA USA). The excised human skin, obtained through plastic surgery, was used similarly to the fluorescent microscope method.

The released and permeated drug concentrations were measured using ultra-high performance liquid chromatography (UHPLC) with a Shimadzu Nexera X2 UHPLC system.

The *in vitro* permeation of diclofenac sodium through the epidermis was calculated based on the cumulative amount, considering the diffusion area, with findings plotted over time to determine the steady-state flux (J), over an incubation period ranging from 1 to 6 hours.

3.3.4.2. Investigation of *ex vivo* drug permeation using Raman Spectroscopy

The confocal Raman spectroscopy can be employed to investigate topical formulations, for both determining permeation and permeation depth.

In my research, Raman microscopy was utilized to capture images depicting the spatial distribution of diclofenac sodium within *ex vivo* human skin. The spectra of diclofenac sodium in the bulk liquid and hydrogel were employed as a basis for comparing treated and untreated skin samples.

Data collection and analysis were carried out using the Dispersive Raman software package OMNICTM 8.2 (ThermoFisher Scientific Inc., Waltham, MA, USA).

3.3.4.3. Statistical analysis

The *in vitro* drug release test results were statistically analyzed using Prism 5.0 software for Windows 10 (GraphPad Software Inc., La Jolla, CA, USA) employing two-way ANOVA with Bonferroni's multiple comparison method, with data representing mean values from six experiments and standard deviations, where significant differences from the foam formulation were observed at $*p \leq 0.05$ and $***p \leq 0.001$ significance levels.

3.4. Methods of experiment part 3 - Assessment of biocompatibility and wound healing potential of foam components and formulations

3.4.1. Composition of the investigated formulations

For cytotoxicity tests, the components were individually dissolved in Dulbecco's Modified Eagle's Medium with high D-Glucose concentration (DMEM-HG) medium in a 100-fold dilution compared to the applied concentrations in the formulations.

The aim of the wound scratch and impedance-based assay was to investigate the effects of the polymers and active ingredients.

On the other hand, the *in ovo* assay targeted the examination of irritation of the promising materials. In these studies, formulations including F-0polymer, F-XANT_0.2, and F-HA_{HMW}_0.2, among the previously blank compositions, as well as formulations containing active ingredients, were also tested. Table 3.

During the experiments, the foam bulk liquids were examined, with pure DMEM-HG medium serving as the control.

Table 3. The composition of active ingredient-containing foam formulations ('–' indicates that the formulation does not contain the excipient).

	F-XANT_0.2-DS_1	F-XANT_0.2-HA_{HMW}_0.2-DEXP_1-NIAC_1	F-HA_{HMW}_0.2-NIAC_5	F-HA_{HMW}_0.2-DEXP_5	F-HA_{HMW}_0.2-DS_1
DS (%) /active ingredient/	1	–	–	–	1
DEXP (%) /active ingredient/	–	1	–	5	–
NIAC (%) /active ingredient/	–	1	5	–	–
HA_{HMW} (%) /polymer/	–	0.2	0.2	0.2	0.2
Xanthan gum (%) /polymer/	0.2	0.2	–	–	–
Caprylocaproyl Polyoxyl-8 glycerides (%) /surfactant/	2	2	2	2	2
Polyoxyl castor oil (%) /surfactant/	2	2	2	2	2
Blend of Phenoxyethanol and Caprylyl Glycol (%) /preservative/	0.5	0.5	0.5	0.5	0.5
Purified water (%) /solvent/	up to 100	up to 100	up to 100	up to 100	up to 100

3.4.2. Cell culturing

Abdominal human keratinocytes and AD-MSCs were isolated via enzymatic digestion and cultured in DMEM-HG supplemented with 10% Fetal Bovine Serum, 1% L-Glutamine, and 1% Antibiotic-antimycotic, followed by incubation at 37°C with 5% CO_2.

3.4.3. Cytotoxicity assay

The impact of the components on cell toxicity was evaluated through MTT assays following the manufacturer's instructions, where AD-MSCs and keratinocytes were seeded into 96-well plates at an initial density of 5×10^3 cells per well and exposed to solutions containing the components, in a 100-fold dilution compared to the applied concentrations.

3.4.4. Investigation of the effect of foam preparations on an *in vitro* wound healing model

3.4.4.1. Wound scratch assay with AD-MSCs

Under *in vitro* conditions, the wound-healing potential of AD-MSCs in response to dermal agents was assessed in 24-well plates, followed by creating uniform wounds on the cell layer using the AutoScratch wound-making device (Agilent/BioTek, Santa Clara, CA, USA). The healing process was observed over 48 hours using an Olympus IX83 (Olympus, Tokyo, Japan) microscope equipped with an Okolab incubation system, with wound area measurements taken at 1-hour intervals and compared to untreated controls for analysis.

3.4.4.2. Impedance-based measurement of wound healing

Under *in vitro* conditions, the wound-healing potential of AD-MSCs in response to dermal agents was investigated in E-Plate WOUND 96 (Agilent/BioTek, Santa Clara, CA, USA) plates and uniform wounds were created using the AccuWound 96 (Agilent/BioTek, Santa Clara, CA, USA) wound-making device. Wound healing was then assessed using the xCELLigence Real-Time Cell Analyzer (Agilent/BioTek, Santa Clara, CA, USA) over a 48-hour period, with impedance measurements recorded via golden electrodes to evaluate cellular responses, followed by data visualization and analysis.

3.4.5. Irritation evaluation by the HET-CAM assay

The potential toxicity on mucosal or skin tissues was assessed using the *in vivo* Hen's Egg Chorioallantoic Membrane Test (HET-CAM), which evaluates irritant effects on the vascular plexus and is adapted to laboratory conditions, following the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommendations, demonstrating its biocompatibility with applications in ophthalmology, cosmetology, and dermatology. Distilled water served as the negative control, whereas SDS at a concentration of 0.5% acted as the positive control.

The evaluation of potential irritative effects on vascularized CAM tissues involved noting the occurrence time, in seconds, of parameters such as hemorrhage (H), vascular lysis (L), and coagulation (C). An irritation score (IS) was then calculated using a specific equation. The IS values, based on Luepke's recommended irritation scale ranging from 0 to 21, classify irritation scores as follows: 0–0.9 (non-irritant), 1–4.9 (weak irritant), 5–8.9 (moderate irritant), and 9–21 (strong irritant).

3.4.6. Statistical analysis

The statistical analysis of the cytotoxicity assay and impedance-based wound healing measurement results was conducted using Prism 5.0 for Windows 10 software, employing the one-way ANOVA analysis of variance test (Dunnett's Multiple Comparison Test). Significant differences from the control were observed at the levels of $***p \leq 0.001$ vs. Control, $**p \leq 0.01$ vs. Control, $*p \leq 0.05$ vs. Control, with data representing the mean values from four (for cytotoxicity) and three (for wound healing) experiments, along with standard error of the means.

4. RESULTS AND DISCUSSION

4.1. Experiment part 1 - Formulation and characterization of the physicochemical properties of foams

4.1.1. Definition of QTPP, CQAs, CMAs, and CPPs for foam systems

Initially, the QTPP and the CQAs of both the bulk liquid and foam system were taken into account, followed by the CMAs and CPPs. Five critical CQAs, including foam volume stability, foam expansion, cross point, bubble size, and number of bubbles, were identified as highly influential attributes, while three moderately influencing CQAs, namely spreadability, foam density, and liquid system viscosity, were recognized during the development of foam formulations. These parameters underwent investigation throughout the research process.

4.1.2. Preformulation study of foams

Preformulation tests were conducted to optimize mixing time and speed crucial for achieving the desired foam consistency. Various stirring speeds (1000 rpm, 1500 rpm, 2000 rpm) and durations (5, 10, 15 min) were evaluated to attain a foam volume twice that of the liquid for testing. Results indicated that a suitable foam consistency for testing purposes was achieved at 2000 rpm for 5 minutes.

4.1.3. Characterization of the critical parameters

4.1.3.1. Macroscopic characterization of foams

Macroscopic analysis revealed that the polymer-free composition exhibited high foam expansion but poor foam stability, resulting in rapid breakdown of the foam structure. Similarly, formulations F-HEC_0.2, F-HA_{LMW}_0.1, and F-HA_{LMW}_0.2 demonstrated high foam expansion but low foam stability. F-HA_{CL} displayed low foam stability at both concentrations tested, with moderate foam expansion. In contrast, formulations F-XANT and F-HA_{HMW} exhibited high foam stability across both concentrations. Formulations with foam expansion exceeding 150% demonstrated optimal foaming characteristics. The most stable formulations were those with foam volume stability above 70%, notably F-XANT_0.2 and F-HA_{HMW}_0.2.

4.1.3.2. Microscopic characterization of foam kinetics and bubble morphology

The analysis of foam kinetic enabled the determination of foam stability, which showed the relationship between the number of bubbles and time. Foams with an initial bubble count exceeding 100 demonstrated microscopic stability, aligning well with results obtained from the macroscopic foam volume stability test using the cylinder method. These were F-XANT_0.1, F-XANT_0.2, F-HA_{LMW}_0.2, F-HA_{HMW}_0.1 and F-HA_{HMW}_0.2.

4.1.3.3. Rheological investigation of the foams (Oscillometric measurements)

Observations revealed two distinct amplitude sweep curves: one with a wider (linear viscoelastic) LVE range and higher elastic modulus, indicating stability, and another where G" dominates, signifying a lack of coherent foam structure. Some foam formulations behaved like liquids with higher G" than G' values in the LVE region, lacking flow points. F-0polymer foam immediately flowed and displayed its highest LVE range limit after 10 minutes due to liquid drainage.

F-XANT_0.1 and F-XANT_0.2 initially displayed better stability than polymer-free foam, but higher concentrations reduced stability after 30 minutes. Increasing polymer concentration in F-HEC_0.2 and F-HEC_0.4 resulted in decreased coherence over time, while F-HA_{LMW} formulations remained stable for 20 minutes and improved with higher polymer content. However, F-HA_{HMW}_0.1 showed enhanced coherence after 30 minutes, contrasting with cross-linked hyaluronic acid, which accelerated foam breakdown at higher polymer concentrations. F-XANT and F-HA_{HMW} systems exhibited promising long-term stability, consistent with FVS% values.

4.1.3.4. Rheological investigation: Flow and viscosity characteristics of foam bulk liquids

All polymer solutions (bulk liquids) typically exhibited shear-thinning behavior due to macromolecular alignment under shear, notably observed with xanthan gum and HEC-containing solutions where viscosity increased with concentration. Low molecular weight hyaluronic acid and cross-linked hyaluronic acid solutions showed similar rheological behavior to polymer-free solutions, while high molecular weight hyaluronic acid exhibited shear-thinning behavior akin to xanthan gum, alongside slight thixotropy.

4.1.3.5. Assessment of spreadability

Polymer-free foam exhibited poor spreadability and quick flow, while polymer content generally enhances firmness, preventing from flowing off the skin. Figure 1 illustrates that higher polymer concentrations require more force for spreading. High molecular weight hyaluronic acid foams demanded the most force, while xanthan gum and high molecular weight hyaluronic acid foams have met spreadability requirements, and correlated with macroscopic findings.

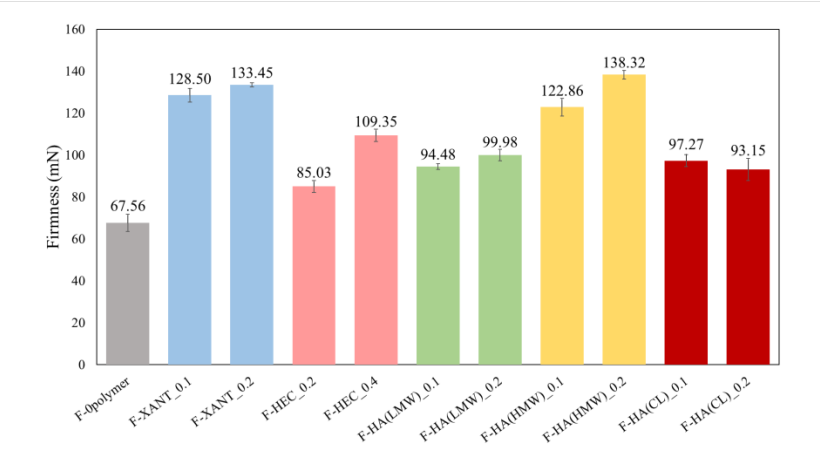

Figure 1. Firmness values of the investigated formulations.

4.1.4. Summary of Experiment 1

The physicochemical and mechanical properties of eleven compositions were analyzed based on an initial risk assessment, focusing on critical CQAs like foam volume stability, expansion, cross point, bubble size, and number, alongside medium-critical attributes such as spreadability, foam density, and liquid viscosity. Polymer concentration and type were identified as highly critical material parameters affecting CQAs, with significant influence on the mechanical properties of foams. Formulations like F-XANT_0.1, F-XANT_0.2, F-HA_{HMW}_0.1, and F-HA_{HMW}_0.2 showed promising foam properties, demonstrating strong correlations across various assessment methods. A protocol combining macroscopic stability assessment, microscopic kinetics examination, and oscillometric measurements offers a novel approach to identifying optimal foam formulations, representing an advancement over previous methodologies.

4.2. Experiment part 2 - Biopharmaceutical analysis of a diclofenac sodium-containing foam drug delivery system compared to foam bulk liquid and hydrogel

4.2.1. Preformulation study of foam formula: *Ex vivo* permeation through fluorescent microscope

The foam was compared with both negative and positive controls in the study, with the negative control involving the examination of untreated skin under a fluorescent microscope, and the positive control consisting of skin pretreatment with a solution containing SLES to enhance permeation. Results depicted a significant increase in light intensity following SLES pretreatment, with fluorescence intensity increasing by 7.81 times after 10 minutes and 6.41 times after 30 minutes compared to untreated skin.

Similarly, the foam exhibited a noticeable increase in fluorescence intensity over time, reaching a relative intensity (RI) comparable to the positive control after 30 minutes, suggesting deep permeation capabilities similarly to the positive control (Figure 2).

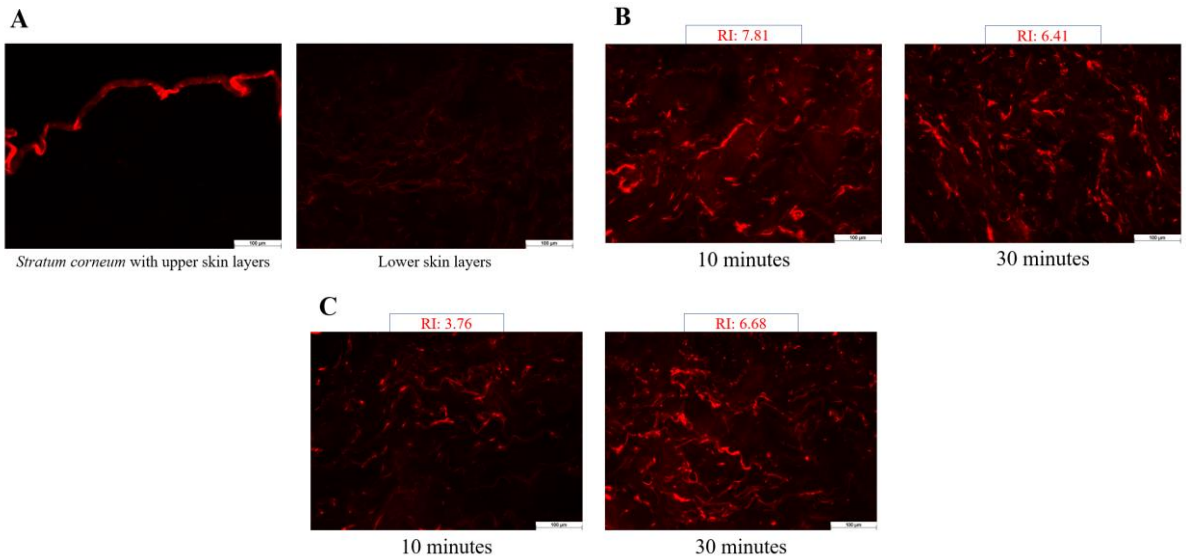

Figure 2. Fluorescent microscopic images of the negative control (A), the positive control (B), and the skin treated with the foam formulation (C).

4.2.2. Rheological properties of the foam formula compared to bulk liquid and hydrogel

The rheological measurements were employed to examine the consistency of the systems. The viscosities of both the initial bulk liquid and the residual liquid film after foam breakdown were analyzed and compared with those of a standard hydrogel formulation. Over time, the foam decayed into a liquid film due to binding forces and chain interactions, leading to a more organized network compared to the initial bulk liquid. This structured network increased the viscosity of the liquid film, resulting in decreased volume filling and higher density. Since the viscosity of the hydrogel increased by 6.5 times compared to the bulk liquid and liquid film, it may lead to a delay in skin permeation due to increased resistance to deformation.

4.2.3. Biopharmaceutical investigation of the foam formula compared to bulk liquid and hydrogel

4.2.3.1. *In vitro* drug release and permeation tests (IVRT and IVPT) using Franz Diffusion Cell System

The results indicated rapid release of diclofenac sodium from the foam, with about 80% released in 30 minutes, compared to approximately 5 hours for the hydrogel (Figure 3).

This rapid release from the foam may be attributed to its porous structure, facilitating easier dispersion of active ingredients and quicker delivery to the target site. While the release kinetics were slower in case of the bulk liquid and hydrogel, diclofenac sodium exhibited slightly faster diffusion from the bulk liquid. This could be due to the lower viscosity of polymer solutions, allowing for easier diffusion of active ingredients. Conversely, hydrogels, with their higher viscosity and more organized structure, may contribute slower release, potentially influenced by the more organized and interconnected structure typical of hydrogels.

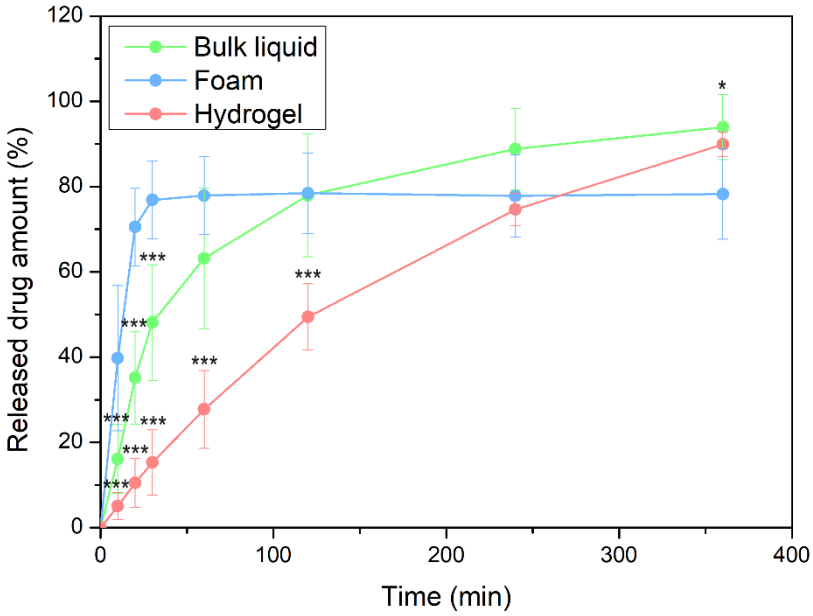

Figure 3. Release profile of diclofenac sodium in various dosage forms (***$p \leq 0.001$ vs. Foam, *$p \leq 0.05$ vs. Foam).

The data of the permeation rate (flux values) indicated that rapid drug release from the foam led to swift drug permeation. The liquid film formed after foam decaying may have become supersaturated, leading to faster permeation compared to hydrogel and bulk liquid formulations.

4.2.3.2. Investigation of *ex vivo* drug permeation using Raman spectroscopy

Ex vivo permeation of diclofenac sodium into human skin samples was investigated using Raman spectroscopy, with Figure 4 illustrating the qualitative distribution of the active ingredient following application of foam, bulk liquid, and hydrogel, where warmer colors on the maps indicate higher concentrations.

In the case of bulk liquid, diclofenac sodium was detectable in deeper skin layers within 10 minutes, becoming more pronounced by 30 minutes.

Conversely, in foam-treated skin sections, gradual foam decay led to liquid leakage between bubbles onto the skin after 10 minutes, creating a supersaturated layer.

Consequently, the increased diclofenac sodium presence in the foam became more prominent, with increased active substance concentrations observed between 20 to 30 minutes. In terms of the hydrogel, the findings suggested that diclofenac sodium permeated only into the outermost epidermal layer during the entire study period. Subsequently, after 1 hour, elevated concentrations were observed only in the upper part of the skin.

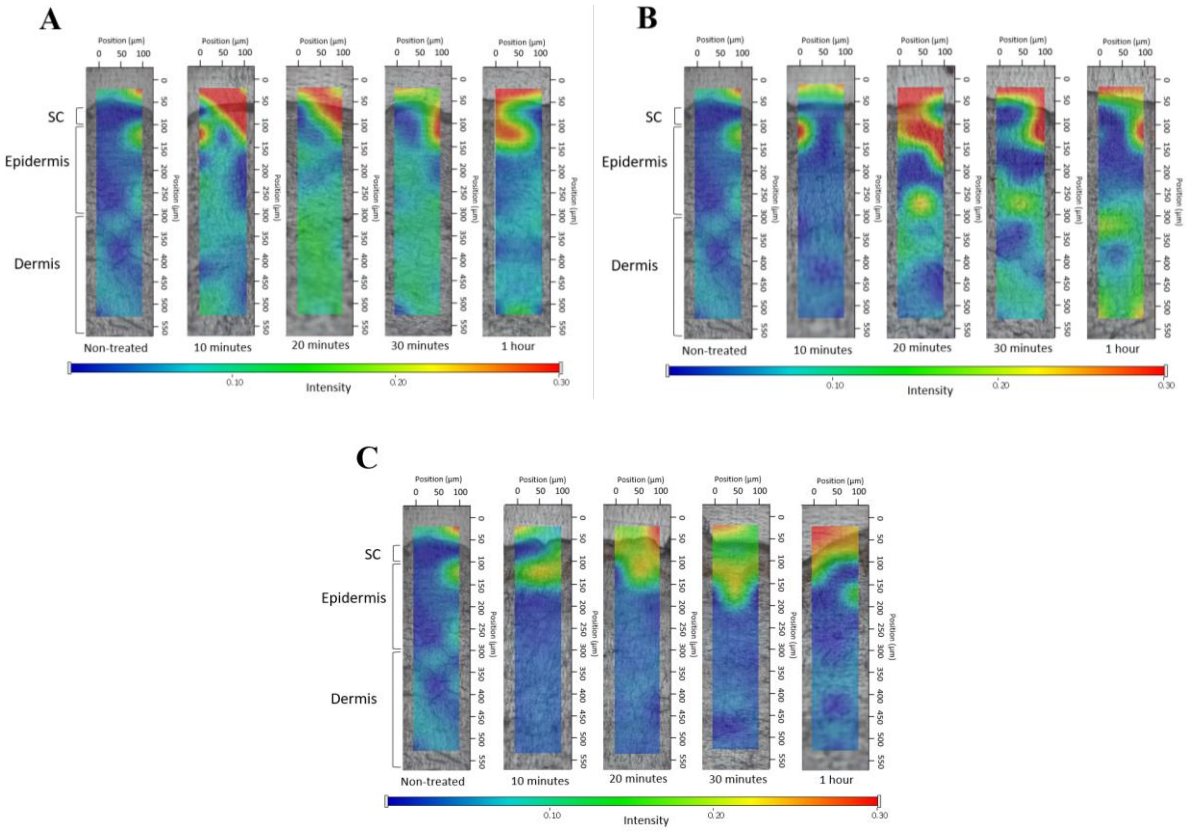

Figure 4. Raman correlation map of bulk liquid (A), foam (B), and hydrogel (C).

4.2.4. Summary of Experiment 2

In this part, the biopharmaceutical characteristics of foams with traditional hydrogels and polymer solutions were compared. Results showed that the foam exhibited faster drug release and deeper skin permeation compared to the hydrogel, with approximately 80% of the diclofenac sodium released within 30 minutes, while the hydrogel took about 5 hours to achieve the same level of release. The rapid release observed from the foam could be attributed to its porous structure, which aids in the efficient dispersion of active ingredients, leading to faster delivery to the target area.

Raman skin permeation studies indicated that the foam initially concentrated in the upper epidermal layers and gradually permeated deeper layers over time, with a supersaturated liquid film observed after 20 minutes. Conversely, the higher viscosity of the hydrogel hindered diclofenac sodium permeation into deeper skin layers even after 1 hour, as supported by Raman mapping.

4.3. Experiment part 3 - Assessment of biocompatibility and wound healing potential of foam components and formulations

In subsequent experiments, active ingredients such as diclofenac sodium, dexpanthenol, and niacinamide were incorporated into the foam formulations. Dexpanthenol, known for its role in wound healing, was included alongside diclofenac sodium, which acts as an anti-inflammatory agent, and niacinamide, contributing to the remodeling phase and also possessing anti-inflammatory properties. Additionally, xanthan gum and hyaluronic acid were utilized as polymers in the foam formulations, potentially offering benefits for minor wound and scar treatment due to their wound healing properties.

4.3.1. Cytotoxicity assay

The MTT assay evaluated the ingredients of the foam formulations (Figure 5), showing that both the polymers, niacinamide, and dexpanthenol had comparable effects on cell viability, with no impact observed when altering concentrations of dexpanthenol and niacinamide on mesenchymal stem cells. Results showed that, in the case of AD-MSCs, components slightly reduced cell viability (except Polyoxyl 40 castor oil), with diclofenac sodium having the most significant reducing effect; yet, all components maintained viability above 70%, adhering to ISO 10993-5 standards for non-cytotoxicity to mesenchymal stem cells.

In keratinocytes, all components, except diclofenac sodium and one non-ionic surfactant, increased cell viability, with Polyoxyl 40 castor oil showing higher viability than active agents and polymers. Because of the higher standard error of the mean values, no more significant differences could be observed in this study. Results, in this case, also met the ISO 10993-5 standard with all components reaching 70% cell viability.

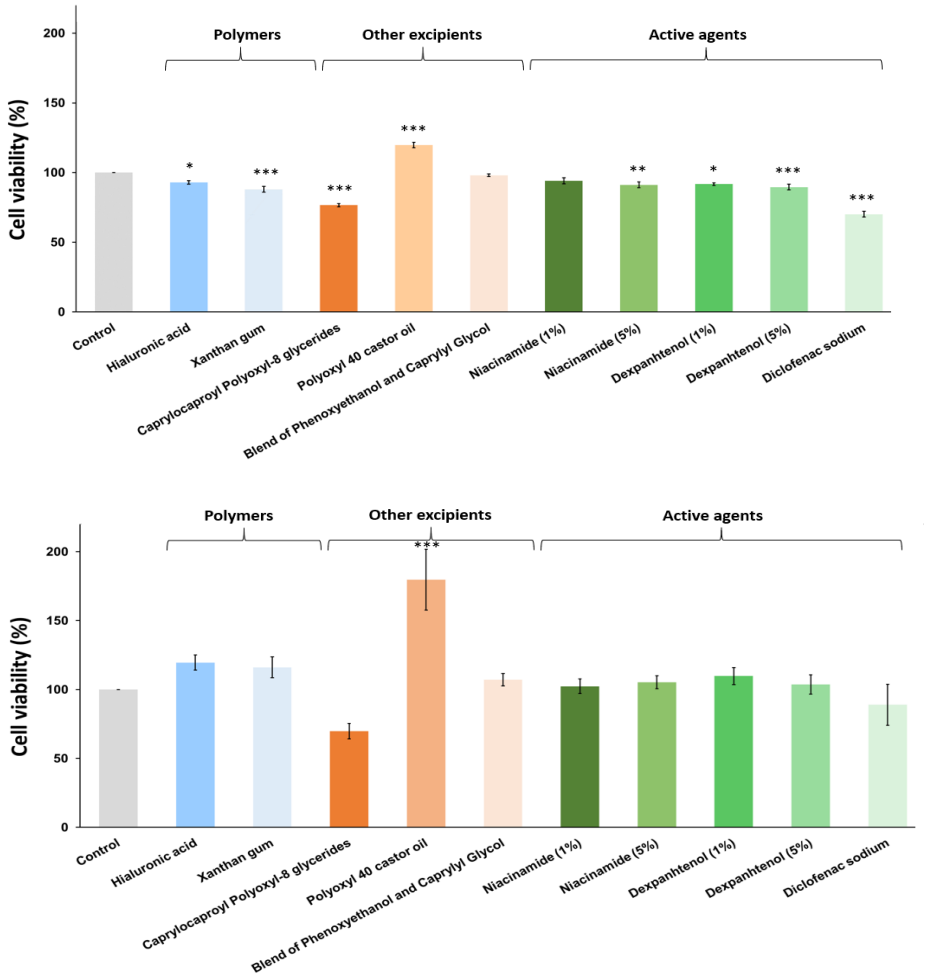

Figure 5. Effect of foam components on cell viability of AD-MSCs (above) and keratinocytes (bottom) (***$p \leq 0.001$ vs. Control, **$p \leq 0.01$ vs. Control, *$p \leq 0.05$ vs. Control).

4.3.2. Comparison of the wound scratch and impedance-based wound healing assays

In this part of the research, the effects of five foam formulae were examined on two widely used wound healing models. The wound scratch assay, a simple yet effective method, analyzes cell migration in two dimensions, while the impedance-based approach, gaining recent prominence, shows promise for three-dimensional proliferation assays.

Most wounds showed effective closure, except for one treated with diclofenac sodium-containing foam, indicating a potential inhibitory effect in the initial phase of wound healing. Previous research suggests that diclofenac has an inhibitory effect on stem cell functions, although in the right combination, it can accelerate wound healing.

According to impedance-based wound healing assay results, polymers like xanthan gum (43.05%) and hyaluronic acid (46.13%) improved cell migration compared to the polymer-free foam (30.9%), while diclofenac sodium-containing foam formulations exhibited a relatively low migration percentage (15.99%).

Although combining polymers with dexpanthenol and niacinamide slightly reduced migration, it still yielded higher values compared to polymer-free formulations, correlating with the results of the wound scratch assas.

4.3.3. Irritation evaluation by the HET-CAM assay

The wound scratch assay showed enhanced wound healing with hyaluronic acid, prompting further investigation of its effects *in ovo* both alone and in combination within foam formulations.

The irritative scores, based on 5-minute observations post-application, indicated no irritation in all samples compared to the positive control. However, after 30 minutes, only the negative control and the F-HA_{HMW}_0.2-DEXP_5 formulation showed no irritation in the measurement.

4.3.4. Summary of Experiment 3

The formulated foam systems offer advantages for maintaining a moist wound environment, aiding in effective wound healing. Incorporating active ingredients like dexpanthenol, niacinamide, and diclofenac sodium addresses various aspects of wound healing, while cytotoxicity assays confirm their safety. Comparative analyses of wound scratch and impedance-based assays emphasize the role of polymer content in promoting cell migration and wound closure. However, foam formulations containing diclofenac sodium show incomplete wound closure, suggesting an inhibitory effect on the initial healing process. The impedance-based measurement also showed the lowest migration value (15.99%) with the foam containing diclofenac.

In ovo examinations have further clarified the effects of hyaluronic acid and dexpanthenol-containing foam formulation, revealing no signs of irritation, indicating their potential in tissue repair.

5. CONCLUSION

The medicinal use of dermal foams is increasingly popular among the public. Their application is aesthetic, non-greasy, and easily removable from the skin, thus enhancing patient adherence. Foams have excellent spreadability on the skin, facilitating immediate absorption of active ingredients without the need for intense rubbing. Despite their numerous advantages, formulating dermal foams poses significant challenges. Meeting user demands for quick spreadability and pleasant skin sensation while ensuring environmental friendliness has become crucial. Additionally, while foams offer many benefits, their availability in the market remains relatively low compared to traditional products like creams and gels.

In my Ph.D. work, novel foam systems were formulated and new investigational methods were established suitable for testing the physicochemical and subsequently the biopharmaceutical properties of foams. Finally, I investigated the biocompatibility and the effects of the formulated products on wound healing.

During my work, I managed to develop testing methods capable of assessing the mechanical properties of foam, and the results indicated that foam, as a dosage form, is suitable for providing rapid drug release, deeper permeation, and may serve as alternatives to conventional carrier systems.

6. FINDINGS AND PRACTICAL RELEVANCE OF THE WORK

- The QbD approach was employed for the first time in foam development, demonstrating its efficacy in identifying and quantifying the critical parameters of these systems.
- A new light microscopy measurement method has been successfully developed for measuring foam kinetics and bubble morphology.
- The effect of stability enhancing polymers used in foams has been demonstrated and the effect of different polymers on the physicochemical properties of foams has been investigated. It has been determined that the combined application of macroscopic, microscopic, and rheological measurement methods allows for the appropriate selection of excipients used in foam formulations.
- The Texture Analyzer device was first employed to determine the physical stability of the foams.

- Through biopharmaceutical investigations, it was demonstrated that foam dosage forms enable immediate drug release and achieving deeper skin permeation. The *in vitro* quantitative IVPT and qualitative *ex vivo* Raman mapping studies were well-correlated. The combination of IVRT, IVPT, and Raman spectroscopy was first employed to determine the biopharmaceutical properties of the foams, establishing that rapid drug release and deep permeation can be achieved.
- The Raman mapping results exhibited a strong correlation with the fluorescent microscopic examination providing additional evidence for the system's rapid permeation.
- The biocompatibility of components in dermally applied foams was demonstrated using human adipose-derived mesenchymal stem cells and keratinocytes extracted from viable human skin, supporting the safety of application on the skin. The correlation between the two MTT tests indicated that, due to their sensitivity, assays conducted on human adipose-derived mesenchymal stem cells are more suitable for determining the cell viability of foam components.
- The wound healing effects of the foam bulk liquids were confirmed in two different models. The combined use of these two models showed good correlation, which may be suitable for selecting the optimal formula for wound treatment.
- The *in ovo* HET-CAM assay was suitable for determining the irritation of foam formulations.

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. **Fanni Falusi**; Mária Budai-Szűcs; Erzsébet Csányi; Szilvia Berkó; Tamás Spaits; Ildikó Csóka; Anita Kovács: Investigation of the effect of polymers on dermal foam properties using the QbD approach.
European Journal of Pharmaceutical Sciences, 173, 106160 (2022) **(Q1, IF:4.6 by JCR)**

II. **Fanni Falusi**; Szilvia Berkó; Anita Kovács; Mária Budai-Szűcs: Application of Xanthan Gum and Hyaluronic Acid as Dermal Foam Stabilizers.
Gels, 8(7), 413. (2022) **(Q1, IF: 4.6 by JCR)**

III. **Fanni Falusi**, Szilvia Berkó, Mária Budai-Szűcs, Zoltán Veréb, Anita Kovács: Foams Set a New Pace for the Release of Diclofenac Sodium.
Pharmaceutics 16, 287 (2024) **(Q1, IF:5.4 by JCR)**

PUBLICATIONS NOT RELATED TO THE SUBJECT OF THE THESIS

I. Anita Kovács, Stella Zsikó, **Fanni Falusi**, Erzsébet Csányi, Mária Budai-Szűcs, Ildikó Csóka, Szilvia Berkó: Comparison of Synthetic Membranes to Heat-Separated Human Epidermis in Skin Permeation Studies In Vitro.
Pharmaceutics 13, 2106 (2021) **(Q1, IF:6.525 by JCR)**

II. Anita Kovács, **Fanni Falusi,** Attila Gácsi, Mária Budai-Szűcs, Erzsébet Csányi, Zoltán Veréb, Tamás Monostori, Ildikó Csóka, Szilvia Berkó: Formulation and investigation of hydrogels containing an increased level of diclofenac sodium using risk assessment tools.
European Journal of Pharmaceutical Sciences 193, 106666, (2024) **(Q1, IF:4.6 by JCR)**

PRESENTATIONS RELATED TO THE SUBJECT OF THE THESIS

Verbal presentations

I. **Fanni Falusi**; Anita Kovács; Erzsébet Csányi
Investigation of foams for topical use
III. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, 2021

II. **Falusi Fanni**
Dermális habok formulálása és vizsgálata
IV. Fiatal Technológusok Fóruma, online, 2021

III. **Falusi Fanni**; Berkó Szilvia; Csóka Ildikó; Kovács Anita
Dermális habok jellemzése és vizsgálata
MKE Kozmetikai Szimpózium, Budapest, 2021

IV. **Falusi Fanni**
Különböző polimer tartalmú habok formulációja és vizsgálata
XIV. Clauder Ottó Emlékverseny, Budapest, 2021

V. **Fanni Falusi**; Anita Kovács; Szilvia Berkó
Formulation and investigation of the effect of polymers on dermal foam properties using the QbD approach
IV. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, 2022

VI. **Falusi Fanni**; Kovács Anita; Budai-Szűcs Mária; Berkó Szilvia
Dermális habok, mint innovatív gyógyszerformák formulálása és vizsgálata
Gyógyszerkémiai és Gyógyszertechnológiai Szimpózium '22, Herceghalom, 2022

VII. **Falusi Fanni**; Kovács Anita; Budai-Szűcs Mária; Csóka Ildikó; Berkó Szilvia
Dermálisan alkalmazott készítmények hatóanyag-felszabadulásának és penetrációjának *in vitro* modellezése
MKE Kozmetikai Szimpózium, Budapest, 2022

VIII. **Fanni Falusi**; Szilvia Berkó; Anita Kovács
Influence of polymers and active substances on foam stability
V. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, 2023

IX. **Falusi Fanni**
A gyógyszeres habok nyújtotta ígéretes lehetőségek a hagyományos gyógyszerhordozó rendszerekkel szemben
VI. Fiatal Technológusok Fóruma, Budapest, 2023

X. **Falusi Fanni**; Berkó Szilvia; Kovács Anita
Dermálisan alkalmazott gyógyszerhordozó rendszerek komparatív vizsgálata
Gyógyszerkémiai és Gyógyszertechnológiai Szimpózium '23, Herceghalom, 2023

XI. **Falusi Fanni**
A dermális habok nyújtotta ígéretes lehetőségek
MKE Kozmetikai Szimpózium, Budapest, 2023

Poster Presentations

I. **Fanni Falusi**; Szilvia Berkó; Mária Budai-Szűcs; Anita Kovács
Formulation and investigation of the effect of polymers on dermal foam properties using the Quality by Design (QbD) approach
9th BBBB International Conference on Pharmaceutical Sciences - Pharma Sciences of Tomorrow, Ljubljana, 2022

II. **Falusi Fanni**; Berkó Szilvia; Budai-Szűcs Mária; Kovács Anita
Hialuronsav és xantángumi, mint habstabilitás növelő komponensek vizsgálata
Gyógyszertechnológiai és Ipari Gyógyszerészeti Konferencia, Siófok, 2022

III. **Fanni Falusi**; Szilvia Berkó; Mária Budai-Szűcs; Anita Kovács
Development and evaluation of stable hydrogel formulations with enhanced diclofenac sodium concentration for effective topical drug delivery
14th Central European Symposium on Pharmaceutical Technology, Ohrid, 2023

PRESENTATIONS NOT RELATED TO THE SUBJECT OF THE THESIS

I. **Fanni Falusi**; Szilvia Berkó; Anita Kovács
Advancements in formulation and investigation of innovative foam-based *in situ* film-forming systems
VI. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, 2024

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