Application of Pickering emulsions in pharmaceutical technology for formulation of water insoluble drugs and essential oils

PhD thesis

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1. Introduction

The pharmacological importance of emulsions is indisputable because they are used in the preparation of many different dosage forms, including the formulation of creams, gels, transdermal patches or infusions. Use of an emulsion form can provide a solution for formulating poorly or non-water-soluble active pharmaceutical ingredients (API). Furthermore, using emulsions in topical preparation, the API bioavailability can be also increased, because the formulation enhances the API penetration through the skin. The emulsions are also suitable for improving the appearance, texture, taste, and shelf life of the API.

However, the emulsion formulation has disadvantages, because for their formulation and stabilization surfactants and tensids are used, which can cause both external and internal irritations, and they have even carcinogenic effects in case of long-lasting treatment.

Using biocompatible solid particles for emulsion stabilization, the harmful effects of surfactants can be eliminated. The solid particle stabilized emulsions called Pickering emulsions [PE]. Several studies show that the bioavailability of the API from PE is greater, than from solutions or conventional emulsions, in case of both oral and topical formulations. Assuming this phenomenon is caused by the preferential binding/adsorption of solid particles to cells or cell membranes. Another advantageous feature of the PE form is, that it also provides controlled API release, which can be altered by the droplet size and the API deposition through the so-called diffuse layer.

The physical chemical properties of PEs (e.g. thermodynamically or kinetically stability, type of emulsion, droplet size) are influenced by several parameters, among others the size and surface properties of particles, the ratio of oil and water phase, the viscosity or density of dispersed phase.

The medical use of essential oils [EOs] is still immensely popular today, they can be used to treat and prevent several diseases, moreover their effect has already been confirmed by studies. Although EOs have a favorable side effect profile, their use can be harmful, as they can cause mild irritation and allergic reactions. It is of outmost importance to study the mode of action of EOs and to find a proper, harmless formulation. Another fundamental problem is the highly lipophilic nature of EOs, which makes it impossible to measure their biological effects in an aqueous environment. The PE form may be suitable for the formulation and testing of EOs. Previous studies have reported decreased

evaporation of EOs from o/w emulsion of nanoparticle-stabilized formulations versus EO–surfactant systems to be a beneficial factor.
By appropriately changing and fine-tuning the above-mentioned parameters of PEs, we can prepare an emulsion based drug delivery system, which can be suitable for the formulation of EOs and water-insoluble APIs, and because of its suitable physical chemical properties a sustained and targeted drug delivery can be achieved.

2. Aims

The main object was to prepare PEs with well-defined properties suitable to formulation and topical application of EOs and lipophilic API. The stability and droplet size of PEs were the most important properties throughout the development of a new drug delivery system, because these determine the characteristics of the API release. In order to achieve the appropriate physicochemical properties of PEs, a systematic and size-controlled SNP synthesis and their surface modification was required. The SNPs with desired size and surface properties have been used as stabilizing agents.

Up to this day, several research groups have been paying attention to develop a simple method for size-controlled SNP synthesis with high reproducibility in the 5-200 nm size range, where the SNP particles are highly monodisperse and spherical. After researching several publications in this theme, we have found that currently there is no paper available, that make possible a tailored-size synthesis of SNPs. This means, that there are no systematic study or results from which the exact parameters of the reaction can be obtained and a synthesis with appropriate reproducibility can be performed. Therefore, I have aimed to carry out a systematic study with the examination its applicability in practice. I used the currently available results of different research groups to design the parameters of my experiments.
Because of the physical chemical properties of EOs and lipophilic API can differ in a wide range (eg. density, viscosity, interfacial tension), the appropriate size and surface modification of SNPs, which are used for stabilization, have been determined in an empirical way.

Our goal was to formulate PEs of chamomile and artemisia EO, and characterize their antimicrobial effect using Gram-positive and Gram-negative bacteria as well as fungal species. To suggest a plausible mode of action of this formulation, experiments about the interaction between the PEs and cell model

have been conducted. Conventional emulsions and solutions have been also prepared to compare the effect of different formulations.

The EO-based commercial products that are used for treatment or prevention of dental diseases are containing surfactants, solvents or co-solvents, which can cause mucous membrane irritation, allergic reaction and other side effects. Our aim was to prepare PEs of EOs with biocompatible SNPs for topical therapy of dental biofilms caused by *Streptococcus mutans*. Taking into account the physical chemical properties of *S. mutans* biofilm, o/w PE with smaller than 0.3 μm droplet size can be an appropriate choice for targeted delivery of EOs. For the formulation of PEs the following EOs with antibacterial effect have been chosen: cinnamon bark (*Cinnamomum verum* J. Presl.), clove (*Syzygium aromaticum* (L.) Merr. and Perry), peppermint (*Mentha x piperita* L.) and thyme EO (*Thymus vulgaris* L.). In vitro biofilm degradation test with *S. mutans* bacteria and in vitro diffusion studies of different EO formulations (PE, conventional emulsion, solution) have been carried out, to determine their antibacterial activity and diffusion properties. In the in vitro diffusion tests *S. mutans* biofilm model membrane was used, which have similar hydrophilic properties and tortuous pore structure.

The other therapeutic application is the topical treatment of onychomycosis. As lipophilic API an azol derivative, tioconazole [TIO] has been chosen, because it has a broad antifungal spectrum for common dermatophytes, which has proved to be efficient for the topical treatment of fungal infections. Nenoff et al. determined that tree tea EO (*Melaleuca alternifolia* EO [MA]) inhibited the growth of several clinical fungal isolates, so they suggested its use in the topical treatment of fungal infections. For the PE formulation the solution of TIO and MA were planned to use, because we assumed, that synergistic effect between the azole derivative and EO can be observed. Using an o/w type PE with appropriate droplet size could penetrate the porous nail structure and retain the antifungal drug on the nail bed for a longer period, which is the main site of reinfection, and thereby a targeted drug delivery can be achieved. The targeted drug delivery has been tested by in vitro diffusion studies, where artificial membranes were used. The aim these studies was to compare drug delivery characteristics of different formulations (PE, conventional emulsions, solutions) in the membranes that have similar porous structure and surface properties as the nail plate and nail bed. The antifungal activity against *Candida*

albicans and *Trichophyton rubrum*—the species mainly responsible for fungal nail infections—has been investigated.
Summary, our aim is to formulate PEs with EOs and lipophilic API, which are non-irritative, stable and they long term application in the topical delivery is feasible.

3. Materials and methods

3.1. Synthesis, surface modification and characterization of silica nanoparticles

The silica nanoparticles [SNP] were prepared by hydrolysis of tetra-ethoxy-silane [TEOS] in ethanol in the presence of ammonium hydroxide as catalyst. The concentration of TEOS was constant in each reaction, c=0.26 mol/dm^3. We changed systematically the concentration of ammonia, water or temperature as follows:

- varied ammonia concentration between 0.29-0.097 mol/dm^3, at constant water concentration (5 mol/dm^3) and constant temperature (25°C)
- varied water concentration between 2-5 mol/dm^3 at constant ammonia concentration (0.29/0.194/0.097 mol/dm^3) and constant temperature (25°C)
- varied temperature between 25-50°C at constant ammonia concentration (0.29/0.194/0.097 mol/dm^3) and constant water concentration (5 mol/dm^3)

The reaction time was 24 h in each cases. The synthesized SNPs were stored until further use in the residual reaction solution. Three parallel synthesis have been performed.
After the synthesis, the surface of hydrophilic SNPs was modified with different silanol precursors (methyl-, ethyl- propyl- and phenyl-triethoxy-silane). In order to a reproducible surface modification process could be carried out, the desired surface coverage of SNPs was calculated with a simple geometrical approach. The reaction time and temperature were 6 hours and 25°C, respectively. The ammonium hydroxide and ethanol content were always removed by fractional distillation from the modified SNP suspensions.
The size distribution, hydrodynamic diameter (d) and polydispersity index (PDI) were determined by dynamic light scattering [DLS]. The size distribution was confirmed and morphology of silica nanoparticles was studied with TEM.

The surface modification of SNPs have been investigated with infrared spectroscopy analysis.

3.1.1. Statistical analysis of SNP synthesis

Statistical analyses were performed using Microsoft Excel® software. Correlations between the studied parameters and diameter of SNPs were assessed by linear regression. The coefficient of determination (R2) was calculated to establish the fit of the models. Finally, confidence intervals for each model were examined to determine the accuracy of the prediction made by the models. For the calculations, the results of three parelell experiments and their standar deviation values were used. Based on the results of calculations, further experiment were planned and performed, to determine the applicability of model in practice.

3.2. Formulation of essential oils for microbiological experiments

3.2.1. Preparation and characterization of Pickering emulsions with chamomile and artemisia essential oils

For PE formulation chamomile and artemisia EOs were used in the 0.1-3.5 mg/ml concentration range. The stabilizing agents of PE were 20 nm, in 50% propyl functionalized [50PR] and in 20% propyl functionalized SNPs [20PR], in case of chamomile and artemisia EO, respectively. The concentration of SNPs was 1 mg/ml. The first step of the emulsification process was sonication for 2 minutes, then emulsification using UltraTurrax for 5 min at 21,000 rpm. The PEs were stored in room temperature (25°C), dark place. Each experiments was made in triplicates.

3.2.2. Characterisation of Pickering emulsions – Droplet size, type of emulsion, stability

The changes in droplet size and stability have been examined in function of EO concentration. The droplet size of the emulsion was determined with DLS. We consider the emulsions to be stable, when the droplet size does not change within at least 1 week and creaming, sedimentation, or disproportionation do not occur in this period. The emulsion type has been determined with conductivity measurements.

3.2.3. Interaction studies between cell models and Pickering emulsions

The unilamellar liposomes, consisting of a single phospholipid, can be used as artificial cells or biological membrane model for studying the interactions

between cells or cell membranes and drugs or biologically active components. Unilamellar liposomes (D=3.5 μm) have been prepared from phosphatidylcholine by the modified method described before by Alexander Moscho et al. The unilamellar liposomes were stored in PBS buffer, in the refrigerator at 8°C until further use.

This study was conducted to determine the intracellular delivery ability of active components from EOs for different formulations, so conventional emulsions with Tween80 stabilizing agent and ethanolic solutions were also prepared. The preparation process of conventional emulsions was the same as in case of PEs.

In the experiments the appropriate amount of unilamellar liposomes and samples were mixed until 24 or 48 hours, at 37°C or 30°C, which measurement parameters are the same as the parameters of microbiological experiments with bacteria or fungi, respectively. The EO content of samples was determined with UV/VIS spectroscopy Each measurements were made in triplicate.

3.2.4. Microbiological experiments

The following microorganism were used in our experiments: *Escherichia coli* PMC 201, *Pseudomonas aeruginosa* PMC 103, *Bacillus subtilis* SZMC 0209, *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* SZMC 0119, *Schizosaccharomyces pombe* ATCC 38366, *Candida albicans* ATCC 1001, *Candida tropicalis* SZMC 1368, *Candida dubliniensis* SZMC 1470, *Candida krusei* SZMC0779. The MIC_{90} and MEC_{10} values of different formulations were determined, furthermore the effect on microbial oxidative balance of different formulations, time-kill kinetics study and live/dead cell viability discrimination studies have been also performed.

3.3. Treatment of Streptococcus mutans biofilm with Pickering emulsions

3.3.1. Formulation of Pickering emulsions with different essential oils

For the experiments the following EOs were used: peppermint (*Mentha x piperita* L.), cinnamon (*Cinnamomum verum* J. Presl.), thyme (*Thymus vulgaris* L.) and clove ((*Syzygium aromaticum* (L.) Merr. and Perry). Their concentration range, which used for the PE formulation, was the same as in the broth macrodilution test, where the minimum inhibitory concentrations [MIC] of EOs have been determined. Based on this, the concentration of EOs were

the following: in case of cinnamon, thyme and clove EO 0.05-2.00 mg/ml, and in case of peppermint EO 0.15-3.25 mg/ml.
The stabilizing agents of PE were 20 nm, in 20% ethyl functionalized SNPs [20ET]. The concentration of SNPs was 1 mg/ml. The first step of the emulsification process was sonication for 2 minutes, then emulsification using UltraTurrax for 5 min at 21,000 rpm. The PEs were stored in room temperature (25°C), dark place. Each experiments was made in triplicates. The influence of EOs concentration on the emulsion droplet size and stability was examined; it was varied until the minimum inhibitory concentration against *Streptococcus mutans* was reached.

3.3.2. Characterization of Pickering emulsions – Droplet size, type of emulsion, stability

The characterization process of PEs were the same, as are described in *part 3.3.2*.

3.3.3. In vitro biofilm degradation experiments

The biofilm inhibition experiments were performed on the base of Peeters and co-worker's study, with the crystal violet assay.

3.3.4. In vitro diffusion studies through biofilm model membrane

The concentration of EOs were MIC/2 values, which was the same concentration as used in the biofilm degradation experiments: 0.98 mg/ml for peppermint EO, 0.4 mg/ml for cinnamon bark EO, 0.51 mg/ml for clove EO and 0.20 mg/ml for thyme EO. To compare the effectiveness of PEs, we have examined the diffusion of EOs in an ethanolic solution and emulsion stabilised with Tween80 surfactant.
The examination of diffusion properties was performed at 37°C in static vertical Franz diffusion cells, six parallel cells were used with 2.1 mm thick, 2 w/w% agar gel membranes with effective penetration area of 2.54 cm^2, the receiver solution was PBS buffer. The diffusion was examined for 6 hours, samples were collected in 30 minutes, and then in every hour. The EO content of samples was determined with UV-Vis spectroscopy.

3.4. Topical treatment of onychomycosis with Pickering emulsion of tea tree oil and tioconaozole

3.4.1. Gas chromatograpy analysis of tea tree essential oil

MA sample preparation using the static headspace solid-phase microextraction technique was carried out (sHS-SPME). The analysis was performed by gas chromatography coupled mass spectrometry (GC-MS). The data were evaluated using MSD ChemStation D.02.00.275 software. The identification of the compounds was carried out by comparing retention data and the recorded spectra with the data of the NIST 2.0 library. The percentage evaluation was carried out by area normalization.

3.4.2. Determination of solubility of tioconazole in tea tree essential oil

After the GC analysis of MA and the indentification of its main components, calculations of Hansen solubility parameters of TIO have been carried out, for the calculations the Hansen Solubility Parameters in Practice (HSPiP) software version 5.0.11 has been used. In the calculations, we used the simplified molecular-input line-entry system (SMILES), the SMILES identifiers has been search from the PubChem database.

The kinetic solubility of TIO in water saturated MA was determined by solvent addition method. Three parallel measurement have been performed at room temperature (25°C). The state of the suspension and the transition between the suspension and the solution were examined by DLS measurements and visual observation. In the case of DLS measurements, I used manual correlation, with which the measurement parameters can be kept constant, so that the transition between the suspension and the real solution can be clearly observed.

3.4.3. Formulation of Pickering emulsions with tea tree essential oil and tioconazole

In the first step, for the PEs formulation I worked with pure MA, and SNPs of different sizes, modified with different functional groups and varying surface coverage. I observed how the concentration of SNPs and the emulsification energy influence the droplet size and stability of PEs. I repeated each experiment at least three times.

The concentration of emulsifiers in the water phase was set to 1 mg/ml and was kept constant for all experiments. Three different sizes of SNPs (20, 50 and 100nm) with the same surface modification (ethyl groups, 20% surface coverage) were used for PE formulation (20ET, 50ET, 100ET). The

concentration of oil phase varied between 0.90 and 17.91 mg/ml, the ratio of TIO and MA was always constant (20 m/m%).
The first step of the emulsification process was sonication for 2 minutes, then emulsification using UltraTurrax for 5 min at 21,000 rpm. Until further use, the PEs were stored in room temperature (25°C), dark place. Each experiments was made in triplicates.

3.4.4. Characterisation of Pickering emulsions – Droplet size, type of emulsion, stability

The characterization process of PEs was the same, as are described in *part 3.3.2.*, with the following additions.
The stability of PEs can be influenced by several parameters, eg. the physical chemical properties of oil phase. That is why I determined how the viscosity of MA changes depending on its TIO content. The measurement was carried out by Ostwald-Fenkse capillary viscometer, at room temperature (25°C).

3.4.5. In vitro diffusion studies through nail model membrane

This study was conducted to determine the diffusion properties of TIO for different formulations, so conventional emulsions with Tween80 stabilizing agent and ethanolic solutions were also prepared. The preparation process of conventional emulsions was the same as in case of PEs.
For in vitro testing 2.1 mm thick 6 m/m% agar gel membrane and the same agar gel membrane combined with 0.8 mm thick cellulose acetate with effective penetration area of 2.54 cm^2 were used. Before each measurement, the agar gel was always freshly prepared.
The examination of diffusion properties was performed at 32°C in static vertical Franz diffusion cells (Hanson Microette Plus); six parallel cells were used and each experiment was made in triplicates. The receptor solution was PBS buffer. The diffusion was examined for 2 hours; the samples were collected after 5, 10, 15, 30, 60, 90 and 120 min. The TIO content was determined with HPLC measurements using UV-Vis detector, the method is based on Bagary et al.

3.4.6. Microbiological tests against Trichophyton rubrum and Candida albicans

The antifungal activity against *Candida albicans* and *Trichophyton rubrum* (T. rubrum) DSM 21146, the species mainly responsible for fungal nail infections, has been investigated.

We followed the methods described previously for *T. rubrum* and *C. albicans* culture preparation. The inoculum *T. rubrum* cell population was adjusted to 0.5-5·10^6 spores/ml, and this value for C. albicans was 1·10^6 cell/ml, the number of cells was determined by using a Bürker cell-counting chamber followed by further turbidity calibration with an UV-Vis spectrophotometer at 520 nm.

To determine the antifungal activity, the minimal inhibitory concentration [MIC] and minimal fungicid concentration [MFC] has been evaluated for *T. rubrum* and *C. albicans*. Beside the ethanolic solutions of TIO and MA, the different PEs were also tested in the 0.5-300 μg/ml concentration range. MFC was evaluated as the lowest drug concentration resulting no growth (≥99.9% growth inhibition). All evaluations were performed in triplicates in six independent experiments

The statistical analyses were conducted using one-way ANOVA test and the significance was set at $p \leq 0.05$.

4. Results and dicussion

4.1. Synthesis, surface modification and characterization of silica nanoparticles

Firstly, we investigated, how influence the ammonium hydroxide concentration the particle size, if the temperature and water concentration are constant, t=25°C and 5 mol/dm^3, respectively. We determined, that under 0.019 mol/dm^3 ammonium hydroxide concentration SNPs are not formed, but in the 0.29-0.097 mol/dm^3 concentration range SNPs between 27.05 and 190.8 nm could be synthesised with high product yield (≥80 %)The SNPs are highly monodisperse, and their shape are spherical.

By the investigation of temperature dependence of SNP synthesis, experiments were carried out in the 25-50°C temperature range. The results show, that with increasing the reaction temperature the size of SNPs decreases (c_{TEOS}=0.26 mol/dm^3, c_{water}=5 mol/dm^3). At different ammonium hydroxide concentration the SNP size decreases in different extent: at 0.29 mol/dm^3 ammonia concentration the particle sizes are between 190.8-69.3 nm; at lower ammonia concentration, 0.194 mol/dm^3 the particle sizes are between in the 99.1-30.2 nm interval, and at 0.097 mol/dm^3 this values are in the 27.1-6.1 nm size range. The values of standard deviations show, that the experiments can be performed with high reproducibility, and all SNPs are monodisperse. The TEM images show that their spherical shape do not change on the effect of temperature. We

found linear correlations between the SPN sizes and reaction temperature, which were used for size-accurate experimental desing. Our results shows, with precise temperature control make it possible to synthesise SNPs with a desired particle size at each ammonia concentration. These calculations in the different SNP size intervals provide possibility for a large-scale SNP size tailoring or fine-tuning. At 0.29 mol/dm^3 ammonia concentration, we can exactly plan the particle size between 65 and 200 nm. If we would like to synthesise SNPs in the 30 and 100 nm particle size range, we should work with constant 0.194 mol/dm^3 ammonia concentration. At 0.097 mol/dm^3 ammonia concentration SNPs under 30 nm size can be produced, where we can tune fine the particle size from 22.51 nm to 6.11 nm.

Next step, we investigated, how influence the water concentration of SNP size. The results show, that the SNP size decreases, if the water concentration decreases. The extent of size decreasing is different at each ammonium hydroxide concentration value: at 0.29 mol/dm^3 the SNP sizes are between 52.18-190.80 nm increase in the 2-5 mol/dm^3 water concentration range. At lower ammonia concentration, 0.194 mol/dm^3 the particle sizes are between 27.81-99.09 nm, and at 0.097 mol/dm^3 this values are in the 6.92-27.1 nm size range. With decreasing water concentration the polydispersity of SNP increases, at 2 mol/dm^3 water concentration the PDI can be more, than 0.250. The TEM images shows, that under 3 mol/dm^3 water concentration the morphology of SNPs significantly change, here the shape of SNP are amorphous, shapeless. Here we also found linear correlation between water concentration and SNPs size, but it has been not used for size-accurate experimental desing, because of the significant shape change.

The native SNPs are hydrophilic because of the high number of free silanol groups at their surface, which has to be modified with organic ligands to achieve appropriate wettability and strong stabilizing effect in PE formulation. Surface modified SNPs with different functional groups (methyl, ethyl, propyl, phenyl) and surface coverage (10-50%) have been synthesized. Based on the results of DLS measurements it can be observed, that the size and polydispersity of SNPs have not been changed on the effect of surface modification process. In the TEM images can be seen, that their morphology have also not been changed, the shape of surface modified SNPs are spherical and they are highly monodisperse.

The surface modification of SNPs was examined with infrared spectroscopy. On the spectra of the native, hydrophilic SNP the characteristic group frequencies of siloxane chain can be indentified. After the surface

modifications, the characteristic bands of symmetric and asymmetric aliphatic methyl and ethyl groups can be seen on the spectra, the intensity of these bands increase with increasing the number of functional groups in the surface. The intensity of peaks belonging to the symmetric and asymmetric vibrational frequency of Si-O-Si group decreasing, if the number of surface modifying functional groups increasing.

4.2. Formulation of essential oils for microbiological experiments

The droplet size of PEs of chamomile and artemisia EO were in the 160-1450 nm size range with 1.2-11.4% standard deviation valeus. We succeeded in formulating stable PEs, the stability depends on the EO concentration, but all PE formulation were at least for three months stable.

The type of emulsions was determined by conductivity measurements. The conductivity values of stabilizing SNPs suspended in distilled water were 223.5 $\mu S \cdot cm^{-1}$ and 193.2 $\mu S \cdot cm^{-1}$ for 20PR and 50PR, respectively, while the conductivity of chamomile and artemisia EO were 0.018 $\mu S \cdot cm^{-1}$ és 0.056 $\mu S \cdot cm^{-1}$. Because the conductivity of PEs were in the 164.0-212.0 $\mu S \cdot cm^{-1}$ range, we can conclude, that all the PEs were o/w type emulsions.

4.2.2. Interaction studies between cell models and Pickering emulsions

This study was performed to determine the intracellular delivery ability of active components from EOs for different formulations.

We have studied the interaction of unilamellar liposomes and different formulations of chamomile EO for 24 h at 35 °C. After 1 h of interaction, 27.2% of EO have penetrated the liposomes from PE, while conventional emulsion and the ethanolic solution did not provide a measurable amount. The next sampling was after 2 h, where PE has delivered 48.3% of EO, conventional emulsion 0.5%, while the amount of EO delivered by the ethanolic solution was not measurable. Final sampling was after 24 h, with 82.2% of chamomile EO found in the unilamellar lipsomes when it was introduced in PE, and this value was 66.8% for conventional emulsion and 32.5% for the ethanolic solution.

In case of artemisia EO we studied in two different circumstances (see *part 3.2.3*). For both experimental circumstances, PE showed the best ability to deliver artemisia EO to the internal water phase of ULs; it was 29.8% after 48 hours at 30 °C, and 59.2% after 24 hours at 37 °C. For the conventional emulsion, these values were 25.0% and 27.3%, respectively, and the lowest values were obtained in the case of ethanolic solutions.

The results obtained from model experiments have highlighted that the PEs of the chamomile and artemisia EO are the most effective form for the intracellular delivery when compared to their conventional emulsion or solution form.
The results of microbiological experiments are part of an other PhD thesis, therefore the detailed result can be read in our relevant publications. Summary it can be concluded, that the PEs have more effective antimicrobial properties, as the other formulations of EOs.

4.3. Treatment of Streptococcus mutans biofilm with Pickering emulsions

4.3.1. Formuation and characterization of Pickering emulsions

The droplet size ranges of PEs of the different EOs were the following: peppermint EO 295-1445 nm, cinnamon EO 196-3100 nm, thyme EO 155-1680 nm, clove EO 180-4300 nm, the values of standard deviation are between 1.8-8.3%. The droplet size increases if the EO concentration increases. We succeeded in formulating stable PEs, the stability depends on the EO concentration, the PEs of cinnamon EO were the most stable, these remain their stability at least until 4 months.
The type of emulsions was determined by conductivity measurements. The conductivity values of stabilizing 20ET SNPs suspended in distilled water was 215.0 $\mu S \cdot cm^{-1}$, while the conductivity of EOs were the follow: peppermint EO 0.071 $\mu S \cdot cm^{-1}$, cinnamon EO 0.043 $\mu S \cdot cm^{-1}$, thyme EO 0.065 $\mu S \cdot cm^{-1}$, clove EO 0.084 $\mu S \cdot cm^{-1}$. Because the conductivity of PEs were in the 110.5-269.0 $\mu S \cdot cm^{-1}$ range, we can conclude, that all the PEs were o/w type emulsions.

4.3.2. Results of in vitro biofilm degradation experiments

The results of biofilm degradation experiments belong to an other PhD thesis, therefore the detailed result can be read in our relevant publications. Summary it can be concluded, that the PE formulation of EOs have the highest biofilm degradation ability, treating the *Streptococcus mutans* biofilm with cinnamon PE, the degradation of biofilm was 85.9%.

4.3.3. In vitro diffusion study through biofilm model membrane

We assumed that there should be a correlation between inhibitory rate and diffusion properties trough biofilm of the EOs in different forms. To confirm this assumption, *in vitro* diffusion tests were performed. The droplet size of the different types of emulsions was similar, so we could assume that the diffusion

properties depend only on the type of the emulsion or surface properties of emulsion stabilising agent.

Generally, we can conclude that the cumulative EO amounts were highest for PEs. Despite of the lowest MIC value of thyme EO, which refers to the most effective antibacterial activity, its biofilm degradation ability is the lowest. This phenomenon could be caused by the diffusion properties of thyme EO, because its ethanolic solution did not provide a measurable amount through model membrane, and the cumulative EO amount in case of conventional emulsions and PE are very low, 9.4 and 18.9%, respectively. The highest cumulative EO amount can be detected in the case of PE of peppermint EO, this value is 80%. In the case of cinnamon EO, these values are only 51.4% for PE, but with this EO higher biofilm degragation effect can be achieved, assuming because of cinnamon EO has lower MIC value, than peppermint EO.

We can conclude that o/w type PEs stabilised by SNPs with appropriate hydrophilic/hydrophobic surface properties, provide a new possibility for the application of EOs in pharmaceutical treatment against *Streptococcus mutans* biofilm formation.

4.4. Topical treatment of onychomycosis with Pickering emulsion of tea tree oil and tioconaozole

4.4.1. Results of GC analysis of tea tree essential oil

The composition of MA was determined by gas chromatography. The components were identified by comparing their retention times and relative retention factors with standards and essential oils of known composition. Two parallel measurements have been performed. The main compounds are p-cymene 35.2% and terpinene-4-ol 32.5%.

4.4.2. Determination of solubility of tioconazole in tea tree essential oil

Based on the results of the calculations of Hansen solubility parameter, it can be concluded, that the TIO can be dissolved in appropriate amount in MA. The result of kinetic solubility determination veryfies the calculations: the maximum solubility of TIO in MA was 0.213 mg/ml (23.8 w/w%). Based on these findings, for the formulation of PEs a 20 w/w% solution of TIO in MA were used.

4.4.3. Preparation and characterization of Pickering emulsions

In these experiments, I investigated how the oil phase concentration, size and surface modification of SNP influence the droplet size and stability of PEs.

As shown by Binks and Horozov, the size of stabilizing particles influences the emulsion droplet size at the same o/w phase ratio. The phenomenon, which they described, can be also observed in our results: we have found that the increment of oil phase concentration and size of stabilizing particles have caused an increment of emulsion droplet size at all examined oil phase concentrations, both at MA and TIO MA oil phase.

For example, where only MA was used for PE formulation, at 4.48 mg/ml concentration value the droplet sizes increase if the size of SNPs increase: at PE 20ET the droplet size is 1400 nm, at PE 50ET is 1700 nm and at PE 100ET is 1850 nm. Examining by any value in the concentration range, the same tendency can be observed, the maximum droplet sizes at 17.91 mg/ml are 1850 nm for PE 20ET, 2300 nm for PE 50ET, and 3080 nm for PE 100ET. Using different size of SNPs also influence the stability of PEs. We found, that using smaller SNP the stability of PE are higher: eg. at 4.48 mg/ml MA concentration the stability is 20 weeks at PE 20ET20, and only 1 week in case of PE 50ET and PE 100ET.

The same tendency in droplet size and stability can be observed, when TIO MA oil phase is used for PE formulation. If we investigate the results at the same concentration values, we can observe that the droplet sizes do not change significantly, but the emulsion stability decreases in case of using TIO MA oil phase instead of MA. For example at 6.27 mg/ml oil phase concentration, the stability PE 20ET decreases significantly: in case of MA oil phase the stability is 20 weeks, but using TIO MA phase is only 8 weeks. The same trend can be found at PE 50ET and PE 100ET at any oil phase concentration value.

Presumably, there is no significant change in PE droplet sizes, if TIO MA oil phase is used instead of MA, because the following parameters did not change: emulsification conditions, the size and surface coverage of stabilizing SNPs, the ratio of oil and water phase, and the visocity of oil phases. The dynamic viscosity value of MA is $9.2608 \cdot 10^{-3}$ Pa·s (25°C), its density is 0.895 g/cm^3 (25°C), while these values in case of TIO MA oil phase are $9.4147 \cdot 10^{-3}$ Pa·s and 0.931 g/cm^3 (25°C).

A linear correlation has been found between the oil phase concentration and droplet size in case of PE 20ET samples. Using MA oil phase the linearity can be observed in the 0.9-7.16 mg/ml concentration range, and at TIO MA oil

phase the linearity is valid for the 0.9-5.37 mg/ml concentration range. We have used this linear correlation to plan PEs with desired droplet size, and based on the calculated results PEs have been prepared. We can conlude, that using 20ET20 SNPs as stabilizing agent, the droplet size of PEs can be precisely planned, which may contribute to the preparation of targeted API drug delivery. The type of the PEs was determined by conductivity measurements. The conductivity values of stabilizing SNPs suspended in distilled water, were

215.0, 211.4 and 268.3 $\mu S \cdot cm^{-1}$, for 20ET, 50ET and 100ET respectively, while conductivity for MA was 0.058 $\mu S \cdot cm^{-1}$ and MA TIO was 0.063 $\mu S \cdot cm^{-1}$. The conductivity values of PEs were in the 139.9- 263.9 $\mu S \cdot cm^{-1}$ range, which means that all the PEs were o/w type emulsions.

4.4.4. In vitro diffusion studies through nail model membrane

Our goal was to formulate an emulsion that is capable of delivering the antifungal drugs through the nail plate and stays retain the drugs in the site of the infection (nail bed) for prolonged time to provide a sustained drug release. For diffusion studies, we applied PEs with different size SNPs, conventional emuslion and ethanolic solution of the same concentration, 17.91 mg/ml as antifungal drug combination, which means 3.58 mg/ml for TIO. Because of the droplet size similarity between the conventional emsulion and PEs we can assume, that only the dosage form determined the diffusion properties of the drug.

We have found that PEs possess better drug delivery properties through agar gel membrane compared to conventional emulsion and ethanolic solution. We have examined the diffusion properties of PEs with different droplet sizes, and have found that the PEs with smaller droplet size (1.85 μm) could deliver as much as 89.9 % of TIO through the agar membrane. In the experiment where the composite membrane was used, we have found that the ethanolic solution has diffused through composite membrane structure, and only a small portion (2.4%) of the drug remained in the composite membrane. The PE 20ET has delivered 89.9% of TIO through the agar gel membrane, and only 5.7% has diffused through composite membranes suggesting that 84.2 % of the applied drug remains in the targeted area. This amount was 61.1% at PE 50ET and 45.13% at PE 100ET. These *in vitro* experimental results suggest that PEs have better on site drug delivery properties.

4.4.5. Microbiological tests against Trichophyton rubrum and Candida albicans

Based on the MIC and MFC values for *T. rubrum* and *C. albicans*, the TIO and MA combination have shown a significant synergistic effect. When *T. rubrum* and *C. albicans* were treated with combination of TIO and MA, both the MIC and MFC values decreased significantly compared to the separately used drugs. Analyzing the antimicrobial data of the different formulations of TIO and MA, it can be clearly shown that the PEs are more effective than conventional emulsion or ethanolic solution against the two pathogens. The PE 20ET100 shows the most effective growth inhibition against both *T. rubrum* and *C. albicans*, and this formulation has the highest fungicidal activity.

5. Summary and novel findings

In the course of our work, we aimed to execute a size-accurate and reproducible synthesis of SNPs followed by the proper extent of surface modification of nanoparticles. Utilizing Ströber reaction from TEOS precursor, we produced SNPs with various reaction conditions, and then looked for a relationship between the reaction parameters and the properties of the nanoparticles. In summary, we achieved nanoparticle synthesis with 6-191 nm size by changing the ammonia concentration, temperature and water concentration. The experiments proved the existence of a linear correlation between ammonia concentration-particle size, reaction temperature-particle size and water concentration-particle size. With the help of linear correlation, the parameters of size- accurate synthesis can be predetermined. Regulating the size, we recommend primarily altering the ammonia concentration/temperature, because in this case spherical and highly monodisperse SNPs are obtainable.
We conducted the surface modification on order to change the hydrophilic-lipophilic characteristics of the SNPs. We used a geometrical approach to calculate the degree of surface coverage before conducting the experiments. In the reactions, we choose different silanol derivatives with different functional groups, such as methyl-, ethyl-, propyl-, and phenyl-triethoxy-silane. The success of the surface modification is proved with infrared spectroscopy, further it is established that the particle size, shape, and polydispersity is not altered by the surface modifications.

Silica nanoparticles are used to stabilize PEs, we aimed for preparing emulsions with adequate droplet size and stability for the route of administration. First,

we formulated chamomile and artemisia EOs for microbiological tests. Throughout my work o/w PEs are prepared with up to 3 months of stability and droplet size ranging from 160 nm to 1415 nm. The SNPs are used in the formulation were 20 nm, their surface modification is performed with propyl functional groups, in case of the chamomile EO in 50%, in case of artemisia EO in 20% coverage, thereby we reached adequate stability in the 0.1-3.5 mg/ml EO concentration range. In both cases, the PE form were significantly more effective against Gram positive, Gram negative bacteria and fungi than the solution or the conventional emulsion systems.
With the interaction between different formulations and cell models, we determined PEs outperformed solutions and conventional emulsion in penetration through cell membrane by orders of magnitude. Assuming that the PE droplets absorbed to the cell membrane, the increased penetration can be caused by two phenomena. The EOs are transported through the cell membrane with passive diffusion caused by the large local concentration gradient resulted by the absorption; or the PE droplets get into the cell with endocytosis. The exact method yet to be researched. Throughout the microbiological test the SNPs were inert there was no observable antibacterial or antifungal effect present, however the ethanol used as solvent and the Tween80 surfactant both showed antimicrobial effects. The more effective penetration of the PEs through the cell membrane and their inert characteristics indicates that PEs possess proper properties for EO formulation.

Through the course of our work we aimed for the local treatment of the *Streptococcus mutans* dental biofilm, o/w PEs were prepared with adequate droplet size and stability using peppermint, cinnamon, thyme and clove EOs. As stabilizing agent, SNPs with 20 nm diameter and with ethyl group modifications with 20% surface coverage showed the best results. The size of the PEs ranged between 210-370 nm, in this scale the penetration in the smaller pores of the biofilm and the EO transport to the deeper layers is obtainable. The stability of the prepared emulsions ranged from one week to four months.
Conducting microbiological test, we concluded an increased biofilm degradability in the case of PE preparations compared to the solutions, conventional emulsions. Assumably the difference is caused by the altered diffusion properties due to formulation. We performed in vitro diffusion tests on model biofilm membranes with all the EOs to prove our thesis. The results of the diffusion tests and microbiological tests show similar outcome. The largest amount of EO diffused through the membrane from the PE, followed

by the emulsion and the solution. The lowest measured MIC value indicated, that the thyme EO has the most significant microbiological effect. Despite showing low biofilm degradational activity, which can be explained given that in case a PE less that 19% of the thyme EO diffused through the model biofilm membrane. The most effective preparation regarding the biofilm degradability was the cinnamon EO PE, in this case the degradation was 85.9% and 51.4% cumulative EO quantity could be measured in the diffusion test. In summary it can be stated, that the o/w type PEs stabilized were more effective formulation in the local treatment of *S. mutans* dental biofilms.

In the course of the preparation and research of PEs for local treatment of nail fungus we found the following results. The coformulation of two lipophilic API TIO and MA is executable. Using the Hansen solubility parameters and investigating the kinetic solubility we concluded that TIO can be dissolved in MA in a great proportion (>20 w/w%) and the solution appropriate as the oil phase of the PEs. The conducted microbiological tests with *Trichophyton rubrum* and *Candida albicans* fungi species concluded that our presumption about the synergic effect of TIO and MA. It was confirmed by the measurement of MIC and MFC values.

Throughout the preparation of PEs, we used constant surface modifications (20% ethyl) and applied three SNPs with different size. The results indicated that the size of the PE stabilizing SNPs influenced the droplet size and stability, using silica with smaller particle size the droplet size decreased and the stability increased. Using 20 nm SNP, the droplet size of the PEs can be adjusted in a linear function of the oil phase concentration. Through the course of our work we aimed for targeted delivery, in this case the lipophilic, large molecular weight TIO transported through the nail plates and penetrates to the nail bed and stays at the place of the action. In vitro diffusion tests were performed to model the nail plate and the nail bed. The diffusion tests resulted in confirming the PE form superiority in targeted delivery compared to the solution or conventional emulsion form of TIO preparations. Further the amount of released API can be controlled by the alteration of emulsion stability, particle size respectively the droplet size. In summary, we confirmed that PE application in onychomycosis topical, targeted treatment is a promising field of research.

6. List of publications

6.1. Articles related to the thesis

Barbara Horváth, Szilárd Pál, Aleksandar Széchenyi: Preparation and in vitro diffusion study of essential oil Pickering emulsions stabilized by silica nanoparticles. *Flavour and Fragrance Journal* ***2018****, 33, 385–396.*
IF: 1.970 DOI: 10.1002/ffj.3463

Barbara Horváth*, Viktória L. Balázs*, Adorján Varga, Andrea Böszörményi, Béla Kocsis, Györgyi Horváth, Aleksandar Széchenyi: Preparation, characterisation and microbiological examination of Pickering nano-emulsions containing essential oils, and their effect on Streptococcus mutans biofilm treatment. *Scientific Reports* ***2019****, 9, 16611.*
*First authors with equal contributions. **IF: 4.525** DOI: 10.1038/s41598-019-52998-6

Sourav Das*, Barbara Horváth*, Silvija Šafranko, Stela Jokić, Aleksandar Széchenyi, Tamás Kőszegi: Antimicrobial Activity of Chamomile Essential Oil: Effect of Different Formulations. *Molecules* ***2019****, 24, 4321.*
*First authors with equal contributions. **IF: 3.060** DOI: 10.3390/molecules24234321

Sourav Das*, Barbara Vörös-Horváth*, Tímea Bencsik, Giuseppe Micalizzi, Luigi Mondello, Györgyi Horváth, Tamás Kőszegi, Aleksandar Széchenyi: Antimicrobial Activity of Different Artemisia Essential Oil Formulations. *Molecules* ***2020****, 25, 2390.*
*First authors with equal contributions. **IF: 3.060** DOI: 10.3390/molecules25102390

Barbara Vörös-Horváth, Sourav Das, Ala' Salem, Sándor Nagy, Andrea Böszörményi, Tamás Kőszegi, Szilárd Pál#, Aleksandar Széchenyi#. Formulation of Tioconazole and Melaleuca alternifolia Essential Oil Pickering Emulsions for Onychomycosis Topical Treatment. *Molecules* ***2020****, 25(23), 5544.*
#Corresponding authors with equal contributions. **IF: 3.267** DOI: 10.3390/molecules25235544

∑IF: 15.882

6.2. Publications not related to the thesis

Viktória Lilla Balázs, Barbara Horváth, Erika Kerekes, Kamilla Ács, Béla Kocsis, Adorján Varga, Andrea Böszörményi, Dávid U. Nagy, Judit Krisch, Aleksandar Széchenyi, Györgyi Horváth: Anti-Haemophilus Activity of Selected Essential Oils Detected by TLC-Direct Bioautography and Biofilm Inhibition. *Molecules* ***2019****, 24, 3301.* **IF: 3.060** DOI: 10.3390/molecules24183301

Salem A., Hagymási A., Vörös-Horváth B., Šafarik T., Balić T., Szabó P., Gősi F., Nagy S., Pál S., Kunsági-Máté S., Széchenyi A. Solvent dependent 4-aminosalicylic acid-sulfamethazine co-crystal polymorph control. *European Journal of Pharmaceutical Sciences* ***2021****, 156, 105599*. **IF: 3.616** DOI: 10.1016/j.ejps.2020.105599

7. Abstracts

7.1. Oral presentations

Barbara Horváth, Szilárd Pál, Aleksandar Széchenyi: Theoretical and practical aspects of essential oil emulsions stabilization with solid nanoparticles. *48th International Symposium on Essential Oils ISEO2017, Pécs, Hungary,* ***10-13 September 2017****.*

Széchenyi A., Horváth B., Šafarik T.,Sándori,D., Nagy S., Pál Sz.: Application of solid nanoparticle as emulsifiers and surface modifiers in controlled drug delivery. *7th BBBB International Conference on Pharmaceutical Sciences, Balatonfüred, Hungary,* ***5-7 October 2017****.*

Horváth Barbara, Nagy Sándor, Balázs Viktória Lilla, Das Sourav, Pál Szilárd, Kocsis Béla, Kőszegi Tamás, Széchenyi Aleksandar. Pickering nano emulziók a gyógyszertechnológiában. *Gyógyszertechnológiai és Ipari Gyógyszerészeti Konferencia 2019, Siófok, Magyarország,* ***2019. szeptember 26-28****.*

7.2. Poster presentations

Horváth, B., Bárdonicsek, N., Takácsi-Nagy, A., Pál, Sz., Széchenyi, A. Pickering emulsion of tea tree oil in water, stabilized with silica nanoparticles for onychomycosis topical treatment. *7th BBBB International Conference on Pharmaceutical Sciences, Balatonfüred, Hungary,* ***5-7 October 2017****.*

K Kvell, B Kollar, B Horvath, A Szechenyi, Sz Pal. Steroid-harboring nanoparticles provide anti-inflammatory response with less adverse effects. 6th

International Conference and Exhibition, Austria, Vienna, ***November 09-11, 2017****.*

Barbara Horváth, Viktória Lilla Balázs; Nikolett Bárdonicsek, Szilárd Pál; Györgyi Horváth; Aleksandar Széchenyi. In vitro diffusion study of tee tree essential oil emulsions stabilized by silica nanoparticles. *11th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Granada, Spain,* ***19-22 March 2018***

Balázs Viktória L, Horváth Barbara, Széchenyi Aleksandar, Varga Adorján, Kocsis Béla, Ács Kamilla, Kerekes Erika, Krisch Judit , Horváth Györgyi. A fahéj és a szegfűszeg illóolajának nanotechnológiai formulálása és biofilm gátló hatásvizsgálata. *Gyógyszer Innováció 2018 Konferencia, Velence, Magyarország,* ***2018. április 9 11****.*

Barbara Horváth, Viktória L. Balázs, Györgyi Horváth, Széchenyi Aleksandar. Preparation and in vitro diffusion study of essential oil Pickering emulsions stabilized by silica nanoparticles for Streptococcus mutans biofilm inhibition. *49th International Symposium on Essential Oils ISEO 2018****, September 13-16, 2018****, Niš, Serbia*.

Viktória L. Balázs, Barbara Horváth, Erika Kerekes, Aleksander Széchenyi, Adorján Varga, Béla Kocsis, Kamilla, Ács, Judit Krisch, Györgyi Horváth. The nanotechnological formulation and anti-biofilm activity of thyme essential oil against Streptococcus pneumoniae. *49th International Symposium on Essential Oils ISEO 2018,* ***September 13-16, 2018****, Niš, Serbia*.

Horváth, B., Nagy, S., Pál, Sz., Széchenyi, A. Application of nanotechnology in formulation of tioconazole and melaleuca alternifolia essential oil for onychomycosis topical treatment. *12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs and Satellite Symposium on Pharmaceutical Biotechnology, Szeged, Hungary,* ***20-22 September 2018****.*

Barbara Horváth, Ala' Salem, Tatjana Šafarik, Szilárd Pál, Aleksandar Széchenyi. Systematic study of reaction conditions for size controlled synthesis of silica nanoparticles. *6th Nano Today Conference, Lisbon, Portugal,* ***16-20 June 2019***.

Horváth Barbara, Nagy Sándor, Balázs Viktória Lilla, Pál Szilárd, Széchenyi Aleksandar. Teafaolajban oldott tiokcoanzol tartalmú pickering emulziók előállítása és alkalmazása körömgomba helyi kezelésére. I. *FKF Szimpózium, Fiatal Kémikusok Fóruma Debrecen, Magyarország,* ***2019. április 3***

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