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Application of new types of surfactants for the development of an adjuvant containing squalene by ultrasonic production

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Abstract: Nowadays, the pandemic events have brought vaccines back into the focus of scientific research. The adjuvants are extremely important for the effectiveness of vaccines. Research and development based on the modernization of ingredients enables the development of more effective adjuvants with a better risk-benefit ratio. In our study, we aimed to develop squalene-containing nanoemulsion adjuvants using modern surfactants. The ultras method was used to develop a squalene-based adjuvant. The size and zeta potential of the created nanoemulsion adjuvant systems and their stabilities were investigated by DLS techniques. Both *in vitro* and *in vivo* harmlessness were demonstrated. The immunoglobulin production-promoting effect (IgG and IgE) was tested. Using the ultrasound size reduction process, the achieved size range was less than 200 nm. The size and zeta potential of the preparation remain stable for several months without significant changes. The developed adjuvant was not toxic in *Galleria mellonella* larvae and in the fibroblast cell line. The formulation achieved similar IgG and IgE production to Addavax, with IgG and IgE levels two orders of magnitude higher after 45 days compared to the OVA dosing alone. In summary, we have successfully developed a modern tenside-containing squalene adjuvant.

Keywords: ultrasonic; squalene; tenside; surfactant; nanoemulsion

We recommend compliance with the Mesh guidelines: https://www.ncbi.nlm.nih.gov/mesh/.

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1 Introduction

Rapid, emerging pandemics provide global challenge to all healthcare systems, and it became apparent, that vaccine developments must be carried out as quickly as possible, which makes it necessary to revert to old recipes and methods in order to react quickly and efficiently. Although mRNA-based vaccines are gaining ground, requiring different immune activation adjuvants, inactivated or fragment-containing vaccines still have a legitimate claim to exist. Their use remains important in childhood and against viruses or bacteria for which it is not worth developing an mRNA-based vaccine.[1] The example of the COVID-19 pandemic has highlighted the vital importance of focused and fast vaccine research programs and the undeniable role of ingredients, especially adjuvants, in increasing the effectiveness of vaccines.[2,3] More than half of the COVID- 19 vaccines still used technology that required adjuvants that have

long been known and used. These adjuvants are extremely important for the effectiveness of vaccines because they help the immune system respond faster and more vigorously to the vaccine.[4,5] For most vaccines against coronavirus disease, gold standards such as aluminium-hydroxide or squalene have been used as adjuvant.[6] Aluminium-based adjuvants have long been widely used in vaccines,[7] but new research and development makes it possible to develop more effective adjuvants that could potentially be more beneficial. Squalene-based nanoemulsions are promising innovations in the field of adjuvants. Squalene, a natural oil found in human skin,[8,9] for example, is necessary for the effective functioning of the immune system.[10,11] Its application in nanoemulsions enables a more effective and targeted immune response, since squalene is divided into smaller droplets and can easily enter the cells.[12,13] Unlike aluminium-based adjuvants, squalene-based nanoemulsions have the potential to reduce local irritation and inflammation, which can severely lower the frequency and degree of adverse effects after vaccination. In addition, nanoemulsion adjuvants can more effectively activate cellular and humoral components of the immune system, which is necessary for an effective immune response.[14] The biological effect of nanoemulsions depends largely on the size of droplets and the surface properties of the droplets formed. The utilized surface-active agents largely determine the above properties of nanoemulsion systems.[15,16] The pandemic has often brought vaccine ingredients to the patient's focus point, in most cases decades old and often containing ingredients that are long out of date. Fear of the ingredients has led to a complete rejection of vaccines in many cases.[17–19]

In our research, we aimed to develop squalene-containing nanoemulsion adjuvants using more modern surfactants than the existing formulations. These adjuvants have been evaluated by a series of tests to demonstrate stability, safety and efficacy, confirming their equivalence to old standard adjuvants. Furthermore, we have applied methods that offer suitable alternative production and manufacturing methods to produce sterile nanoemulsions on an industrial scale, which is currently a huge challenge for the pharmaceutical industry.

2 Materials and methods

2.1 Materials

Squalene, Tween 80, Span 85 and *α*-tocopherol-acetate were purchased from Sigma Aldrich Kft. (Budapest, Hungary). Other solubilizers were gifts from Gatefossé (Lyon, France), whose HLB values are shown in Table 1. All other reagents were purchased from Sigma Aldrich Kft. (Budapest, Hungary) in analytical grade. The balb/c mouses were bred by AnimaLab Hungary Kft. (Vác, Hungary).

2.2 Production of nanoemulsion

The nanoemulsions with the different composition of surfactants were prepared by the following method (Figure 1). Each emulsion contains 5 % ($^{v}/_{v}$) squalene, 0.5 % ($^{v}/_{v}$) low HLB tenside, 94 % ($^{v}/_{v}$) citrate buffer and 0.5 % ($^{v}/_{v}$) high HLB tenside (Table 2), so that the final volume was 10 mL. The 50 µl of low HLB surfactant was added to squalene phase, meanwhile the high HLB ones were dissolved in citrate buffer at pH 6.5. These two phases were mixed with a vortex mixer for 2 min at room temperature to produce a total of 24 unique compositions (Table 2), of which the composition designated T1 is a tenside pair already present in an existing adjuvant (Addavax). The created microemulsions' droplet sizes were further decreased by sonication. The nanoemulsion formulation was performed at 50 % amplitude for 2 min in every case. During sonication the emulsion was kept on an ice bed to avoid overheating.

2.3 Dynamic light scattering evaluation of nano-emulsions

The colloidal properties of nanoemulsions were measured by dynamic light scattering. The created nanoemulsions

Table 1: Applied surfactants and solubilizers, their chemical names and their hydrophilic-lipophilic balance (HLB) values.

Brand name	Chemical name	HLB[a]
Capryol 90	Propylene glycol monocaprylate	5
Labrafac lipophile WL1349	Medium-chain triglycerides of caprylic and capric acids	1
Maisine CC	Glyceryl monolinoleate	1
Peceol	Glyceryl monooleate	1
Plurol Oleique CC	Polyglyceryl-3 dioleate	3
Transcutol HP	Diethylene glycol monoethyl ether	Not applicable
Span 85	(Z,Z,Z)-Sorbitan tri-9-octadecenoate	1,8
α-tocopherol-acetat	*α*-tocopherol-acetat	0,0
Kolliphor RH40	Macrogolglycerol hydroxystearate	14–16
Kolliphor EL	Macrogolglycerol ricinoleate	12–14
Tween 80	Polyethylene glycol sorbitan monooleate	15

[a]The HLB values given are based on the manufacturer's specifications, which are available on the manufacturer's website.

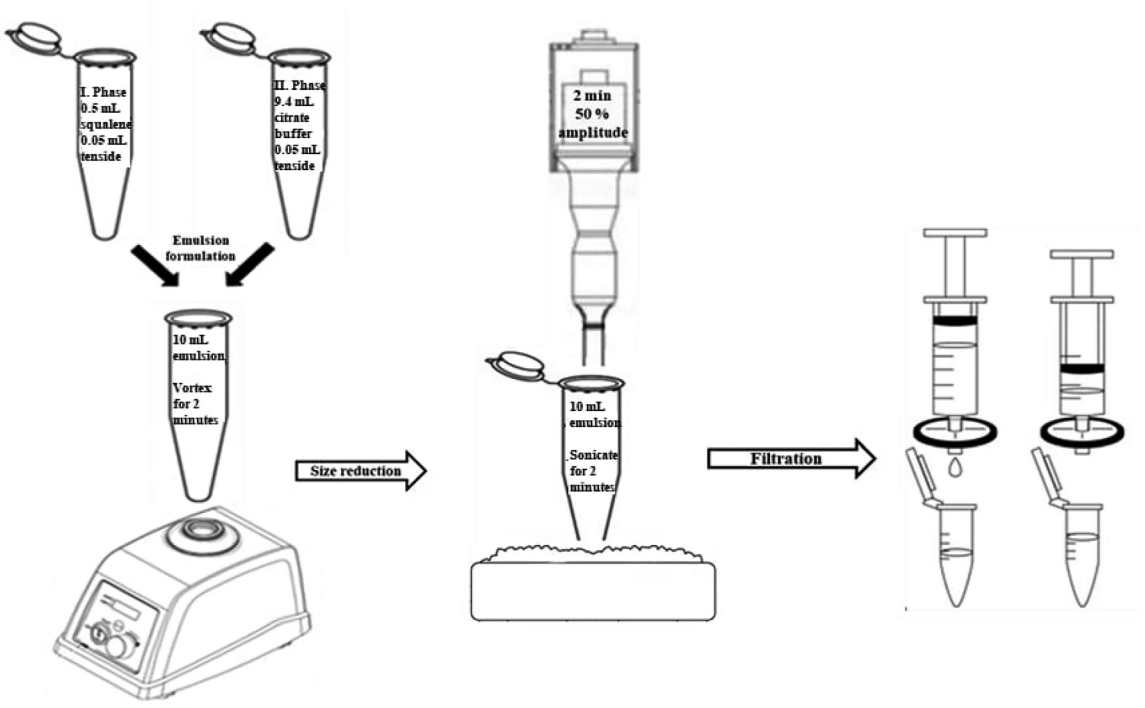

Figure 1: Step-by-step formulation of nanoemulsion. Firstly, the surfactants were dissolved at the right phases, then two phases were mixed by vortex, after that ultrasonic was used to decrease the droplet size on an ice bed. Finally, the nano emulsions were filtered by 0.2 µm PES membrane filter.

Table 2: Final compositions. All of samples initialled T contained Tween 80 tenside, all of compositions initialled R contained Kolliphor RH40, and all of samples initialled E contained Kolliphor EL. The variables within these groups of compositions were the low HLB tensides.

		Citrate buffer		
		Tween 80	**Kolliphor RH 40**	**Kolliphor EL**
Squalene	Span 85	Addavax (T1)	R1	E1
	Peceol	T2	R2	E2
	Capryol 90	T3	R3	E3
	Transcutol HP	T4	R4	E4
	Labrafac™ lipophile WL 1349	T5	R5	E5
	Maisine CC	T6	R6	E6
	Plurol olieque CC 497	T7	R7	E7
	α–tocopherol	T8	R8	E8

Letter and number combinations indicate samples, as described in the table title, with only T1, as a marketed positive control, being added to the table with a name.

were diluted with citrate buffer (0.02 mL nanoemulsion and 1.98 mL citrate buffer) and a Malvern Nano-ZSP Zetasizer (Malvern Instruments, UK) was used to determine the size distribution and zeta potentional of the droplets. In case of each sample, five parallel measurements were performed at 25 °C.

2.4 Stability study

To evaluate the formulation stability, a modified ICH stability guidelines were used.[20–22] The long-term stability studies were carried out at 4 °C for just 3 months. 10 mL of the final compositions were placed into a fridge for 3 months placed in a way to avoid freezing. 1 mL of samples were taken after 1, 2 and 3 months and the size distributions and polydispersity of the nanoemulsion were measured.

2.5 MTT assay

The cytotoxic effects of nanoemulsions were evaluated using MTT test. The NIH-3T3 cell line was obtained from the ATCC (Manassas, USA). Cells were grown in Nunc™ EasyFlask™ (Thermo-Fisher, Darmstadt, Germany) surface-treated 75 cm^2 plastic cell culture flasks in Dulbecco's Modified Eagle's Medium with 4.5 g L^{-1} D-glucose, 0.584 g L^{-1} L-glutamine

and phenol red (Sigma-Aldrich), supplemented with 3.7 g L^{-1} $NaHCO_3$ (Sigma-Aldrich), 10 % ($^{v}/_{v}$) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1 % ($^{v}/_{v}$) non-essential amino acids solution (Lonza, Basel, Switzerland), 100 IU mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin solution (Lonza) at 37 °C in an atmosphere of 5 % CO_2. The cells were routinely maintained by regular passaging and glutamine was supplemented by GlutaMax™ (Thermo-Fisher). The cells were subcultured twice per week at 80 % confluence and the passage number was kept under 20 times. NIH-3T3 cells were seeded on 96-well plates in 20,000 cells/well concentration. After 24 h, the DMEM medium was removed, and the cells were treated with nanoemulsions (T6, Addavax) in 0.1 % ($^{v}/_{v}$) at 37 °C for 30 min. The nanoemulsions were removed and then the cells were washed gently twice with PBS. The cells were then incubated with 0.5 mg mL^{-1} MTT solution for 3 h at 37 °C, after which the dye was removed and 0.1 mL of a solution of isopropanol and 1 M hydrochloride acid (25:1 ratio) was used to dissolve the formed formazan crystals. The absorbance of the dissolved formazan crystals was measured at 570 nm and the background was measured at 690 nm by Thermo-Fisher Multiskan Go (Thermo-Fisher, USA) microplate reader. Triton-X at the concentration of 0.5 % ($^{v}/_{v}$) was used as positive control and PBS was used as a negative control.

2.6 *G. mellonella* larvae survivability test

Larvae of the sixth developmental stage of *Galleria mellonella* were obtained from Bugs World Inc. (Budapest, Hungary). After arrival, the larvae were stored at 10 °C and in a dark environment for 12 h. Groups consisting of 12 larvae were selected from the original stock and placed into sterile vented Petri dishes. The size of the members was between 2 and 3 cm, they showed no sign of melanization and the weight of each group was equalized. The final formulation and Addavax were diluted with citrate buffer (1:1 ratio) for injection and 20 µL of each sample was injected into the *G. mellonella* haemocoel through the last pro-leg using a 29 G needle. The injected larvae were incubated at 30 °C for 48 h in dark environment in humid air. For the assessment of larval viability, larvae were gently probed with a blunt-ended needle and if no response was observed, the larvae considered were dead. Viability was observed at 15, 24, 39, 48 h.

2.7 *In vivo* antibody production

The *in vivo* antibody production study was conducted according to regulations of the Hungarian Scientific Ethical Committee on Animal Experimentation (approval number: 6/2023/DEMÁB). 20 balb/c female mice (12–36 weeks old) were involved in the experiment. Animals were kept in autoclavable PC cages (3 animals per cage), under 12-12 h of light and dark cycles. Temperature was set 22 ± 2 °C, and relative humidity to 55 ± 10 %, through continuous mechanical air exchange with filtered air blowing. Water and food were available to the animals *ad libitum*.

Animals were immunized subcutaneously with appropriately prepared 100 µL sterile adjuvant systems, that contained 1 µg ovalbumin with Addavax® or T6, and we used as control 1 µg ovalbumin, citrate buffer, and free T6 300 µL of blood were collected from the animals after immunization after 15, 30, 45 days to prove the antibody production. Blood was collected from the tail vein under anaesthesia (ketamine:xylazine 50:10 mg kg^{-1}). Antibody titers (IgG and IgE) were determined from the serum using ELISA kits.

2.8 IgG and IgE level

Anti-Ovalbumin IgG1 and IgE (mouse) ELISA Kit (Cayman Chemical, Ann Arbor, USA) was used to determine the level of the OVA-IgG and OVA-IgE in the mouse serum after the immunization. First, the standard solutions were created in Assay puffer. In the case of T6, OVA and the positive/negative control the samples were diluted to a ratio of 1:10, while in case of OVA + Addavax and OVA + T6 to a ratio of 1:100 (1 µL adjuvant + OVA and 99 µL Assay buffer). The dilution rates were determined in preliminary experiments. 100 µL of standards and diluted samples were added to the wells of the ELISA plate and incubated for 2 h at room temperature on an orbital shaker. Then the wells were washed four times with wash buffers and residual wash buffers were removed. Detection antibody working solution (100 µL) was added to each well and incubated again at room temperature for 1 h. Before the incubation with 100 µL TMB substrate, we washed four times again with the appropriate wash buffers. After 30 min, the redox reaction was stopped by 100 µL HRP solution. The color change was detected by a Multiskan Go plate reader at a wavelength of 450 nm. In case of calibration curve, quadratic equation was used.

2.9 Statistical analysis

For statistical analysis GraphPad Prism 6 software was used. Dates are presented as means ± SD. Comparison of two groups

was performed by unpaired *t*-test. During the *in vivo* antibody production, Kruskal-Wallis test and Dunn post-test were used and differences were considered significant at $p < 0.05$.

3 Results

3.1 Production of nanoemulsion

The compositions that are shown in Table 2 were prepared using the method described above. The Tween 80 paired well with low HLB surfactants to produce nanoemulsions in almost all cases, except for *α*-tocopherol (T8). In the cases of the Kolliphors RH 40 and EL, the Span 85, Peceol and *α*-tocopherol surfactants were suitable to produce nanoemulsions. The Kolliphor RH 40 and Maisine CC were also successful together to reach lower size than 1 µm. For Capryol, Plurol Oleique CC, Transcutol HP and Labrafac Lipophile WL1349 with higher HLB values, phase separations were observed after formulation.

3.2 Dynamic light scattering evaluation of nano-emulsions

The size distributions of the squalene droplets of the successful formulations T1 to E8 were determined by dynamic light scattering method and the results are presented in Figure 2. Based on our preliminary experiments, we can successfully filter emulsions under 300 nm through a 0.2 µm membrane. This range of sizes was considered the upper limit and was used to select those formulations these were considered successful during development and suitable for further testing. In the case of Peceol and Maisine CC, the combination with Tween 80 (T2 and T6) or Kolliphor RH 40 (R2 and R6) resulted <300 nm droplets after preparation. Tween 80 formed with Capryol 90 (T3) and Plurol Olieque 497 (T7) a significantly larger droplet size nanoemulsion than T2 and T6. In the case of Kolliphor EL, only the *α*-tocopherol (E8) sample shows good results. Samples T4; T5; E1, E2 and R8 were excluded from further experiments, because, although their size is in the nanoscale, they are several times larger than the limit to filter.

The size distribution after sterile filtration is shown in Figure 3. For R1 and E8, filtrations of the emulsions were not possible, the oil droplets in the emulsion clogged the pores of the filter, preventing filtration and they were excluded from further tests. For compounds with droplet sizes larger than 200 nm, sterile filtration led to a reduction in size. In these cases, except for T3, an average droplet size below 200 nm was obtained.

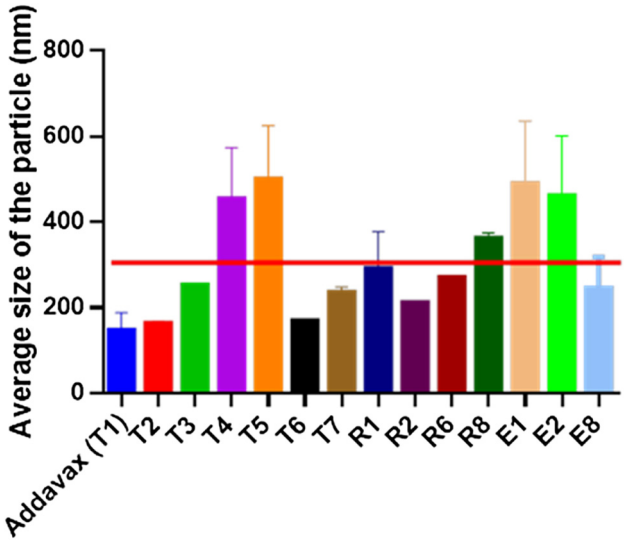

Figure 2: Results of size distribution of the compositions. The red line shows the upper limit of the sterile filtration. (Data is presented as means ± SD; $n = 5$).

Determining the zeta potential is necessary to predict long-term stability and the result is shown in Figure 4. The zeta potential of samples R6 and R2 did not reach – 20 mV, indicating that the formed nanoemulsion is not sufficiently stable and they were excluded from further experiments. The additional emulsions have sufficiently high zeta potential in absolute value to remain stable.

3.3 Stability study

Figure 5 shows the stability of the samples, the average droplet size and polydispersity were measured. The polydispersity changed significantly for sample T7, as it was excluded from further experiments. Of the remaining two

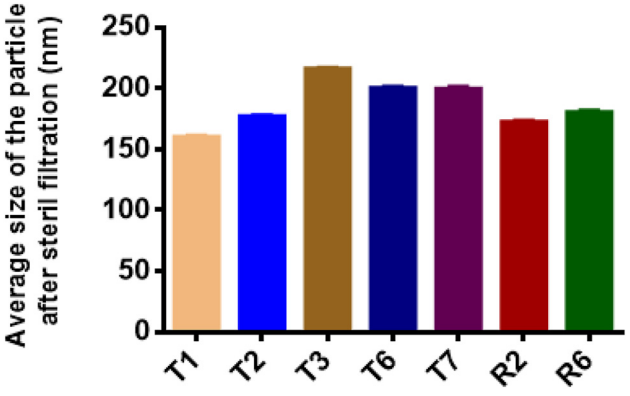

Figure 3: Results of size distribution after sterile filtration. (Data is presented as means ± SD; $n = 5$).

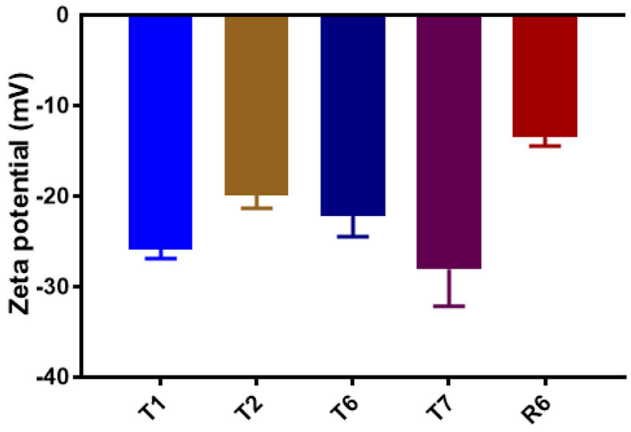

Figure 4: Results of zeta potential. (Data is presented as means ± SD; *n* = 5).

samples (T2, T6), T6 was the best, as its droplet size did not change over time, and it has the lowest polydispersity of the two samples.

3.4 MTT assay

Based on the MTT tests, neither T6 nor Addavax caused significant cell death, as the cell viability values are above 90 % (Figure 6). The results confirm the biocompatibility and safety of the sample in NIH-3T3 cell line.

3.5 *G. mellonella* larvae survivability

In vivo larval tests have demonstrated the harmlessness of the adjuvants before they were used in animal studies. *G. mellonella* larvae were injected with both Addavax and the T6 formulation. During our experiment no larvae died from the injected materials. The results confirm the biocompatibility of the samples.

3.6 *In vivo* antibody production

The IgG immunoglobulin blood concetration is presented in Figure 7. In animals receiving OVA alone, IgG levels against OVA were produced in some animals, but were not statistically different from control animals until day 45. Significant increases in IgG level were already observed on day 45 after a single subcutaneous administration of adjuvants compared to OVA-only animals. Compared to the Addavax group, no significant difference could be observed when using the T6 formulation.

Figure 7 also shows that IgE immunoglobulin production increased significantly 45 days after injection in the T6 sample. However, when using Addavax, IgE levels were not significantly different than T6. IgE production was much lower than IgG in all cases.

4 Discussion

In the present study, we aimed to prepare a squalene-containing nanoemulsion system, which uses newer, modern tensides instead of the old traditional ones and prove similar applicability of new formulations while improving the benefit-risk value of adjuvants. We first created o/v emulsions of different compositions, which were reduced in size to the appropriate extent by ultrasonication, one of the most common high-energy processes.[23–25] Based on the results obtained so far, the size of the stable nanodroplets that can be produced by ultrasonic technology is 30–300 nm.[26,27] Previous studies have already compared different options for the production of nanoemulsions for parenteral administration, measuring the droplet size and polydispersity of the emulsions produced by each method. Of the methods used, the microfluidisation technology produced the emulsion with the smallest droplet size (110 nm), but its stability was not satisfactory. Stable nanoemulsions were also obtained by ultrasound and premix membrane emulsification.

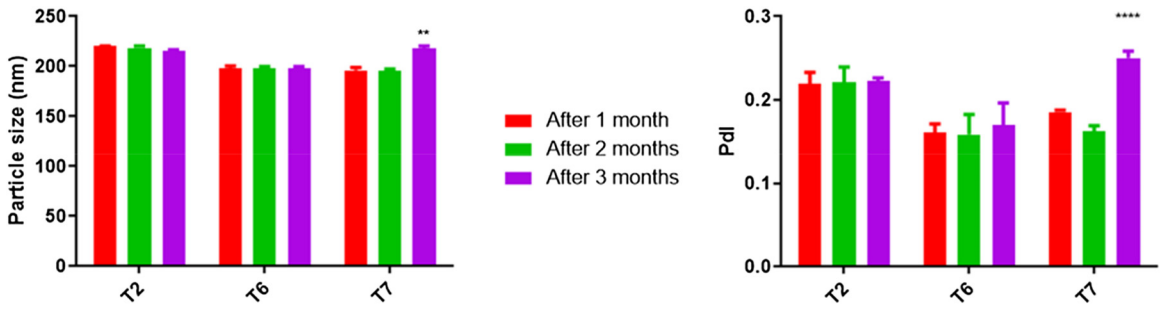

Figure 5: Change in size distribution and in polydispersity during the stability test after 1, 2 and 3 months. (Data is presented as means ± SD; *n* = 5, **, **** indicate statistically significant differences from the 0. time at p < 0.0001 and 0.01).

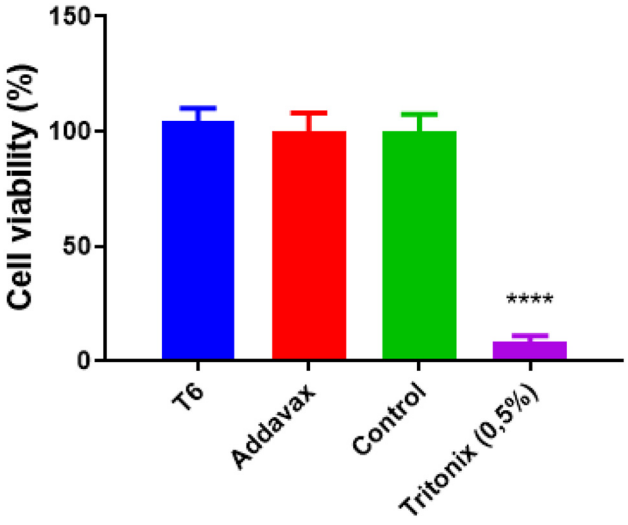

Figure 6: Results of MTT test after 0.5 h incubations. The Addavax and T6 samples are 0.1 % ($^{v}/_{v}$) while Triton-X is 0.5 % ($^{v}/_{v}$). (Data is presented as means ± SD; n = 3, **** indicates statistically significant differences from buffer control at $p < 0.0001$).

However, in terms of size, the nanoemulsion produced by ultrasound has a much smaller droplet size (173 nm) than the nanoemulsion produced by premix membrane emulsification (335 nm).[28] Also for nanoemulsions containing squalene, systems with droplet sizes around 180–200 nm have been successfully prepared by various methods.[29–32] The developed nanoemulsion system has proven to be applicable and effective *in vitro* and *in vivo*. The methods and tests used in the research, such as dynamic light scattering (DLS) analysis, helped to determine the droplet size and stability of nanoemulsions. Based on the results of DLS studies, nanoemulsions with too large droplet size were excluded and systems with appropriate droplet sizes were focused on. Although microemulsions can be administered parenterally after appropriate sterilisation procedures, the effect of adjuvants is greatly reduced in non-nano systems.[33–35] The size of the stable emulsions we have created using ultrasound have the average droplet size of 190–200 nm after sterile filtration. That size range is similar to the well-known adjuvants already on the market.[36–38] The tenside pairs used in our emulsions have not been used in this form yet. Our results show that for oleate derivatives, a good size reduction is obtained with the addition of any tenside pair. For the caprylic acid, tocopherol and deitylene glycol derivatives, the size reduction was not satisfactory, presumably as a result of the hydrophilic groups, and for squalene, the use of oleate derivatives is preferable to achieve nano-sized droplets. Microemulsions have been successfully created using Maicine CC- Tween 80- Transcutol as previously described by others,[39] but to date, no nano-scale emulsions have been formed for parenteral use. In addition, Safia S. et al. in one of their studies also investigated whether the cytotoxicity of the newer types of tensides differed from the older ones and found that the newer types (Gattefose tensides and self-emulsifying excipients) were less cytotoxic, making them safer to use as adjuvants.[40,41] Nanoemulsions can be considered stable at zeta potentials higher than 20 mV absolute, as even at such high surface voltages there is little chance of droplet fusion,[42–44] but for human parenteral use the target value is −30 mV, at which stability is assured and no harmful interaction with cellular elements is observed due to the negative surface charge. Thus, the successful formulations obtained by us can be considered stable, since values between −20 and −30 mV were obtained. During the stability studies, we found that the developed nanoemulsion adjuvants remained stable over the long term, which is an important aspect in the production of vaccines. Based on literature data, nanoemulsions in the 200 nm or lower range are stable, remaining unchanged in size and homogeneous for up to 6 months.[45] Based on the literature reviews and the results obtained, the nanoemulsions we have prepared can be considered stable. The adjuvant size of commercially available AS03 and MF59 are 160 nm.[46] The size of Addavax

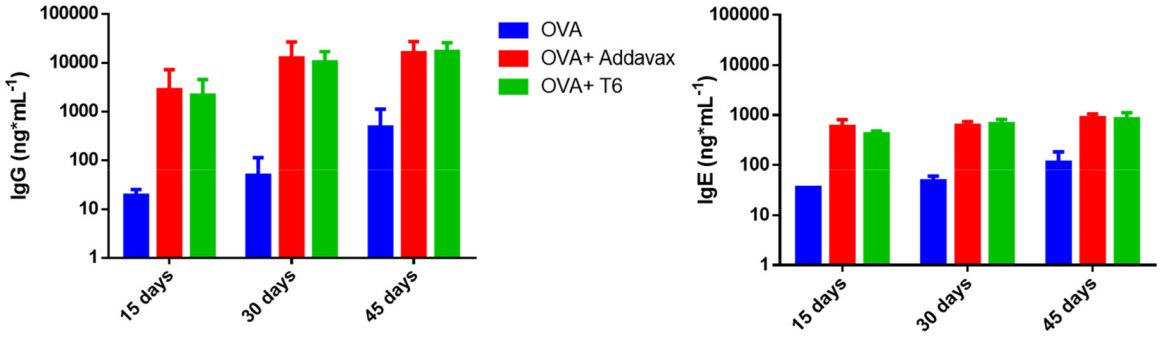

Figure 7: IgG and IgE levels after injection. The amount of antibody raised by samples containing adjuvant was compared to that raised by OVA at the same time point. (Data is presented as means ± SD; n = 4).

used in preclinical studies is also ~160 nm. However, adequate information on their zeta-potential value cannot be found so we could only use our own measured data for comparison, which showed that the adjuvant developed and purchased did not differ significantly. In the cell viability studies, the NIH-3T3 fibroblast cell line was chosen because most vaccines administered in contact with connective tissue cells.[47,48] We found that neither the adjuvant we developed nor Addavax, which is already on the market, caused cell death, so we can say that the adjuvants are safe to use. A survival test of *G. mellonella* caterpillars allowed systems to be tested for *in vivo* compatibility and survival chances, and the results were positive.[49–51] *In vivo* immunological studies have shown that the developed adjuvants effectively stimulated the immune system response, and Anti-Ovalbumin IgG1 and IgE ELISA tests have also confirmed efficacy.[52] Comparing our results with the Addavax adjuvant, we found that the nanoemulsion adjuvants developed were at least as effective as the Addavax used as a comparison. This represents extremely promising results in vaccine development and suggests that squalene-based nanoemulsions are potentially competitive in the market for adjuvants.[53,54] The tensides used are gaining ground in parenteral use and are replacing the old tensides, allowing the development of products with a higher level of safety and a favourable benefit-risk profile.

In addition, the method of preparation developed by us makes the production of nanoemulsion adjuvants much easier and, as the above results show, the emulsions obtained remain sufficiently stable. The results of this research are of great importance for further development and human studies. New formulations and human trials will allow us to develop even more effective and safer vaccines that can contribute to fighting pandemics and reducing serious health damage and deaths in the future.

5 Conclusions

In summary, the present study successfully developed a squalene-based nanoemulsion system using modern surfactants, offering a safer and more effective alternative to traditional adjuvants. The nanoemulsions produced by ultrasound achieved stable droplet sizes within the 190–200 nm range, comparable to those of commercial adjuvants such as Addavax and AS03. The novel surfactant combinations demonstrated favorable droplet size reduction and stability, with zeta potential values within the acceptable range for parenteral use. *In vitro* and *in vivo* studies confirmed the safety and immunostimulatory efficacy of the developed formulations, with no observed cytotoxic effects and comparable or superior immune responses relative to established adjuvants. The simplified production method and favorable stability profile highlight the potential of these formulations in vaccine development. These findings lay the groundwork for future human studies and offer promising opportunities for the creation of safer, more effective vaccines with enhanced benefit-risk profiles.

Research ethics: The *in vivo* antibody production study was conducted according to regulations of the Hungarian Scientific Ethical Committee on Animal Experimentation (approval number: 6/2023/DEMÁB, 2023).

Informed consent: Not applicable.

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