Turpentine oils as a basis for the creation of nanostructured lyosols: physicochemical characteristics and stability

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Abstract. Currently, one of the most common methods for the synthesis of dispersions based on turpentine in aqueous media is the preparation of its solutions in water using multi-stage chemical and/or physical treatment. In this case, the chemical composition is selected in such a way as to ensure the complete dissolution of hydrophobic substances in water. An example of solving such a problem is the use of an aqueous solution of the following composition: gum turpentine 43.0%; oleic acid 25.0%; castor oil 18.0%; caustic soda 3.0%. However, such methods do not allow setting and controlling dimensional parameters in the final product. In addition, in many cases, such a lyosol requires the addition of stabilizers and emulsifiers, which is determined by the purpose of the synthesized lyosol. Here we show the possibility of influencing the size parameter of the resulting particles in lyosol and their resistance to sedimentation in an aqueous solution. This result is achieved by homogenization using ultrasonic dispersion of a low-concentrated aqueous solution of turpentine, bubbling with various gases and subsequent filtration of the resulting emulsion through a membrane with a suitable pore size. This approach makes it possible to obtain at least 95% of nanoparticles from the total dry matter fraction. Compounds based on such lyosols, as a rule, have immunoreactogenic properties and can be used as an adjuvant component that increases the immunogenicity of inactivated vaccines, including for veterinary use.

1. Introduction

The development of new, more effective and safer vaccines, as well as the improvement of existing ones, capable of forming a stable and longer lasting immunity, has been and remains the primary task of immunology [1,2, 3]. But an equally urgent task is the search for effective adjuvants to stimulate the immunogenic activity of veterinary vaccines, which is associated with the epidemiological, economic and partly geopolitical situation in Russia, as well as the issue of reducing the content of antibiotics necessary to reduce biological contamination of the internal organs of farm animals, including birds [4, 5]. The weak immunogenicity of some biopreparations used today indicates the need to use adjuvants in their composition that have an immunostimulating effect [6, 7, 8].

Today there are more than a dozen substances of inorganic and organic nature that are used for such purposes [9, 10, 11]. Today, special attention is paid to research devoted to the creation of antigen and adjuvant delivery systems based on bio- and synthetic polymers. Currently, the main adjuvants used in
Medical and veterinary practice are mineral compounds such as aluminum oxide gel and aluminum phosphate hydrate, bacteria and bacterial components (BCG vaccine extracts), polymer substances, complex chemical mixtures (protein-lipopolysaccharide complexes, lipopolysaccharides, muramyl dipeptide and its derivatives), lipids and emulsifiers (lanolin, arlacel); substances that cause an inflammatory reaction (saponin, turpentine, squalene) and others [12].

However, the issue of regulating and unifying the size parameter of particles formed by the components of these compounds remains outside the focus of basic research, despite the fact that both the physicochemical parameters of the lyosol itself and its biological effect depend on the particle size. Therefore, it was suggested that the regulation of the size range of the particles of turpentine oil in the composition of lyosol, and, therefore, with a high probability, the surface charge of the particles plays an important, if not key, role in the formation of adjuvant properties. Thus, an attempt was made to work out the modes of obtaining lyosol with the given granulometric characteristics.

2. Materials and methods

2.1. Materials
All materials and reagents were used according to the manufacturer's instructions and recommendations. The solvent used was water obtained at a MilliQ water treatment plant (Millipore, USA) with a resistance of 18.2 MΩ.

For the manufacture of the adjuvant substance, various types of turpentine oils (essential oil of needles, turpentine) were used as raw materials: transparent volatile liquid, with a characteristic odor without sediment and water, density at an ambient temperature of 20 °C - from 0.855 to 0.863 g/cm³, the refractive index at an ambient temperature of 20 °C is from 1.465 to 1.472, the volume fraction of distillate within the temperature range of 155-170 °C at a pressure of 101-325 Pa is 92 ± 1%, the total mass fraction of α- and β-pinene is not less than 60%, acid number - not more than 0.5 mg of KOH per 1 g of product, mass fraction of non-volatile residue - not more than 0.5%.

2.2. Methods
Granulometric control of the starting components and the final product was carried out using the method of dynamic light scattering using a Zetasizer Nano instrument (Malvern Instruments, UK), which allows detecting the molecular diameter in the range 0.3 nm - 10 μm, sensitivity to protein concentration from 0.1 mg/ml and a modular dynamic spectrometer and statistical light scattering Photocor Complex (Photocor, Russia) with similar characteristics according to the method described in [13, 14].

A helium-neon laser with a wavelength of 632.8 nm and a maximum power of 4 mW was used as a radiation source. The data obtained were processed using the Malvern Instruments software (Malvern Instruments, UK).

2.3. Equipment
Sparging was performed using a Millivac membrane pump (Merck, USA). An ultrasonic disintegrator Sonicator Q500 (Qsonica, USA) was used as a source of ultrasound. A PowerPac HC device (Bio-Rad, USA) was used as a source of electric current. Filtration was performed using syringe filters with pore diameters of 0.22 μm, 0.45 μm, and 1.5 μm (Merck, United States).

2.4. Method of obtaining lyosol
In all cases, the base emulsion was obtained by mixing 450 ml of distilled water and 10 ml of the oil phase (pine essential oil, turpentine). With stirring, the mixture was sonicated with a frequency of 26 kHz, an amplitude of 80% using an ultrasonic disintegrator for 2-5 minutes. After that, the base emulsion was placed in a special bath and exposed to a direct electric current with a voltage of 120 V for 30 min. The resulting base emulsion was filtered through a filter with an appropriate pore size, added dropwise with constant stirring in distilled water and bubbled with atmospheric air or nitrogen at an air flow rate
of 1 l/min for 30 minutes. The volumes of water and base emulsion for mixing were selected so as to obtain an emulsion with an organic phase content of 1% at the outlet.

3. Results

3.1. Obtaining lyosols based on the essential oil of pine needles and turpentine.

An ultra-emulsion of the essential oil of needles and an emulsion of turpentine using air bubbling were obtained and filtered through filters with a pore diameter of 0.22 μm, 0.45 μm, and 1.5 μm. For all the resulting emulsions, the particle size was measured (table 1).

| № | Sample name              | Filter diameter used | Particle size, time and conditions of incubation after synthesis | Particle size after 17 days |
|---|--------------------------|----------------------|------------------------------------------------------------------|-----------------------------|
| 1 | Needle essential oil ultraemulsion | 0.22 μm | 26 nm and more than 2 microns, 1 day at RT | 469 nm |
| 2 | Needle essential oil ultraemulsion | 0.22 μm | More than 6 microns, 3 days at +4 °C | 57 nm, more than 6 μm |
| 3 | Ultra-emulsion turpentine | 0.22 μm | 70 nm and more 6 microns, 1 day at RT | more than 6 microns |
| 4 | Turpentine emulsion         | 0.45 μm | 1-1.4 microns, 1 day at RT | 1.2-1.4 μm |
| 5 | Turpentine emulsion (glass) | Not filtered, only ultrasonic treatment and electric impact | 58 nm, 1 day at RT | 50 nm, 6-7 μm |
| 6 | Turpentine base emulsion     | 1.5 μm | 40 nm, 1 day at RT | 62 nm |

For a sample obtained using essential oil of needles and filtered through pores with a diameter of 0.22 μm after one day at room temperature, the presence of particles with a size of 26 nm was revealed, which is comparable to the pore size of the filter and particles larger than 2 μm. And after 17 days, the presence of particles with a size of 469 nm was detected, which is significantly larger than the pore size of the filter, but less than the size of the initially recorded micron-sized particles. Storage of this sample at + 4 °C led to the formation of particles with a size of about 70 nm and more than 6 μm after three days, and on the seventeenth day the formation of large aggregates was revealed. Thus, the sample obtained on the basis of essential oil of needles is not stable, regardless of storage conditions.

Therefore, further experiments were carried out using a sample obtained on the basis of turpentine. For this, the synthesized samples were filtered through pores with a diameter of 0.22 μm, 0.45 μm, and 1.5 μm, the stability of which was estimated only at room temperature. After one day of incubation under such conditions, the presence of nanosized particles was revealed only for the emulsion filtered through pores with a diameter of 0.22 μm and 1.5 μm. Moreover, in the first case, all the registered particles had a size larger than the pore diameter of the filter, which indicates their partial aggregation during the first day, and after 17 days their complete aggregation was recorded. The use of a glass filter with a pore diameter of 1.5 μm led to the formation of particles with a size of 58 nm and only partial aggregation after 17 days. In this case, the size of the particles present in the base emulsion after a day was about 40 nm, and after 17 days this value increased only to 58 nm, which indicates its potential stability during storage under normal conditions, which is of great both practical and scientific interest. In addition, it is obvious that the filter material has a significant effect both on the structure of the resulting particles and on the stability of the resulting emulsion sample.

3.2 Preparation of lyosol containing particles of the nanoscale range using various methods of bubbling

Since the greatest stability of the granulometric composition was revealed for the sample of the base emulsion that was not subjected to filtration, then only such a sample was used, which was treated not only with a flow of atmospheric air, but also with nitrogen for three hours. Thus, if the bubbling method
affects the surface charge of the lyosol particles, then this will indirectly affect the stability of the particle size distribution of the resulting final emulsion. Therefore, at the next stage, in addition to evaluating the particle size distribution, the surface charge of the lyosol particles was also controlled (table 2).

Table 2. Recorded values of the zeta potential of lyosols.

| №  | Sample name                                           | Zeta potential, mV | Average zeta potential, mV |
|----|------------------------------------------------------|--------------------|---------------------------|
| 1  | Turpentine base emulsion sparged with a stream of atmospheric air | -12.5, -12.0, -10.4, -13.5, -12.3 | -12.1±1.1 |
| 2  | Turpentine base emulsion sparged with nitrogen flow   | -8.59, -9.93, -8.78, -10.8, -8.04 | -9.2±1.1 |

Two samples were used for comparison. The first was a base emulsion bubbling with atmospheric air, the second was a similar abrasive treated with a stream of nitrogen. The zeta potential of the sample of the base turpentine emulsion sparged with a stream of nitrogen is, on average, -9.2 ± 1.1 mV, which is slightly higher than the sample of the base turpentine emulsion sparged with a stream of atmospheric air (-12.1 ± 1.1). At the same time, the size of particles detected after treatment with a nitrogen flow was 162.9 nm, 603.7 nm and over 6 μm (figure 1), and after treatment with an atmospheric air flow 126.2 nm, 312.5 nm and over 6 μm (figure 2). It is possible that in both cases we see aggregates or the result of merging smaller objects.

Figure 1. The result of evaluating the granulometric composition of lyosols. Distribution of the intensities of the maximum particle sizes present in the base emulsion of turpentine, bubbling with a stream of atmospheric air.
Figure 2. The result of evaluating the granulometric composition of lyosols. the distribution of the intensities of the maximum particle sizes present in the base emulsion of turpentine, bubbling with a stream of nitrogen.

4. Conclusion
The production of lyosols based on turpentine oils is an urgent applied and fundamental problem, and the possibility of obtaining emulsions containing particles of the nanometer range may be a trend in the field of medicine, pharmacology, biotechnology, and veterinary medicine. The results obtained indicate the possibility of influencing the basic physicochemical characteristics of the obtained lyosol during its synthesis. It was found that the components of the equipment, in particular the filter material, as well as the composition of the gas mixture, with the help of which the bubbling is carried out, has a significant effect on the stability of the resulting lyosol.

In this case, the exclusion of the emulsion filtration step is expressed in the formation of particles with a size less than 100 nm, which have a stable size for at least 17 days of incubation at room temperature. An increase in the duration of bubbling up to 3 hours with both atmospheric air and a stream of nitrogen, leading to an enlargement of the fractional composition and the appearance of aggregates, the size of which reaches several microns, which is most likely due to the interaction of gas constituents with emulsion components. Despite the fact that nitrogen is very inert, in its pure form it has a more pronounced destabilizing effect on the lyosol components, which is expressed in the formation of colloids of a larger size than in the case of bubbling with atmospheric air, while the zeta potential of such colloids is somewhat higher. It is possible that aggregates of smaller objects are formed in both cases, but in the emulsion obtained using a nitrogen flow, this result is more pronounced due to the difference in charge.

However, the flow of pure nitrogen is not capable of not having a significant effect on the components of the emulsion, since this gas is largely inert, therefore, it should not be excluded that the fractional composition may be influenced by mechanical action, which can be investigated in a separate series of experiment.

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