**Nanoemulsions for bioactive molecules: loading and application**

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## Introduction

Lipid or surfactant aggregates of different structure are well-known and wide used carriers for proteins (enzymes) and other bioactive substances [1-5]. There is long history of using micelles (normal and reverse) (called also microemulsions or nanoemulsions) as well as liquid crystalline structures (lamellar, hexagonal, cubic, etc.) for enzyme regulation, protein modification applying the fundamental research to chemical synthesis, analysis, medical needs, and so on [1,5,6]. According to the phase diagram of a ternary system 'Lipid (Surfactant)-water-oil (organic solvent)' (one of the widely used examples is shown on Fig.1 [4]), different ordered structures (varying by size and shape) can be formed spontaneously depending on the nature and component's ratio in the system. As seen, in diluted aqueous solutions a surfactant forms up normal spherical micelles ($L_1$), the core of which is constituted of apolar chains (tails), while the outer shell contacting with water consists of polar (and often charged) heads. In oil or apolar organic solvents a surfactant also forms spherical – but inverted (or reverse) – micelles ($L_2$); here, the core consists of polar (charged) heads while non-polar tails are oriented outside into the solvent. Normal micelles are capable to solubilize apolar compounds while reverse micelles solubilize polar compounds, primarily water. Hydration of reverse micelles is accompanied by an increase in their sizes and formation of an independent aqueous phase inside the micelle. Surfactants, both low molecular mass and polymeric, lipids, polysaccharides and polyelectrolytes, all can be used as matrix building material, and the size and properties of the matrix can be easily varied by changing the nature and concentration of components. General methodology, techniques, and potentialities of using such systems have been analyzed in recent reviews [3,5-7]. The size of such particles lies in nano-diapason, and recently the systems were 'rediscovered' as nanoemulsions becoming widely spread in new direction of drug delivery as drug carriers (for recent reviews see [3,7-9]). The important issue of the use of nanoemulsions for enzyme (protein) regulation is that the systems with bioactives wrapped tightly by nanosized matrix play an important role both on activity and conformational level. In this case, matrix become determining the enzyme (protein) behavior.

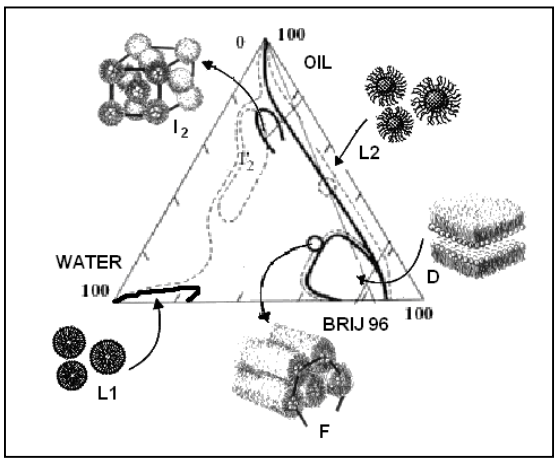


**Fig. 1. Phase diagram of ternary system Brij 96-water-oil and schematic representation of normal and reverse micelles ($L_1$ and $L_2$), lamellar (D), reverse hexagonal (F) and cubic ($I_2$) phases [10]**

In the work presented, different surfactant or lipid additives both in aqueous and non-aqueous media were used to study the activity and stability of bacteriolytic enzyme. The enzyme, called Lysin, is the phage-associated enzyme (PAE) found to be able to lyse group A Streptococcus cells [10]. Pathogenic Streptococci appear to be etiological agents of the most widespread infections of bacterial nature. Group A (S. pyogenes) Streptococci can cause infections of upper respiratory tract (tonsillitis, pharyngitis, etc.), skin (impetigo), scarlet fever (see, for example [11]). Bacteriophages representing

viruses of bacteria were discovered as a source of lytic enzymes that can destroy the host bacterial cells with a potential to be an alternative to antibiotics treatment (see, for example [12]). According to [13], effective against Streptococcus pyogenes cells Lysin is N-acetylmuramoyl-L-alanine amidase, which hydrolyzes the amide bond connecting the sugar and peptide constituents of the cell wall peptidoglycan.

The enzyme has a potential use both for treatment and prevention (prophylactics) of Streptococcal infections as a component of mouthwashes, gargles, sprays, etc. In the work presented, the components of typical mouthwashes and spray products met on the market were tested in their compatibility with Lysin efficiency. The main drawback of using PAE in buffer solution is low enzyme stability at elevated temperatures (even at room temperature). In the work presented, different additives were examined in their effectiveness to stabilize Lysin and to employ them as components of liquid preparations of mouthwashes/gargles (aqueous solutions) and sprays (aqueous and non-aqueous solutions).

**Materials and methods**

Recombinant Lysin (Lot O and Lot A) has been prepared by Dr. Surekha Valhyam and provided by New Horizons Diagnostic Co, Columbia MD, USA (www.nhdiag.com) as a part of IPP Project. Both preparations were stored in 20 mM phosphate buffer, pH 7.0 at $4^{o}$C; protein content was 2.18 and 12.76 mg/ml for Lo A and Lot O, respectively.

Bacterial strains used: Streptococcus pyogenes M29 type, strain D-28/11 N62/59 (Prague Collection), and T5 type, strain 12344 ATCC. Rifampicin-pretreated T5 cells were kindly provided by Prof. V.M. Bondarenko and Dr. A.N.Kuzikov (GIEM, Moscow, Russia).
M29 cells were grown at 37˚C using Todd-Hewitt broth ("Difco", USA). In each series of experiments, stored frozen at –20˚C ampouled culture (with preliminary passages on a blood) was adapted to a liquid culture media. 1% of overnight culture was used to inoculate broth. Cell culture was sedimented by low-speed centrifugation at 3,000 g for 15 min, washed twice with sterile 0.02 M phosphate buffer, pH 6.0-7.5, and used as a substrate for Lysin activity assay.
Measurement of Lysin activity in routine experiments was based on turbidimentric determination of cell lysis. Gentamicin- or rifampicin-pretreated and lyophilized M29 or T5 Streptococcus pyogenes cells, respectively, were used. A continuous assay was conducted to measure Lysin activity. Cell lysis was followed spectrophotometrically measuring of OD decrease at 600 nm for 15-30 min at $37^{o}$C.
For a spot-test, 0.1ml of the night M29 Streptococci broth culture ($2^{nd}$ passage) grown on blood agar was placed onto a Petri dish with agar. The mixture was evenly distributed on the surface of agar, and the dish was left for Streptococci growth for 18-20 hours at $37^{o}$C. The dishes with culture lawn were used to evaluate the lytic aGAS activity in different compositions. For this, the clearing zone was measured at 1,5,10, and 30 min of incubation at $37^{o}$C after the enzyme addition, first, and culture growth of the samples withdrawn from the zone of lysis (after 30 min) and transferred through the broth onto Petri dishes with blood agar was checked 24 hours after, second.

**Results and discussion**

Compounds of different origin: proteins, salt at high concentrations, sugars (mono-, di-, and poly-), lipids, polyelectrolytes, and micelle-forming low- and high molecular mass surfactants, were tested as additives to Lysin to find out stabilizing ones. Lysin performance in the presence of additives, mostly micelle-forming in water (o/w type) or in oil (w/o type) or combinations of micelle-forming surfactants with polymers that can play a stabilizing role in Lysin functioning was examined in a spot-test (clearing zone in Streptococci lawn) and in the Streptococci growth ability after enzyme treatment test (number of colonies calculation after 24h). The data are shown in Table 1.

| No | Additive | Clearing zone, mm/Treatment time, min | | | | CFU/24h |
|---|---|---|---|---|---|---|
| | | 1 | 5 | 10 | 30 | |
| M2* | Tween 80** | 0 | yes[(a)] | yes | 7 | 100-500 |
| M4 | Brij 30 | 0 | yes | yes | 10 | 1 |
| M7 | F 127 | 0 | yes | 9 | 9 | 50-100 |
| M9 | Chitosan | 0 | ? | ? | 4 | 100-500 |
| M10 | Heparin | 0 | ? | yes | 7 | >500 |
| M11 | PAA | 0 | yes | yes | 9 | 50-100 |
| M16 (H16) | PAA + Brij30 | ? | 7 | 8 | 8 | 0 (4) |
| M17 | PAA + F127 | ? | yes | 8 | 8 | 5 |
| M24 | AOT in oil | ? | ? | ? | ? | 0 |
| M27 | Brij96 in oil | ? | ? | ? | ? | 100-500 |
| M30 | L61 in oil | ? | ? | ? | ? | >500 |
| H42 | Chitosan + Brij30 | nd | nd | nd | yes | 11 |
| H43 | Lecithin in oil | nd | nd | nd | yes | 10 |
| **Control_1** | **Lysin in buffer, No additives** | **0** | **4** | **5** | **10** | **40** |
| **Control_2** | **Buffer, No enzyme** | **0** | **0** | **0** | **0** | **>500** |
| | | | | | | |

*Numbers of series of experiments, M, N, etc., remained;
**Lysin final dilution was 1/102000 in all experiments;
[(a)] Yes – observed, but not measured in mm; nd – not determined; ? – not clear.

**Table 1. Microbiological test of the effect of additives on the efficiency of Lysin in Streptococci M29 cells lysis expressed as the zone of lysis on the Streptococci lawn (clearing zone measured in mm) at 1, 5, 10, and 30 min of the enzyme action, and as number of colonies (CFU) calculated after 24h incubation of samples taken from the lysis zone at 30 min**

As seen, in the presence of many additives, Lysin gives large clearing zone on Streptococci lawn (as large or close to that Lysin in buffer shows (Control_1)). In the presence of several additives or their combinations Lysin showed higher performance (was working faster than the enzyme in buffer). Note that buffer solution without enzyme did not give any clearing zone (negative Control_2). The efficiency of the lysis has been checked also in the experiment of the Streptococci growth. Number of colonies counted after 24h incubation at 37$^{o}$C of the samples taken from zones of lysis at 30 min of the enzyme action is shown in the last column of Table 1. As seen, more than 500 colonies were found from the sample without enzyme (negative Control_2). The enzyme in buffer was quite effective (positive Control_1) giving big clearing zone. However, still 40 colonies were found on the blood agar after 24h of incubation of the sample taken from the lysis zone. At the same time, in the presence of some additives or their combinations, few or no colonies were found that makes these compounds promising as matrices for Strep A bacteriolytic enzyme.

One of the important features of adjusting Lysin to work as a component of a mouthwash or spray is to stabilize the preparation for a long-term storage at room temperature (RT). Lysin performance in the presence of several additives shown in Table 1 was tested in time scale: 2 days, 2 weeks, and 2 months after preparation. We found that combination of surfactants and polyelectrolytes was giving best results (Fig.2). As shown in Fig.2, Lysin with these additives quite effectively lysed Streptococci lawn giving both big clearing zone (A) after preparation (black), in 2 days (light gray), in 2 weeks (gray), and in 2 months (lightest gray) and almost no colonies (B). The efficiency of Lysin in water dropped fast; in 2 days, still 4mm clearing zone was observed but the number of colonies grown from the sample taken from clearing zone became high (200).

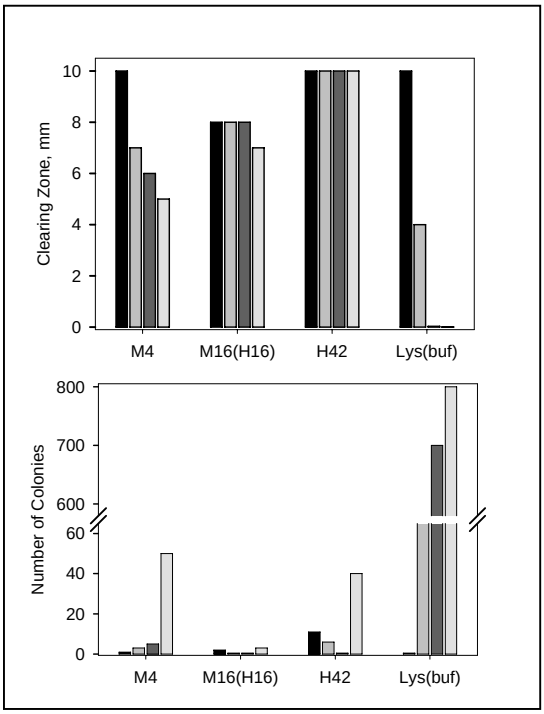


**Fig. 2. Lysin stability at RT in the presence and absence of additives (M4, M16, H42) expressed as a clearing zone (mm) (A) and number of colonies counted (B) in time scale: 30 min after preparation (black), after 2 days (light gray), after 2 weeks (gray), after 2 months (lightest gray)**

## Conclusion

To our opinion, the micellar approaches to enzyme (and, in general, to bioactives) modification and stabilization are rather prospect and fruitful in the view of final drug formulation useful for practice.

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