BIO Web of Conferences **116**, 03016 (2024) https://doi.org/10.1051/bioconf/202411603016
EBWFF 2024

Thermal stability of water-in-oil microemulsions containing solubilized nutritional protein gelatin

Mariya Klimovitskaya[1,2], *Polina* Skvortsova[1], *Olga* Zueva[3], and *Yuriy* Zuev[1,*]

[1]Kazan Institute of Biochemistry and Biophysics, FRC Kazan Scientific Center of RAS, Lobachevsky St., 2/31, Kazan 420111, Russia
[2]Butlerov Chemical Institute, Kazan Federal University, Kremlevskaya St., 18, 420008 Kazan, Russia
[3]Kazan State Power Engineering University, Krasnoselskaya str. 51, 420066 Kazan, Russia

Abstract. To develop new food and pharma technologies, various combinations of encapsulation and delivery of biological macromolecules are used. Proteins, polysaccharides, fats and lipids must be conveyed inside living organism, protecting them during the stages of storage and preparation from exposure of aggressive external environment. Some of the most common food protein compositions are various gels and emulsions. In the present study, we focused our attention on the influence of protein molecules on the properties and dynamical stability of water-in-oil microemulsion. Microemulsions, the oil dispersion of surfactant-based reverse micelles, each carrying nanosized water core with embedded protein. We studied the result of protein encapsulation in the water core of surfactant reverse micelles, namely, the fish and mammalian gelatin. The method of electric conductivity was explored to detect the properties of reverse micelles as containers for food proteins. We have shown that a rather high protein content does not destroy microemulsion structure, which retain reverse micelles, though the properties of the system undergo definite alterations, in particular, it substantively lost thermal stability accelerating exchange processes between reverse micelles at lower temperatures which have to be taken into account in nutritional and pharmacy objectives.

1 Introduction

Gelatin is the semi-structured protein obtained by hydrolytic degradation of native fibrillar protein collagen, the crucial component of bones, cartilages, connective tissue and skin of fish and mammals, which plays a considerable role among food hydrocolloids in modern nutritional and pharmaceutical technologies [1-3]. A large bank of data regarding the structures and properties of gelatin in different environments and states is known [4-6]. However, in many cases these knowledges are insufficient, since the appearance and development of novel technological ideas. One of relatively novel approaches in gelatin usage, processing and treatment is gelatin-based oleogels or organogels [7,8]. Oleogelation is rather novel approach used to design soft and hard oil-based products. The known

* Corresponding author: yufzuev@mail.ru

oleogels are based on the formation of semi-crystalline or crystalline networks by adding such oleogelators as surfactants, proteins or their joint compositions to texture and modify oils. Oleogels are considered as semisolid smart systems, with a continuous phase formed by hydrophobic liquid medium where a self-assembled network operates as physical armoring of oil liquid phase [9-11]. Oleogels, being the multi-component heterogeneous systems at nano and micro scales are not so simple for modeling and study on molecular and supramolecular level, which is extremely useful in engineering design of novel nutritional technologies. Unfortunately, many natural compounds are not stable in their content bringing some difficulties in modeling and study of their structure and properties. Sufficiently convenient systems for this purpose are surfactant-based microemulsions [12-14].

Among different types of microemulsions, which have various applications, in the present study we are interested in the water-in-oil (W/O) microemulsions or dispersions of reverse micelles (water droplets stabilized by surfactant monolayer and dispersed in organic solvent), having challenging potential in different protein applications, for instance in "non-aqueous" enzymology to give new properties to enzymes [15,16]. However, to use microemulsions as the protein reservoir the conditions of definite thermodynamic stability are necessary, in particular under the counter action of guest protein molecules. It is known that varying the dispersed water fraction or temperature it is possible to evoke a phase transition from a dispersion of discrete reverse micelles to the infinite cluster of water droplets, leading to a sharp alteration of various physical properties of microemulsion, particularly the dramatic rise of electrical conductivity of the system as a consequence of percolation process [17,18]. It is generally considered that, during percolation the reverse micelles come into close contact resulting in the formation of "infinite" clusters containing water droplets. Therewith the channel pathways appear through which the charge carriers (mainly surfactant counterions [18]) propagate by hopping between droplets or by exchange of droplet content under coalesce and fission of reverse micelles. The formation of clusters and the rate of contents exchange turned out to be influenced not only by temperature, droplet size and concentration but also by different additives present in the water phase [19].

In the present study, we focus our attention on the influence of gelatin molecules on the properties and dynamical stability of microemulsion medium. We studied the result of protein encapsulation in the water core of surfactant reverse micelles. The fish and mammalian gelatin were used as fillers of microemulsion water phase. The method of electric conductivity was explored to detect the stability of reverse micelles to temperature action as containers for food proteins.

2 Materials and methods

Water-in-oil microemulsions were prepared using the anionic surfactant sodium bis(2-ethylhexyl) sulfosuccinate (AOT), produced by TCL (Belgium), product number S0139. Surfactant in concentration 0.35 M was dissolved in 99% decan (Acros Organics, Russia). Then, the appropriate amount of water or gelatin water solution was added to keep molar ratio water/AOT $W = 35$ with intensive shaking. The characteristic radius of the reverse micelle R_M is the function of water/surfactant ratio W [20]:

$$R_M\,(\text{nm}) = 0.15W + L_{AOT}, \tag{1}$$

where L_{AOT} is the length of AOT molecule (~1.4 nm). As a result, we prepared the dispersion of reverse micelles in decan with volume fraction of micelles about 0.25, each having the radius about 6.7 nm with the micelle water core radius of 5.3 nm.

In the present research we used two gelatins of type A, one from porcine skin (Sigma—Switzerland, G6144, Lot # BCBR5299V) and second from cold water cod skin. Fish gelatin was extracted from the skin of Atlantic cod in Murmansk Arctic University following the standard procedure [6]. Samples of gelatin water solutions with 140 mg/ml concentration were prepared according to following procedure. The required volume of water was added to protein sample. After 24 hours of swelling at 20 °C the samples were heated to 50 °C and stirred at this temperature until being fully dissolved. The laboratory-extracted fish gelatin was purified from salt ions and small fragments of peptide chains by dialysis with the seven times change of solvents with the help of cellulose SnakeSkin Dialysis Tubing (Thermo Scientific), 3.5 kDa MWCO. Purified gelatin was frozen in liquid nitrogen and freeze-dried to constant weight.

The concentration of gelatins 140 mg/ml was chosen two attain the maximum level of protein occupancy of reverse micelles. According to our practice it resulted in 1 protein molecule per 2 micelles (1/2), saving optical transparency of microemulsion.

The low-frequency electrical conductivity was measured in the temperature range 10-50 °C using a Radelkis OK102/1 conductometer at 100 Hz and 3 kHz.

Hydrodynamic radius of gelatins studied was obtained with the help of protein self-diffusion coefficients [21], determined using NMR spectrometer (AVANCE-III, Bruker, USA) operating at 600.13 MHz and equipped with a standard z-gradient inverse probe TXI (5 mm) capable of producing magnetic gradients up to a maximum strength 0.557 $T \cdot m^{-1}$.

Alternatively, the size of gelatin molecules was obtained by the DLS Photocor Complex (Photocor, Russian Federation), equipped with He–Ne laser (λ = 632.8 nm) [22]. Cylindrical glass cuvettes with 1 cm radius were used for measurements. Protein solutions were cleaned from dust using the 0.2 μm membrane filters.

3 Results and discussion

We choose the value of water-to surfactant molar ratio $W = 35$ to provide enough place for gelatin molecule inside water pool of surfactant reverse micelle. According to Eq. (1) this W value corresponds to the micelle water core radius of 5.3 nm. It closely corresponds to hydrodynamic radius obtained from NMR and DLS experiments with the help of Stokes-Einstein relation [23] as ~5-6 nm both for porcine and fish gelatins (Table 1).

Table 1. Diffusion coefficients, obtained from NMR and DLS experiments, and hydrodynamic radius of gelatin molecules

Gelatin sample	Diffusion coefficient, $m^2 \cdot s^{-1}$		Hydrodynamic radius, nm	
	NMR	DLS	NMR	DLS
Porcine, 30°C	5.0 ± 0.6	4.0 ± 1.8	5.6 ± 0.7	6.7 ± 2.9
Fish, 35°C	4.9 ± 0.6	5.7 ± 0.8	6.4 ± 0.8	5.5 ± 0.8

For illustration of influence of gelatin molecules on the properties and dynamical stability of reverse micelles we have chosen the experiments on electrical conductivity of our system. The physical characteristics that most clearly reflects the specific behavior of W/O microemulsion is its electrical conductivity. The conductivity of the oil phase is significantly lower than the conductivity of dispersed phase. Two main sources of free electric charges are the ionized surfactant molecules (AOT in our case) and their Na^+ counterions. In general, their statistic ensemble is electrically neutral, but due to thermal

fluctuations, microdroplets can carry definite charge. The electrical conductivity of W/O microemulsion is determined by the mobility of micelles having an uncompensated electrical charge. The diffusion of micelles in a viscous medium obeys the Stokes' law, and the electrical conductivity of microemulsion σ is determined by the expression [17,24]

$$\sigma = \frac{\varepsilon_0 \varepsilon k_B T}{2\pi\eta} \frac{\phi_M}{r^3}, \quad (2)$$

where $\varepsilon_0 = 8.85 \cdot 10^{-12}$ F·m^{-1} is the dielectric constant, ε is the dielectric constant of organic medium, k_B is Boltzmann's constant, T is the absolute temperature, η is the viscosity of organic medium, ϕ_M is the volume fraction of dispersed phase. During thermal movement microdroplets collide with each other, exchange surfactant ions and aqueous content containing counterions, and charge carriers are transferred in the volume of microemulsion. With increasing temperature, the probability of ionization of surfactant head groups increases, the mobility of microdroplets growths which increase the probability of their contacts, the entanglement of neighboring micellar aggregates by surfactant hydrocarbon radicals and the exchange of micelles content through the coalescence mechanism rises. In this case, clusters of microdroplets are formed, uniting an ever-increasing number of individual units. At a certain temperature the clusters of microdroplets are formed, which creates a permanent path for charge transfer during deformation of micelle surface and formation of short-lived water channels between neighbors, causing a sharp increase in electrical conductivity of microemulsion (Fig. 1). The formation of conducting chains in an insulating material of oil phase is called electrical percolation [25].

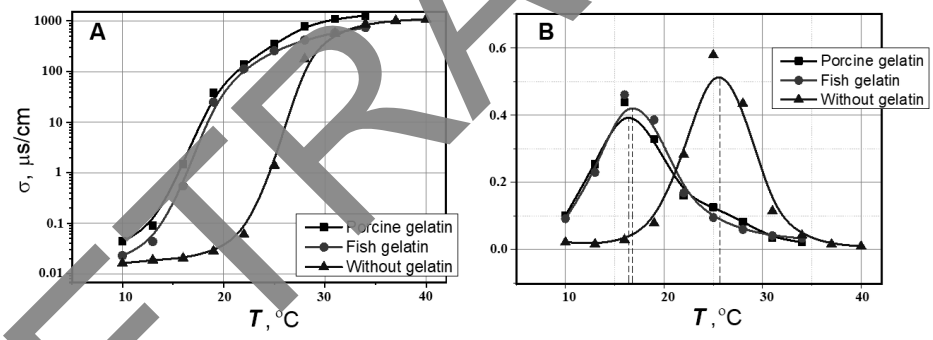

Fig. 1. A – Temperature dependence of electrical conductivity of microemulsions with polar phase containing pure water (triangles), porcine (squares) and fish (circles) gelatin. B - 1-st derivative of electrical conductivity dependences shown at the left – maximum positions correspond to percolation threshold 26 °C for "pure" reverse micelles (triangles), 16.3 °C containing porcine gelatin (squares) and 16.8 °C containing fish gelatin (circles).

The process of formation of clusters from micellar microdroplets is the dynamical in its nature; they are formed and destroyed in the dispersion bulk under the influence of thermal motion, but above a certain temperature T_P, called the percolation threshold, at least one "infinite" cluster always presents in the microemulsion volume, providing high electrical conductivity of the system [26-28]. Protein molecules solubilized in water core of reverse micelles interact with surfactant shell and affect its "ruggedness" and alter the percolation phenomenon. Thus, studying electric conductivity under the action of encapsulated protein can provide information about dynamics and stability of W/O microemulsion, being useful in different dairy and pharma technologies.

The results on electrical conductivity of microemulsions show significant, about ten degrees, shift of percolation transition temperature for gelatin-loaded microemulsions in which water core of reverse micelles contain protein molecules (according to our preliminary estimations every second reverse micelle is occupied by gelatin molecule). Fig. 2 depicts the snapshot of dynamical process taking place in the system studied.

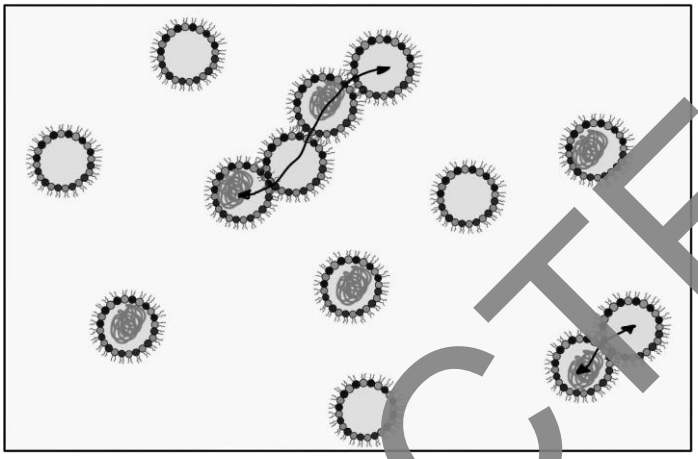

Fig. 2. Snapshot of reverse micelles collisions and intermicellar exchange of water phase.

The conditions for percolation threshold depend on the properties of reverse micelles – on the probability to realize of the contact between reverse micelles (depend on micelle size and concentration) and the strength of micelle surfactant shell (depend on micelle size and provocative impurities). To rouse the definite microemulsion system (adjusted micelle size and concentration) to action the necessary energy deposit is required to form large micellar clusters, to disturb surfactant shells and promote ion transport across oil bulk. It seems that gelatin molecules entrapped in the water core of reverse micelle disturb surfactant shell, making it less crashproof under direct and strong contacts of micelles. This can be easily explained by the opposite sign of protein charge and surfactant interface. Our results on gelatin zeta potential obtained from DLS experiment (data not shown) gave slightly positive values: 1 – 2 mv for fish gelatin and 2 – 4 mv for porcine gelatin, argumentative of the positive sign of protein charge [13,29]. The AOT-based shell of reverse micelles has the negative charge which attracts the positively charged gelatin molecules disturbing the stability of reverse micelles.

Thus, our results on the percolation transition in water-in-oil microemulsions have shown that protein molecules entrapped in water core of reverse micelles does not destroy microemulsion structure, though the properties of the system undergo definite alterations, in particular, it substantively lost thermal stability accelerating exchange processes between reverse micelles at lower temperatures which have to be taken into account in nutritional and pharmacy objectives. These findings are in accord with modern tendencies of engineering design of food, pharmacy and biosensors formulations [30,31].

4 Conclusion

The present study was devoted to the thermal stability of water-in-oil microemulsions containing solubilized nutritional protein gelatin. We studied the effect of protein (fish and mammalian gelatins) encapsulation in the water core of surfactant reverse micelles using the electric conductivity of microemulsion as the marker of electric charge transfer across

the oil-continuous microemulsion via the hopping of surfactant ions and counter-ions between colliding micelles. Our results have shown that in the presence of protein in reverse micelles the shift of about 10°C towards lower temperature is observed for the percolation threshold of electric conductivity. We suppose it a result of disturbance of negatively-charged surfactant shell of reverse micelles under the interaction with positively charged protein. The obtained result may be useful for nutritional and pharmacy objectives.

Acknowledgments

M.A.K., P.V.S. and Y.F.Z. acknowledge support of the Russian Science Foundation, project No. 23-64-10020) for finance provision of NMR, DLS and conductivity experimental study of gelatin systems. O.S.Z. thanks Strategic Academic Leadership Program "PRIORITY-2030" of KSPEU.

Authors thanks professor S.R. Derkach from Murmansk Arctic University for kind gift of fish gelatin extracted from the skin of Atlantic cod.

References

1. I. J. Haug, K. I. Draget, Gelatin, in *Handbook of Hydrocolloids*, 2nd ed., edited by G. O. Phillips, P. A. Williams (CRC Press, Boca Raton, 2009), pp. 142–163.
2. J. Alipal, N. A. S. Mohd Pu'ad, T. C. Lee, N. H. M. Nayan, N. Sahari, H. Basri, M. I. Idris, H. S. Abdullah, Mater. Today Proc. **42**, 240 (2021). https://doi.org/10.1016/j.matpr.2020.12.922
3. O. V. Mikhailov, Int. J. Mol. Sci. **24**, 3583 (2023). https://doi.org/10.3390/ijms24043583
4. M. Yang, L. Yang, J. Xu, Y. Nie, W. Wu, T. Zhang, X. Wang, X. J. Zhong, Food Chem. **368**, 130818 (2022). https://doi.org/10.1016/j.foodchem.2021.130818
5. V. N. Izmailova, S. R. Derkach, M. A. Sakvarelidze, S. M. Levachev, N. G. Voron'ko, G. P. Yampol'skaya, Polymer Science C **46**, 73 (2004). https://doi.org/10.1134/S1061933X14020021
6. Yu. F. Zuev, S. R. Derkach, L. R. Bogdanova, et al., Gels **9**, 990 (2023). https://doi.org/10.3390/gels9120990
7. E. D. Co, A. G. Marangoni, J. Am. Oil Chem. Soc. **89**, 749 (2012). https://doi.org/10.1007/s11746-012-2049-3
8. K. Chen, H. Zhang, ACS Appl. Mater. Interfaces **12**, 7795 (2020). https://dx.doi.org/10.1021/acsami.9b21435
9. J. M. Franco, R. Sánchez, M. A. Delgado, C. Valencia, C. Gallegos, Grasas y Aceites **62**, 328 (2011). https://doi.org/10.3989/gya.113410
10. A. R. Patel, D. Schatteman, W. H. De Vos, A. Lesaffer, K. Dewettinck, J. Colloid Interface Sci. **411**, 114 (2013). https://doi.org/10.1016/j.jcis.2013.08.039
11. N. Pal, Y. Alzahid, A. M. AlSofi, M. Ali, H. Hoteit, Energy&Fuels **37**, 858 (2023) https://doi.org/10.1021/acs.energyfuels.2c03148
12. P. Szumała, J. Kaplińska, B. Makurat-Kasprolewicz, S. Mania, Mol. Pharmaceutics, **20**, 232 (2023). https://doi.org/10.1021/acs.molpharmaceut.2c00599
13. Yu. F. Zuev, N. N. Vylegzhanina, N. L. Zakhartchenko, Appl. Magn. Reson. **25**, 29 (2003). https://doi.org/10.1007/BF03166964

14. N. L. Klyachko, A. V. Levashov, Curr. Opp. Coll. Interface Sci. **8**, 179 (2003). https://doi.org/10.1016/S1359-0294(03)00016-5
15. A. Shome, S. Roy, P. K. Das, Langmuir **23**, 8, 4130 (2007). https://doi.org/10.1021/la062804j
16. E. A. Ermakova, Y. F. Zuev, Mendeleev Commun., **15**, 166 (2005). https://doi.org/10.1070/MC2005v015n04ABEH002062
17. A. E. Aferni, , M. Guettari, I. B. Naceur, Chemistry Africa 5, 793 (2022). https://doi.org/10.1007/s42250-022-00356-x
18. B. K. Paul, R. K. Mitra, J. Colloid Interface Sci. **295**, 230 (2006). https://doi.org/10.1016/j.jcis.2005.07.072
19. J. Dasilva-Carvalhal, L. García-Río, D. Gómez-Díaz, J. C. Mejuto, P. Rodríguez-Dafonte, Langmuir **19**, 5975 (2003). https://doi.org/10.1021/la026857m
20. P. M. Geethu, I. Yadav, S. K. Deshpande, V. K. Aswal, D. K. Satapathy, Macromolecules **50**, 6518 (2017). https://doi.org/10.1021/acs.macromol.7b01521
21. A. M. Kusova, A. E. Sitnitsky, B. Z. Idiyatullin, D. R. Bakirova, Yu. F. Zuev, Appl. Magn. Res. **49**, 35 (2018). https://doi.org/10.1007/s00723-017-0957-y
22. A. M. Kusova, A. K. Iskhakova, Yu. F. Zuev, Eur. Biophys. J. **51**, 375 (2022). https://doi.org/10.1007/s00249-022-01605-0
23. Yu. F. Zuev, A. M. Kusova, A. E. Sitnitsky, Biophysical Reviews **15**, 1111 (2023). https://doi.org/10.1007/s12551-023-01108-y
24. H. Lin, M.V. Majji, N. Cho, J.R. Zeeman, J.W. Swan, J.J. Richards, PNAS **119**, e2203470119 (2022). https://doi.org/10.1073/pnas.2203470119
25. A. van Bommel, G. MacIsaac, N. Livingstone, R. Palepu, Fluid Phase Equilibria, **237**, 59 (2005). https://doi.org/10.1016/j.fluid.2005.08.015
26. Y. Hattori, H. Ushiki, L. Courbin, P. Panizza, Phys. Rev. E **75**, 021504 (2007). https://doi.org/10.1103/PhysRevE.75.021504
27. A. E. L. Aferni, M. Guettari, T. Tajouri, et al., J. Mol. Liq. **12**, 795 (2020). https://doi.org/10.1016/j.molliq.2020.114012
28. S. Chatterjee, R. K. Mitra, B. K. Paul, S. C. Bhattacharya, J Colloid Interface Sci. **298**, 935 (2006). https://doi.org/10.1016/j.jcis.2005.12.061
29. S. M. Ahsan, C. M. Rao, Int. J. Nanomedicine **12**, 795 (2017). https://doi.org/10.2147/IJN.S124938
30. N. Haribi, A. Mauser, Y. Ko, J. Lahann, Adv Sci (Weinh) **9**, e2104012 (2022). https://doi.org/10.1002/advs.202104012
31. M.-L. Arsene, I. Răut, M. Călin, M.-L. Jecu, M. Doni, A.-M. Gurban, Processes **9**, 345 (2021). https://doi.org/10.3390/pr9020345