POZNAN UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY
INSTITUTE OF CHEMICAL TECHNOLOGY AND ENGINEERING

Ph.D. THESIS

Biomimetic systems studied by Langmuir and Langmuir-Blodgett techniques

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NATIONAL SCIENCE CENTRE
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Scientific activity

Publications:

1. Katarzyna Dopierała, **Martyna Krajewska**, Krystyna Prochaska, Binding of α-lactalbumin to oleic acid monolayer and its relevance to formation of HAMLET-like complexes, International Dairy Journal - 2019, vol. 89, p. 96-104, Impact Factor: 2.512, Ministry points: 100

2. **Martyna Krajewska**, Katarzyna Dopierała, Marek Weiss, Krystyna Prochaska, Temperature, pH, and molecular packing effects on the penetration of oleic acid monolayer by α-lactalbumin, Langmuir - 2019, vol. 35, iss. 8, p. 3183-3193, Impact Factor: 3.557, Ministry points: 100

3. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Lipid–protein interactions in Langmuir monolayers under dynamically varied conditions, The Journal of Physical Chemistry B - 2020, vol. 124, iss. 1, p. 302-311, Impact Factor: 2.991, Ministry points: 140

4. **Martyna Krajewska**, Katarzyna Dopierała, Paweł Wydro, Marcin Broniatowski, Krystyna Prochaska, Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study, Journal of Molecular Liquids - 2020, vol. 319, p. 114089-1-114089-9, Impact Factor: 6.165, Ministry points: 100

5. Katarzyna Dopierała, **Martyna Krajewska**, Marek Weiss, Physicochemical characterization of oleanolic acid–human serum albumin complexes for pharmaceutical and biosensing applications, Langmuir - 2020, vol. 36, iss. 13, p. 3611-3623, Impact Factor: 3.882, Ministry points: 100

6. Katarzyna Dopierała, **Martyna Krajewska**, Krystyna Prochaska, Study on pH-dependent interactions of linoleic acid with α-lactalbumin, Food Hydrocolloids - 2021, vol. 111, p. 106217-1-106217-9, Impact Factor: 11.504, Ministry points: 140

7. Mateusz Szczygiełda, **Martyna Krajewska**, Lei Zheng, Long D. Nghiem, Krystyna Prochaska, Implementation of forward osmosis to concentrate alpha-ketoglutaric acid from fermentation broth: Performance and fouling analysis, Journal of Membrane Science - 2021, vol. 637, p. 119593-1-119593-8, Impact Factor: 10.530, Ministry points: 140

8. Adam Andrzejewski, **Martyna Krajewska**, Jagoda Nowak-Grzebyta, Mateusz Szczygiełda, Ewa Stachowska, Krystyna Prochaska, Concentration of pectin solution: Forward osmosis performance and fouling analysis, Journal of Membrane Science - 2022, vol. 653, Impact Factor: 10.530, Ministry points: 140

9. Mohammad Zohurul Islam, **Martyna Krajewska**, Sheikh Imamul Hossain, Krystyna Prochaska, Azraf Anwar, Evelyne Deplazes, Suvash C. Saha, Concentration-dependent effect of the steroid drug prednisolone on a lung surfactant monolayer, Langmuir - 2022, vol. 38, iss. 14, p. 4188-4199, Impact Factor: 4.331, Ministry points: 100

10. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, The biomimetic system of oleanolic acid and oleic acid at the air-water interface–interactions in terms of nanotechnology-based drug delivery systems, Membranes - 2022, vol. 12, iss. 12, p. 1215-1-1215-15, Impact Factor: 4.562, Ministry points: 100

11. Katarzyna Dopierała, Marek Weiss, **Martyna Krajewska**, Justyna Błońska, Towards understanding the binding affinity of lipid drug carriers to serum albumin, Chemistry and Physics of Lipids – 2023, vol. 250, p. 105271-1-105271-12, Impact Factor: 3.570, Ministry points: 100

Publications under review:

1. Pablo Simón Marqués, **Martyna Krajewska**, Bradley D. Frank, Markus Antonietti, Krystyna Prochaska, Lukas Zeininger, Morphology-dependent aggregation-induced emission of Janus emulsion surfactants, manuscript accepted for publication in Chemistry - A European Journal

2. Lei Zheng, Yanfang Xiong, Yimeng Gao, Fengjun Yin, Mateusz Szczygiełda, **Martyna Krajewska**, Phong H. N. Vo, Changsheng Jiang, Hong Liu, Tailoring the bidirectional diffusion in the integrated electro-Fenton and forward osmosis for enhancing emerging contaminants removal, manuscript submitted to Science of The Total Environment

3. Mateusz Szczygiełda, **Martyna Krajewska**, Adam Andrzejewski, Lei Zheng, Long D. Nghiem, Piotr Oleskowicz-Popiel, Daria Szymanowska, Krystyna Prochaska, Dewatering fermentation broth for keto carboxylic acid enrichment by forward osmosis: A techno-economic analysis, manuscript submitted to Journal of Membrane Science

Oral presentations at international conferences:

1. Katarzyna Dopierała, **Martyna Krajewska**, Krystyna Prochaska, Study on biologically important phenomena using Langmuir monolayer technique, Nano Tech Poland International Conference & Exhibition & 1st Symposium on Polydopamine, 6-9.06.2018, Poznań, Poland

2. Adam Andrzejewski, **Martyna Krajewska**, Mateusz Szczygiełda, Krystyna Prochaska, Concentration of aqueous pectin solution using forward osmosis, 13th Scientific Conference – Membranes and membrane processes in environmental protection, MEMPEP 2021, 10-11.06.2021, Zakopane, Poland

Oral presentations at national conferences:

1. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Oddziaływanie lizozymu z kwasem oleinowym na granicy faz woda/powietrze, II Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg, 02.12.2017, Poznań, Poland

2. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Interaction of oleic acid with milk proteins at the air-water interface, XIIIth Summer School for Ph.D. Students and Young Researchers Interfacial Phenomena in Theory and Practice, 25-29.06.2018, Sudomie, Poland
   AWARD FOR THE BEST ORAL PRESENTATION

3. **Martyna Krajewska,** Katarzyna Dopierała, Aleksandra Koperska, Krystyna Prochaska, Thermodynamic and rheological properties of oleanolic acid monolayers in the presence of proteins, XIVth Summer School for Ph.D. Students and Young Researchers Interfacial Phenomena in Theory and Practice, 24-28.06.2019, Sudomie, Poland

4. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Oddziaływania między cząsteczkami kwasu oleinowego i kwasu oleanolowego na granicy faz, III Edycja Studenckiej Konferencji Nauk Ścisłych im. Prof. Antoniego Hoborskiego, 14.11.2020, Kraków, Poland
   1ST AWARD FOR THE BEST ORAL PRESENTATION IN SESSION B - CHEMICAL SCIENCES

Poster presentations at international conferences:

1. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Miscibility of the oleanolic and oleic acids within the Langmuir monolayer, Geneva Colloids, 8-9.04.2021, Geneva, Switzerland

2. Mateusz Szczygiełda, **Martyna Krajewska**, Adam Andrzejewski, Krystyna Prochaska, Concentration of alpha-ketoglutaric acid solutions using forward osmosis process, 13th Scientific Conference – Membranes and membrane processes in environmental protection, MEMPEP 2021, 10-11.06.2021, Zakopane, Poland

3. **Martyna Krajewska**, Shang Gao, Jan Zawala, Gerald Fuller, Investigation on the Polyacrylate Emulsions Stabilization Behaviors at the Air-liquid Interface, The 10th Annual Stanford Polymer Collective Poster Symposium, 19.05.2022, Stanford University, USA

4. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Interactions between molecules of oleic and oleanolic acid in the pharmaceutical formulations - physicochemical perspective, 36th Conference of the European Colloid and Interface Society ECIS 2022, 4-10.09.2022, Chania, Greece
   AWARD FOR THE BEST POSTER PRESENTATION FROM LANGMUIR, ACS PUBLICATIONS

Poster presentations at national conferences:

1. Katarzyna Dopierała, Monika Rojewska, Marta Skrzypiec, **Martyna Krajewska**, Aleksandra Bartkowiak, Krystyna Prochaska, Monowarstwy Langmuira i warstwy Langmuira-Blodgett jako narzędzie do badania oddziaływań w układach biologicznych, X Poznańska Konferencja Naukowa „Chemia - Nauka i Przemysł", 30.11.2018, Poznań, Poland

2. Julia Olejarska, **Martyna Krajewska**, Katarzyna Dopierała, Oddziaływanie kwasu linolowego z białkami na granicy faz woda/powietrze, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg, 07.12.2019, Poznań, Poland

3. Aleksandra Bagińska, Justyna Błońska, **Martyna Krajewska**, Katarzyna Dopierała, Monowarstwy Langmuira jako narzędzie do oceny oddziaływań międzycząsteczkowych w układach o znaczeniu farmaceutycznym, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg, 07.12.2019, Poznań, Poland

4. Aleksandra Koperska, **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Badanie oddziaływania kwasu oleanolowego z albuminą osocza w filmach Langmuira-Blodgett, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg, 07.12.2019, Poznań, Poland

5. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Badanie właściwości reologicznych lipidowo-proteinowych monowarstw Langmuira, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg, 07.12.2019, Poznań, Poland

6. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Dwuskładnikowe monowarstwy kwasu oleinowego i kwasu oleanolowego na granicy faz woda-powietrze, Ogólnopolska studencka konferencja naukowa Bliżej Chemii, 09-10.01.2021, Kraków, Poland

7. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Technika monowarstw Langmuira w badaniach biomedycznych i farmaceutycznych, II Pomorskie Studenckie Sympozjum Chemiczne, 20-21.03.2021, Gdańsk, Poland
   AWARD FOR THE BEST POSTER PRESENTATION

8. Paulina Andrzejewska, Adam Andrzejewski**,** **Martyna Krajewska**, Mateusz Szczygiełda, Katarzyna Dopierała, Analiza foulingu membran filtracyjnych w procesie zatężania roztworu pektyn poprzez badanie zwilżalności i energii powierzchniowej, II Pomorskie Studenckie Sympozjum Chemiczne, 20-21.03.2021, Gdańsk, Poland

9. Adam Andrzejewski, **Martyna Krajewska**, Mateusz Szczygiełda, Krystyna Prochaska, Wydzielanie i zatężanie pektyn ze stałych pozostałości po przetwórstwie jabłek w wieloetapowych układach separacji membranowej, X Kongres Technologii Chemicznej, 11-14.05.2022, Wrocław, Poland

10. Mateusz Szczygiełda, **Martyna Krajewska**, Adam Andrzejewski, Marcin Pytel, Krystyna Prochaska, Zatężanie roztworów pofermentacyjnych techniką wymuszonej osmozy, Trzecie Seminarium Praktyczne Aspekty Inżynierii Chemicznej PAIC-2022, 07-08.06.2022, Zaniemyśl, Poland

11. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Monowarstwy Langmuira jako modelowy surfaktant płucny do badania oddziaływań z lekami, IV Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg, 03.12.2022, Poznań, Poland

12. Adam Andrzejewski, **Martyna Krajewska**, Mateusz Szczygiełda, Krystyna Prochaska, Wpływ procedury obróbki wstępnej na rezultaty zatężania rzeczywistego roztworu wodnego pektyny techniką wymuszonej osmozy, IV Ogólnopolskie Sympozjum Chemii, Organicznej i Biomateriałów BioOrg, 03.12.2022, Poznań, Poland

Research projects:

1. 4-month grant for the academic year 2021/2022 at Stanford University in the Kosciuszko Foundation Exchange Program to the United States

2. "The lipid fraction of the pulmonary surfactant enriched with fatty acids as a biomimetic drug carrier from the physicochemical perspective", 2021/41/N/ST4/01289, PRELUDIUM 20 for 12 months, National Science Centre, Poland, 01.07.2022-30.06.2023

Internships and scientific stays:

1. Max Planck Institute of Colloids and Interfaces, Responsive Soft Materials & Interfaces Team, 3 months, 01.05.-31.07.2021, Potsdam, Germany

2. Stanford University, Professor Gerald Fuller Team, 4 months, 01.02.-31.05.2022, Stanford, California, USA

Summer Schools and Short Courses:

1. XIIIth Summer School for Ph.D. Students and Young Researchers Interfacial Phenomena in Theory and Practice, 25-29.06.2018, Sudomie, Poland

2. XIVth Summer School for Ph.D. Students and Young Researchers Interfacial Phenomena in Theory and Practice, 24-28.06.2019, Sudomie, Poland

3. 1st Summer School and Workshop on Pharmaceutical Preformulation and Processing (Orbis Project) 12-14.06.2019, Dublin, Ireland

4. 2nd Summer School and Workshop on Oral Dosage Forms: Fundamentals, Challenges and Future Opportunities (Orbis Project), 18-20.09.2019, Helsinki, Finland

5. International scholarship exchange of Ph.D. candidates and academic staff (including research workshop and research skills training, hands-on sessions on the development of research publications and research collaboration activities), 24.11-07.12.2019 at University of Technology, Sydney, Australia

6. Supramolecular and Colloid Chemistry and Physics for the Life Sciences – Online summer school and workshop by ECIS (European Colloid and Interface Society), 27-29.07.2020, University of Rijeka, Croatia

7. Short Course: Advanced Characterization and Modelling techniques for Colloids (during 36th European Colloid & Interface Society Conference) 2-3.09.2022, Crete, Greece

Awards and scholarships:

1. Scientific scholarship awarded by the Rector of the Poznan University of Technology for the Ph.D. students 2018/2019, 2020/2021, 2021/2022, 2022/2023

2. Award for the best oral presentation at XIIIth Summer School for Ph.D. Students and Young Researchers Interfacial Phenomena in Theory and Practice, 25-29.06.2018, Sudomie, Poland

3. 6th Award for the best student performances on Supramolecular and Colloid Chemistry and Physics for the Life Sciences – online summer school and workshop, ECIS, 27-29.07.2020, University of Rijeka, Croatia

4. 1st Award for the best oral presentation in session B - Chemical sciences at III Edycja Studenckiej Konferencji Nauk Ścisłych im. Prof. Antoniego Hoborskiego, 14.11.2020, Kraków, Poland

5. Award for the best poster presentation at II Pomorskie Studenckie Sympozjum Chemiczne, 20-21.03.2021, Gdańsk, Poland

6. Award for the best poster presentation from Langmuir ACS Publications at 36th Conference of the European Colloid and Interface Society ECIS 2022, 4-10.09.2022, Chania, Greece

Author's impact:

- Total impact factor according to the Journal Citation Reports (JCR) from the year of the article publication: **64.134**
- Ministry points: **1260**
- Total number of citations according to the Web of Science (on 08.01.2023): **43**
- Hirsch index, according to Scopus: **5**
- Hirsch index, according to Web of Science: **4**

LIST OF PUBLICATIONS CHOSEN FOR THE BASIS OF THE Ph.D. PROCEDURE

PUBLICATION 1. **Martyna Krajewska**, Katarzyna Dopierała, Marek Weiss, Krystyna Prochaska, Temperature, pH, and molecular packing effects on the penetration of oleic acid monolayer by α-lactalbumin, Langmuir - 2019, vol. 35, iss. 8, p. 3183-3193, Impact Factor: 3.557, Ministry points: 100

CONTRIBUTION: conceptualization, investigation (Langmuir technique, Langmuir-Blodgett technique, contact angles measurements, surface free energy calculation), visualization, writing - original draft (contact angles and surface free energy analysis

PUBLICATION 2. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, Lipid–protein interactions in Langmuir monolayers under dynamically varied conditions, The Journal of Physical Chemistry B - 2020, vol. 124, iss. 1, p. 302-311, Impact Factor: 2.991, Ministry points: 140

CONTRIBUTION: conceptualization, investigation, data analysis, visualization, writing - original draft

PUBLICATION 3. Katarzyna Dopierała, **Martyna Krajewska**, Krystyna Prochaska, Study on pH-dependent interactions of linoleic acid with α-lactalbumin, Food Hydrocolloids - 2021, vol. 111, p. 106217-1-106217-9, Impact Factor: 11.504, Ministry points: 140

CONTRIBUTION: investigation, methodology, visualization, data curation

PUBLICATION 4. **Martyna Krajewska**, Katarzyna Dopierała, Paweł Wydro, Marcin Broniatowski, Krystyna Prochaska, Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study, Journal of Molecular Liquids - 2020, vol. 319, p. 114089-1-114089-9, Impact Factor: 6.165, Ministry points: 100

CONTRIBUTION: conceptualization, investigation, methodology, visualization, data curation, writing - original draft

PUBLICATION 5. Katarzyna Dopierała, **Martyna Krajewska**, Marek Weiss, Physicochemical characterization of oleanolic acid−human serum albumin complexes for pharmaceutical and biosensing applications, Langmuir - 2020, vol. 36, iss. 13, p. 3611-3623, Impact Factor: 3.882, Ministry points: 100

CONTRIBUTION: investigation (ISR, contact angles measurements, surface free energy calculation), visualization, writing - review & editing

PUBLICATION 6. **Martyna Krajewska**, Katarzyna Dopierała, Krystyna Prochaska, The biomimetic system of oleanolic acid and oleic acid at the air-water interface–interactions in terms of nanotechnology-based drug delivery systems, Membranes - 2022, vol. 12, iss. 12, p. 1215-1-1215-15, Impact Factor: 4.562, Ministry points: 100

CONTRIBUTION: conceptualization, investigation, writing—original draft preparation; corresponding author

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Abstract

The Langmuir technique is a simple and versatile tool to study surface phenomena at the molecular level. Observing nature has enabled the implementation of its solutions on many levels of life and technology. The progress of science has moved the drawing inspiration from nature to the nanoscale when studying monomolecular structures or interactions between molecules. Research in biomedicine seems to be the next step toward understanding the mechanisms of disease formation and developing novel therapeutic methods. Therefore, based on the background mentioned above, in the presented dissertation, the research utilizing the Langmuir technique is undertaken to characterize qualitatively and quantitatively biomimetic systems of therapeutic potential, with a particular focus on fatty acid-protein complexes, triterpenic acid-protein systems, and triterpenic acid-fatty acid binary monolayers.

The first part of this doctoral thesis is devoted to an extensive literature review (Chapter 1) concerning the Langmuir methodology applied in studying the monolayers of natural substances. The literature review emphasizes the significance of surfaces and interfaces, defines the Langmuir monolayers, and introduces the basis of the Langmuir and Langmuir-Blodgett techniques. A brief outlook of the application fields of the Langmuir monolayers of biomimetic character is also included.

The essential part of the thesis presenting the publications selected for the basis of the Ph.D. procedure is divided into three chapters (Chapters 2-4), focused on different research systems. Each research chapter starts with a short introduction and finishes with a concise summary, highlighting the most significant results and fields of application. The crucial step of the studies includes the formation of mixed monolayers composed of lipids and additional substances. The influence of various factors on the binary structures at the air-water interface is investigated, and the monolayers morphology is characterized. Studies using the Langmuir technique have been supplemented with Brewster angle microscopy (BAM), surface potential (SPOT), dilatational and shear rheology, and PM-IRRAS. Some systems are transferred onto a solid substrate to examine the structure of the films. Studies on solid-state films transferred by the Langmuir-Blodgett and Langmuir-Schaefer techniques include

wettability, surface free energy (SFE), and topography measurements by atomic force microscopy (AFM).

Chapter 2 describes the factors governing the formation of the HAMLET-like complexes at the air-water interface based on research systems composed of oleic acid with α-lactalbumin and linoleic acid with α-lactalbumin. The role of temperature, pH, molecular packing, protein concentration, and calcium ions presence on the HAMLET-like complexes features at the air-water interface is insightfully investigated.

Chapter 3 discusses the interactions of the monolayer of oleanolic acid as an active substance of therapeutic potential with human serum albumin. Human serum albumin, as a possible carrier in pharmaceutical formulations, reduces the toxicity of the drugs and controls the final therapeutic efficiency. Thus, via the Langmuir monolayer methodology, a molecular-scale insight into studies was introduced for designing improved drug delivery systems.

Chapter 4 focuses on the physicochemical characterization of the two-component monolayers composed of oleic acid and oleanolic acid to solve the issue of the limited solubility of triterpenoids as active pharmaceutic compounds in novel nanotechnology-based drug delivery systems.

The dissertation ends with the general conclusions of the conducted research and Chapter 5, which contains a literature overview of the possible future applications of the Langmuir methodology.

Particular attention is paid in this dissertation to specifying the type of interactions between monolayers and additional components in the biomimetic systems studied via the Langmuir and Langmuir-Blodgett techniques. The results confirmed the usefulness of the monolayer studies on the substances of the therapeutic potential and provided physicochemical insight into novel pharmaceutical dosage forms. Thus, the conclusion about the possible application fields of the examined biomimetic systems was drawn.

Streszczenie

Technika Langmuira jest prostym i wszechstronnym narzędziem do badania zjawisk powierzchniowych na poziomie molekularnym. Obserwacja natury umożliwiła wdrożenie jej rozwiązań na wielu płaszczyznach życia i technologii. Postęp nauki umożliwił czerpanie inspiracji z natury w skali *nano*, dzięki badaniu struktur monomolekularnych i interakcji między cząsteczkami. Badania z zakresu biomedycyny stanowią kolejny krok w kierunku zrozumienia mechanizmów powstawania chorób i opracowywania nowych metod terapeutycznych. Bazując na powyższym, w prezentowanej rozprawie podjęto badania z wykorzystaniem techniki Langmuira w celu jakościowego i ilościowego scharakteryzowania systemów biomimetycznych o potencjale terapeutycznym. Skupiono się na kompleksach typu kwas tłuszczowy-białko, układach typu kwas triterpenowy-białko oraz monowarstwach dwuskładnikowych złożonych z kwasu triterpenowego i kwasu tłuszczowego.

Pierwsza część rozprawy doktorskiej poświęcona jest obszernemu przeglądowi literatury (Rozdział 1) dotyczącemu metodologii Langmuira w badaniach monowarstw substancji naturalnych. W przeglądzie literatury podkreślono znaczenie powierzchni materiałów i granic faz, zdefiniowano monowarstwy Langmuira oraz przedstawiono podstawy technik Langmuira i Langmuira-Blodgett. W tej części zawarto również krótki przegląd obszarów zastosowań monowarstw o charakterze biomimetycznym.

Zasadnicza część pracy dotycząca opisu publikacji wchodzących w skład niniejszej rozprawy doktorskiej została podzielona na trzy rozdziały (Rozdziały 2-4) poświęcone odmiennym układom badawczym. Każdy rozdział opisujący wyniki badań rozpoczyna się krótkim wstępem i kończy zwięzłym podsumowaniem, podkreślającym najważniejsze wyniki i obszary zastosowań. Kluczowym etapem badań każdego z układów jest utworzenie mieszanych monowarstw ze składników lipidowych z dodatkowymi substancjami. W ramach pracy badany jest wpływ różnych czynników na struktury dwuskładnikowe na granicy faz woda-powietrze oraz charakteryzowana jest morfologia monowarstw. Badania z wykorzystaniem techniki Langmuira zostały uzupełnione technikami pomocniczymi: mikroskopią kąta Brewstera (BAM), potencjałem powierzchniowym (SPOT), reologią dylatacyjną i ścinającą oraz PM-IRRAS. Część

układów badawczych została przeniesiona na podłoże stałe w celu zbadania struktury filmów. Badania nad filmami na ciele stałym, przeniesionymi technikami Langmuira-Blodgett i Langmuira-Schaefera obejmowały zwilżalność, swobodną energię powierzchniową (SFE) oraz badania topografii mikroskopem sił atomowych (AFM).

W Rozdziale 2 opisano czynniki determinujące powstawanie kompleksów typu HAMLET na granicy faz woda-powietrze złożonych z kwasu oleinowego z α-laktoalbuminą oraz kwasu linolowego z α-laktoalbuminą. Zbadano wpływ temperatury, pH, upakowania monowarstw, stężenia białka i obecności jonów wapnia na cechy kompleksów typu HAMLET na granicy faz.

W Rozdziale 3 omówiono interakcje monowarstwy kwasu oleanolowego, jako substancji czynnej o potencjale terapeutycznym, z albuminą surowicy ludzkiej. Albumina surowicy ludzkiej, jako potencjalny nośnik w preparatach farmaceutycznych, zmniejsza toksyczność leków i kontroluje ich skuteczność. Zastosowanie techniki Langmuira umożliwiło wgląd w interakcje między składnikami monowarstwy i badania w skali molekularnej w celu projektowania ulepszonych systemów dostarczania leków.

W Rozdziale 4 skoncentrowano się na charakterystyce fizykochemicznej dwuskładnikowych monowarstw złożonych z kwasu oleinowego i kwasu oleanolowego w celu rozwiązania problemu ograniczonej rozpuszczalności triterpenoidów, jako aktywnych związków farmaceutycznych w nowoczesnych systemach dostarczania leków opartych na nanotechnologii.

Rozprawę zamykają wnioski końcowe z przeprowadzonych badań oraz rozdział 5, zawierający przegląd literatury naukowej dotyczącej innowacyjnego wykorzystania metodologii Langmuira oraz możliwych przyszłych obszarów jej zastosowań.

W rozprawie szczególną uwagę poświęcono określeniu rodzaju oddziaływań między monowarstwami a dodatkowymi składnikami w systemach biomimetycznych, badanych technikami Langmuira i Langmuira-Blodgett. Wyniki potwierdziły przydatność badań nad monowarstwami substancji o potencjale terapeutycznym oraz zapewniły charakterystykę fizykochemiczną nowych postaci farmaceutycznych. Dzięki temu wyciągnięto wnioski o możliwych obszarach zastosowań badanych systemów biomimetycznych.

CHAPTER 1. The Langmuir monolayer technique for studying natural substances. State of the art

1.1. The significance of surfaces and interfaces

The homogenous, bulk part of the system, which is physically distinct and separated from other parts of the system is distinguished as *a phase*. Physical properties within a phase, including, among others, the density, chemical composition, concentration of some solutes, index of refraction, magnetization, solubility, compressibility, and conductivity, are essentially uniform. Where two phases of the system meet, there is a boundary of the finite thickness called *an interface*. Among interface, the material properties differ markedly from the adjacent phases. Within the interfacial region, the change of mentioned physical and electrochemical properties may follow a smooth monotonic transition or can reveal values significantly different from those for bulk phases. Due to the character of the surrounding bulk phases, interfaces are classified as: fluid interfaces (gas-liquid, liquid-liquid) and non-fluid or solid (gas-solid, liquid-solid, solid-solid). If gas is one of the phases, the boundary is often called *a surface* [1–5]. Processes driven by the interfacial phenomena (like diffusion) occur in a narrow region of a few lattice spacing order. Although the surface may be commonly considered insignificant because of the negligible thickness, the interfacial properties may determine the behavior of an entire system. To reveal the interfacial structure it is necessary to apply experimental techniques working with atomic resolution [4].

Interfaces are involved in countless systems, phenomena, and processes starting from the human body, through the world of microbes, plants, soil, and atmosphere, to the food, cosmetic, and manufacturing industries. Some surface phenomena are observed in everyday life, and the colloids and surfaces science provide a deep understanding of their nature. For instance, *tears of wine* were noticed in antiquity but explained only in the XIXth century. The ring of clear liquid inside the glass of high alcohol-content wine occurs just above the liquid level due to the capillary action and surface tension gradient. Wine climbs along the walls of glass forced by the capillary action, but the alcohol contained in the wine evaporates more rapidly than water. As a result, there are some regions among the film richer in water and thus characterized

by increased surface tension (the surface tension of water is much higher than the surface tension of ethyl alcohol). The surface tension gradient created draws wine from the bulk because it has lower surface tension due to the alcohol content. The collected liquid is subject to gravity forces and flows downward. The patterns formed on the glass resemble tears. The transport of liquids due to the surface tension gradient called the Marangoni convection is considered a significant effect in colloid science [1,2].

What is more, the capillary action acts as a driving force in the spontaneous rise of the water through the tree trunk or in spreading the water in the soil from wet to dry areas. It is the adhesion forces between the solid and liquid and the surface tension of the liquid that are responsible for the capillary rise. Beyond natural processes, the capillary action phenomenon underlies advanced analytical techniques such as thin-layer chromatography and is utilized for the production of functional clothing [1,2].

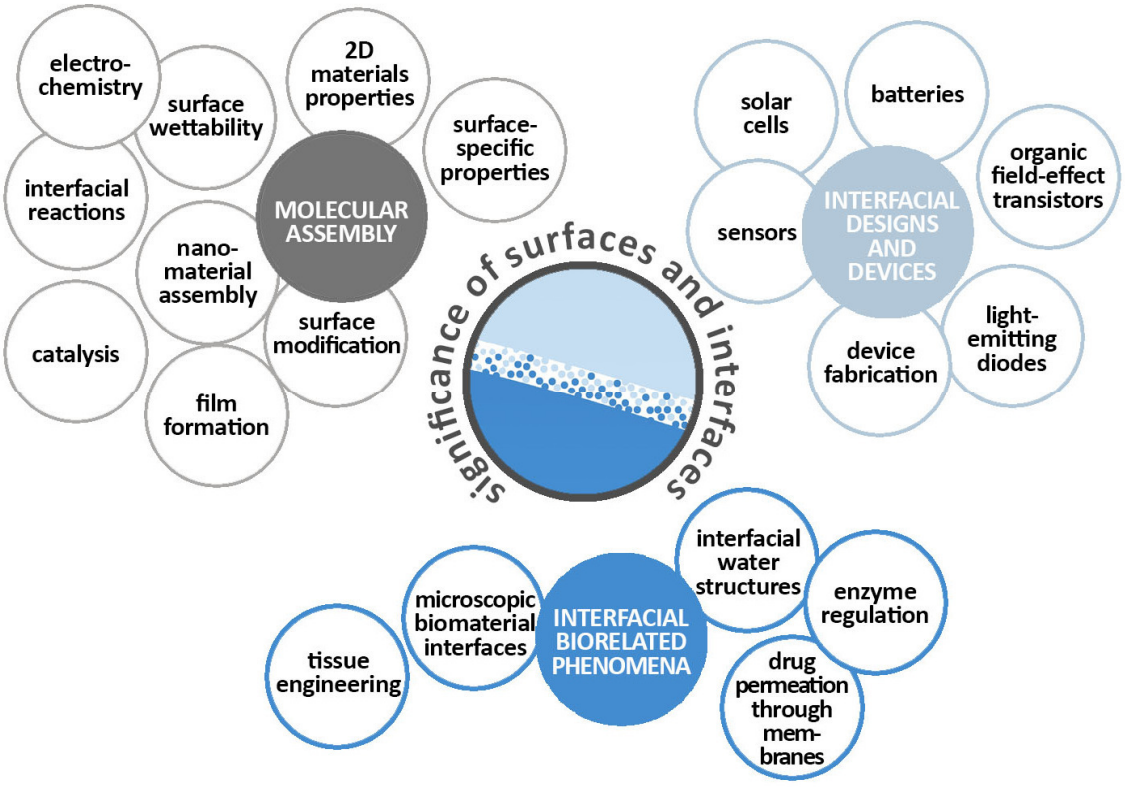

Figure 1. The significance of surfaces and interfaces recognized in research systems. Based on [6]

Due to the increasing demand for the miniaturization in technology, determining the surface properties of materials and their modification gains importance. Still, the

fast-growing field of nanotechnology relies on the processes occurring on the solid surfaces of mesoporous materials and nanoparticles. Therefore, surface science is relevant to numerous developments and technological improvements and is applied in practice.

Biological sciences are an important and growing field of application for research on surfaces and colloids. A good example of the interface created by nature is pulmonary surfactant coating the inner layer of the lung alveoli. Lung surfactant is a complex mixture of phospholipids (80%), neutral lipids (like cholesterol, 10%), and surfactant-associated proteins (10%), which is secreted by the type II cells of the alveolar epithelium. Although lung surfactant is one molecule thick layer, it plays a crucial role in the breathing process because it reduces the surface tension preventing the collapse of the alveoli during exhalation. The deficiency of the pulmonary surfactant associated with the incomplete development of the respiratory system in premature infants is known as respiratory distress syndrome. This fatal disorder is usually treated by surfactant replacement therapy based on the administration of exogenous surfactant preparations. Understanding the physicochemical properties of the lung surfactant layer and interactions between its components is essential for the development of effective therapies and strategies for drug delivery [2,7–10].

The possible field of application of biological surface science lies in medical implants inserted into a human body, tissue engineering, diagnostics biochips, biosensors, bioelectronics, artificial photosynthesis, and biomimetic materials. The focal point of this field of science is characterizing the mutual interactions between surfaces and biological environments (Figure 1) [11]. Moreover, the significance of interfaces is identified in multiple research systems, including basic phenomena based on molecular assembly, device designing, and interfacial bio-related processes [6].

1.2. Amphiphilic substances at the interface - Gibbs vs. Langmuir monolayers

Surface-active agents, abbreviated as *surfactants,* belong to the class of amphiphilic substances. When surfactant in low concentration is present in the system it is strongly adsorbed at the interface, decreasing the surface tension. The amphiphilic nature causes the adsorption. Among the molecule of the amphiphilic structure, there

are two segments combined: water-insoluble (hydrophobic) and water-soluble (hydrophilic). Due to the balance between those segments, the solubility of amphiphilic molecules varies from almost completely insoluble to highly soluble in water [2,3,12]. In a simple structure, the surfactant hydrophilic moiety is covalently bound to the hydrophobic group, but many variations of the basic structure occur [13].

Due to the hydrophobic effect, at the gas-liquid interface, some surfactant tails are expelled from the solution. Molecules adsorb at the interface until an equilibrium distribution between the surface and bulk solution is established. The same effect forces surfactant molecules to form micelles at higher bulk concentrations [13].

When the hydrophilic head of the water-soluble surfactant is immersed in the aqueous solution, and the hydrophobic tail is out of the water, the film adsorbed at the interface is called a *Gibbs monolayer.* The balance between the hydrophilic and hydrophobic parts of the molecule determines the water solubility of the surfactant. Usually, amphiphilic molecules composed of stronger hydrophobic moieties are less soluble or even insoluble in water. Insoluble amphiphiles at the air-water interface form insoluble monomolecular films called *Langmuir monolayers* [2,14].

The features of the monomolecular films, like molecular packing or monolayer fluidity, are determined by the structure of the amphiphilic molecules, the degree of hydrocarbon chain unsaturation, the head group size, and charge. Moreover, the structure of the lipid molecules affects the intermolecular interactions that exist between the components at interfaces [15,16]. Surfactants of the simpler structures are fatty acids, fatty esters, fatty alcohols, amines, sulfates, and sulfonates [17,18]. In biological systems, there are plenty of more complex surfactant structures like phospholipids, di- and triglycerides, phosphoglycerides, triterpenoids, or sterols. Numerous groups of amphiphiles can be listed, but the most important for this thesis are fatty and triterpenic acids.

The molecular structure of fatty acids consists of a –COOH head group of relatively small size and one hydrocarbon chain of varying length but having at least three carbon atoms [15,19]. The long-chain fatty acids (hydrocarbon chain length over 12 carbon atoms) are surface active and form interfacial films [17]. Fatty acids constitute

a source of energy for mammalians and influence cardiovascular, nervous and immune systems. A key factor responsible for the beneficial effect on human health is fatty acid unsaturation. *Cis* mono- and poly-unsaturated fatty acids (MUFAs and PUFAs respectively) are widely known for their strongly positive effect on human health [20–22]. Saturated fatty acids and unsaturated fatty acids with *trans* double bond generally increase the level of plasma cholesterol and lead to serious heart diseases [23,24]. Unsaturated fatty acids are abundant in biological systems, acting as a component of biological membranes. The differentiation of unsaturated hydrophobic chains in terms of their number, position, and configuration introduces the diversity of functions and properties of these membranes. In turn, hydrocarbon chain length and unsaturation degree affect the hydrophobic nature of the entire moiety [20]. Unsaturated fatty acids are frequently utilized in the pharmaceutical, cosmetics, and food industries [22].

The structure of molecules and the influence of unsaturation on the behavior of systems formed by unsaturated fatty acids continuously attract the attention of researchers. Langmuir himself pioneered the research on the unsaturated fatty acids at the air-water interface. It was found that the unsaturation within the hydrophobic chain affects the thermodynamic properties of the monomolecular films analogously as the chain shortening. Due to the geometrical configuration, for compounds of the same chain lengths, the presence of the unsaturated bond causes a monolayer expansion. Moreover, the expansion is more pronounced for the *cis* than *trans* conformations. The *cis* double bond in a chain prevents mutual rotation in the 2D structure and thus interferes with the tight packing of the long hydrophobic chains in a monolayer. The hindering effect exacerbates when the unsaturated bond is in the middle position of the chain [22]. It was also revealed, that the increase of the hydrophobic chain length in compounds of the same number of unsaturated bonds leads to a slight increase in the monolayer molecular areas. Upon the over-compression of the monolayer and significant reduction of the surface area available for a single molecule, the monolayer collapses. For PUFAs, with an increasing unsaturation degree of the apolar chain, the monolayer stability decreases, which is manifested by a reduction in the value of surface

pressure of the monolayer collapse. The hydrophobic chain extension increases the collapse surface pressure, evidencing improved monolayer stability [20].

On the other hand, triterpenoid compounds at the phase boundaries exhibit even more complex behavior. The terpenoids represent a large class of plant-origin secondary metabolites derived from C30 carbon atom precursors. They are primarily collected in the tissues of the leaf epidermis, in the bark or resin. Numerous terpenoid compounds are of pronounced pharmacological activity and thus are interesting for medicine [25,26]. Several groups of compounds can be identified among terpenoids: triterpenes, steroids, quassinoids, limonoids, and steroidal and triterpenoidal saponins. Most of the triterpenic molecules are tetra- and pentacyclic. Triterpenoids, especially steroids, sterols, and saponins, are extensively characterized in terms of biological activity. The pentacyclic triterpenes are divided into three classes: lupane-type (lupeol and betulinic acid), ursane-type (α-amyrin and ursolic acid), and oleanane-type (β-amyrin and oleanolic acid), due to the structure of the hydrocarbon skeleton. This group of compounds is biosynthesized by plants in the squalene cyclization process [25,26]. Lupeol, α- and β-amyrin are isomers described by the general formula $C_{30}H_{50}O$ with –CH_3 group at carbon 28 atom, while betulinic, ursolic and oleanolic acids are isomers of $C_{30}H_{48}O_3$ with –COOH group at carbon 28 atom. The lupane-type compounds comprise one cyclopentane and four cyclohexane rings, while both ursane-type and oleanane-type are composed of five cyclohexane rings. Compounds of each group differ in *cis* or *trans* conformations and the presence of double bonds within the cycloalkane skeletons, locations and types of functional groups, and a number of chiral carbon atoms. All the described examples of pentacyclic triterpenes are surface active and form insoluble monolayers at the air-water interface. However, their behavior at the interface and surface activity is strongly affected by the molecular structure. Reduced terpenes (lupeol, α- and β-amyrin) are simple surfactants with –OH polar group as the hydrophobic moiety. Oxidized terpenes show bolaamphiphilic character as they have two polar groups (-OH and -COOH) localized on the opposite sites of the molecule. As a consequence, a molecule at the interface takes one of two possible orientations depending on the thermodynamic conditions [25].

1.3. The basics of the Langmuir and Langmuir-Blodgett technique

Spread films with their unique properties have become the subject of observation and research in the past. According to the inscriptions on clay tablets, in Hammurabi's period, patterns formed by oil poured on water or water onto the oil acted as a basis for divination and rituals. Thin films of ink floating on the water's surface were considered an artistic expression in a technique known as *Suminagashi.* The distinctive patterns created by placing the ink on the water surface were developed in Japan in the 12th century BC. The resulting paintings resembling the marble structure on the water are transferred to the paper by touching it to the ink layer [14,27]. In ancient times Aristotle described the calming effect of oil spilling onto rough sea water near ships. The first scientific consideration of this phenomenon was done in 1765 by Benjamin Franklin, who spread a teaspoon of oil on the waters of Clapham Pond in London. He noticed the oil spread immediately over a large part of the surface. Although, it was Lord Rayleigh who, in 1889, estimated the thickness of spread films to be 1.6 nm based on the area covered by the oil of known volume [14,27,28].

The author of the first direct experiments on the monolayers was Agnes Pockles. Her simple apparatus became the basis of equipment that is currently known as the Langmuir trough. She developed a measurement method to investigate surface tension. Without any professional and scientific background, this inspiring young female had studied the behavior of the contaminated greasy water surface for ten years. For the experiments, she applied a rectangular tin tray filled with water, equipped with a tin strip laying on the liquid's surface. By moving the tin barrier she could increase or decrease the degree of contamination, adjust the area and clean the water surface. According to previous reports of Ludwig Wilhelmy, she utilized a simple balance to measure the force needed to lift a small wooden disk above the surface of the water, which was in proportion to surface tension. Thanks to the letter addressed to Lord Rayleigh and his assistance, she published the results of her research in Nature in 1891 [14,27–29]. In 1917, Irving Langmuir adapted the trough concept by Pockles and introduced theoretical considerations on the films at the air-water interface. His achievements turned out to be so significant that monomolecular layers of a surface

active substance on the surface of the aqueous subphase are called *Langmuir monolayers* [14,27–30]. Moreover, in 1932, Langmuir was awarded the Nobel Prize in Chemistry due to his work in surface chemistry. He investigated the pure substance's behavior at the air-water interface and proved that the films are indeed monolayers [14].

To create a monolayer, in a typical Langmuir experiment, a known amount of the amphiphilic substance of known molecular mass is dissolved in a volatile, water-immiscible, organic solvent of a high spreading coefficient like chloroform or ethanol. The material is then gently placed on the cleaned water surface. After 10-15 minutes of solvent evaporation, the monolayer is formed by self-assembly and is compressed or expanded with movable barriers of inert material. The hydrophilic headgroups of the surface-active molecule stay in contact with the water subphase while the hydrophobic hydrocarbon tails point to the air. The polar heads of the amphiphiles are anchored to the water due to electrostatic interactions, like ion−dipole, dipole−dipole, and hydrogen bonds. When the adhesion forces dominate over cohesion, molecules occupy the entire area available at the interface, such as a gas taking up the entire possible volume. At the same time, the hydrophobic tails show repulsive interactions with the water. Molecular ordering is favored by nonpolar chain/chain interactions driven by the van der Waals forces, which are weak enough to prevent lateral aggregation and ensure the spontaneous spreading of the molecules [14].

Usually, the structure of the monolayer forming is monitored via surface pressure – area per molecule isotherms (π-A isotherms). The standard Langmuir trough is made of a hydrophobic material like Teflon (Figure 2). One or two symmetrical barriers slide over the water surface, limiting the area of the film and thus causing its compression. A device to measure surface pressure is a platinum, mica or filter paper Wilhelmy plate connected to an electronic microbalance [27,31,32].

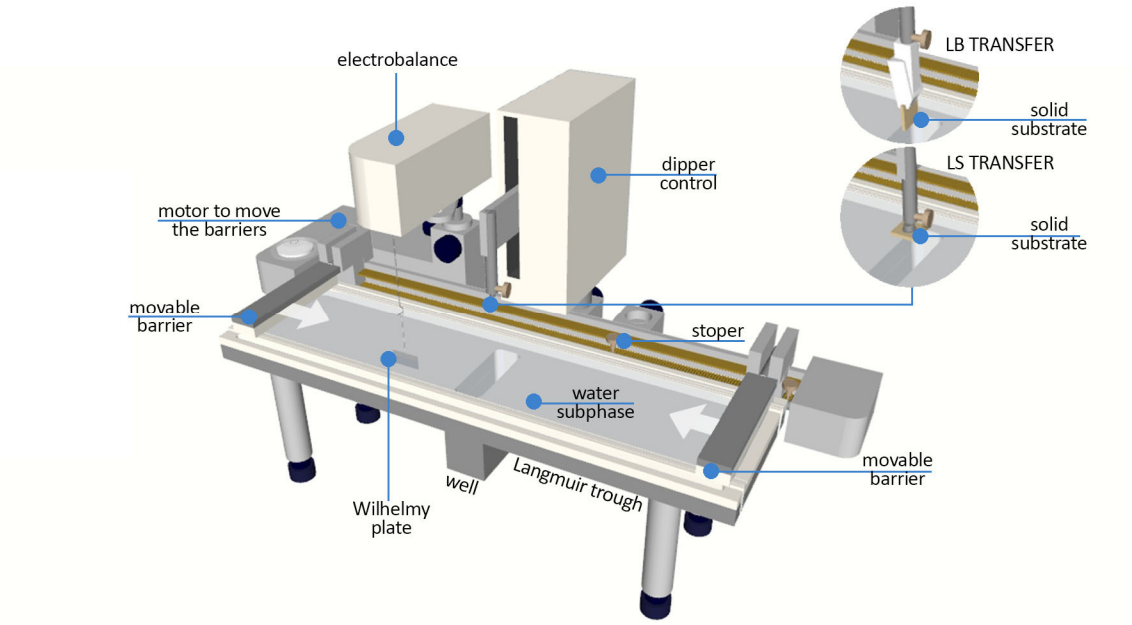

Figure 2. Schematic representation of the Langmuir-Blodgett trough

When conducting a π-A isotherm experiment, due to the area available between the barriers, the molecules undergo several orientation changes (Figure 3). At the initial stage of compression, when the molecules are relatively distant, they poorly interact with each other by analogy to the three-dimensional states of matter described as the two-dimensional gaseous state. Further reduction of the surface area induces interactions between the head groups. However, the tails are still chaotically oriented, which is called a two-dimensional liquid phase. The monolayer can be compressed until the heads and tails of the molecules are closely packed and ordered (two-dimensional solid phase). The monolayer collapses if further compressed. Shape of the π-A isotherm in Langmuir technique is specific for particular amphiphilic substance in given conditions [24]. For monolayer reproducibility, appropriate conditions must be provided, like precise preparation of pure materials and spreading solvents, application of ultrapure water, and a clean, dust and vibration-free environment [27,31].

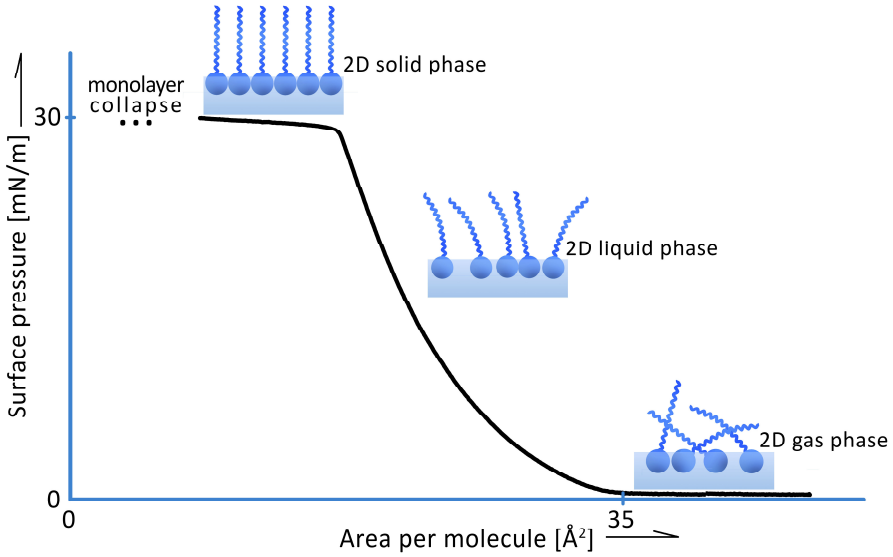

Figure 3. Typical π-A isotherm for oleic acid monolayer at the air-water interface with a schematic orientation of molecules

Based on the π-A isotherm data, it is possible to determine the characteristic parameters of the monolayer like $A_{lift\text{-}off}$, A_{lim}, and π_{coll}. The graphical definition is in Figure 4A. $A_{lift\text{-}off}$ refers to the area per lipid molecule at which the surface pressure of the compressed monolayer rises. A_{lim} is obtained by extrapolating the steep part of the isotherm curve to π=0 mN/m, which corresponds with the minimal area occupied by the molecule within the monolayer. π_{coll} is the surface pressure during the film collapse [2,14,24].

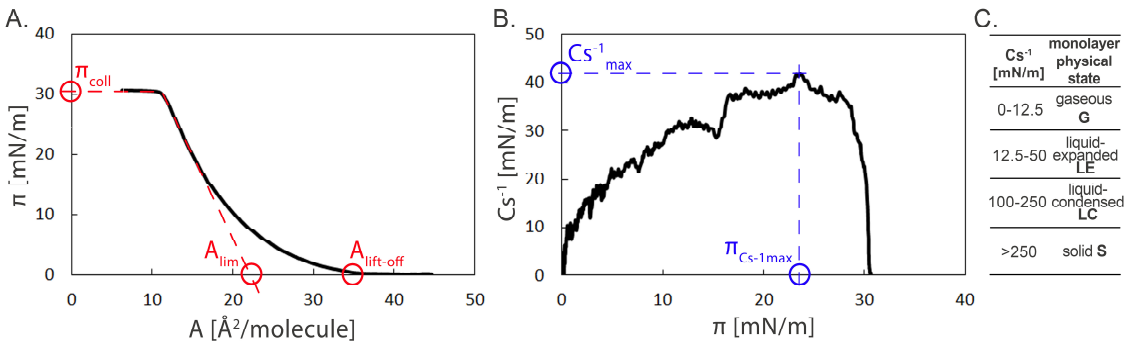

Figure 4. An exemplary graph of the π-A isotherm (A), the Cs^{-1} vs. π (B) with the characteristic values highlighted, and (C) physical states of the monolayer with corresponding Cs^{-1} values

According to monolayer compression, there are several phase transitions due to the changes in the molecular packing. The monolayer compressibility expressed as

compressibility modulus (Cs^{-1}) can be utilized to characterize the physical state of the monolayer:

$$Cs^{-1} = -A\left(\frac{d\pi}{dA}\right)_{p,T} \quad (1)$$

Since Davies and Rideal classified the monolayer physical states, the compression modulus provides information on the stages of compression, where higher Cs^{-1} values correspond to condensed monolayer (Figure 4B, C) [5,24,33].

A few years after reporting of the Langmuir trough, the idea was developed into a system for transferring the already-formed monolayer onto a solid substrate (Figure 2). The Langmuir trough was additionally equipped with a dipper system and a well, and named as Langmuir-Blodgett trough (LB deposition) in honor of the female scientist working alongside Langmuir on the monolayer deposition. In 1934, Katherine Blodgett improved the methodology of vertical film deposition on one substrate, sequentially one after another until the formation of the multilayer coating [14,30]. The transfer of films onto a hydrophilic-hydrophobic solid substrate was further developed by Langmuir's team. Vincent J. Schaefer developed horizontal deposition carried out as stamp transfer, known as Langmuir-Schaefer (LS) procedure. The LS transfer is particularly recommended for rigid monolayers [14,31,34].

Basic information on the quality of the film transfer comes from a transfer ratio value. The transfer ratio is a simple calculation of the area that barriers moved over during the transfer process divided by the area of the plate covered with a film. For an ideal deposition, the transfer ratio is 1 [14,31]. The quality of the LB and LS deposition depends on multiple factors such as the type of amphiphilic molecule, subphase composition (pH and ionic strength), solid substrate properties, monolayer thermodynamic properties during the deposition, and dipping rate [14].

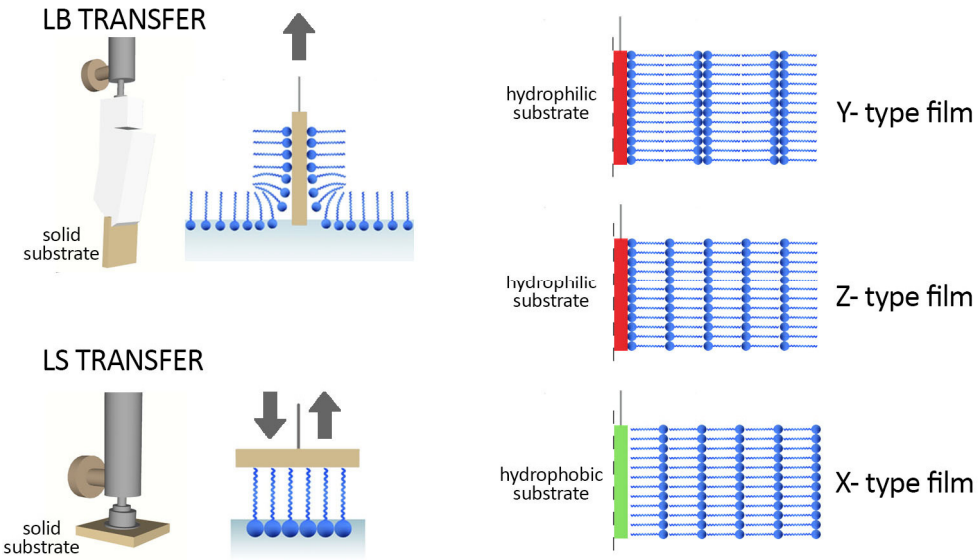

Figure 5. Representation of the schematic LB and LS transfer and different types of films deposition

Depending on the substrate surface character and amphiphilic substance, a transferred film can be of a different type (Figure 5). The Y-type film creates when the monolayer transfers during both the insertion and withdrawal process. Z-type film forms if the transfer is during withdrawal only. However, the X-type film creates when the transfer is during insertion only. Multilayers in the LS technique are performed by repeating the touching and lifting procedure [14,30]. Nowadays, many manufacturers produce Langmuir troughs with sophisticated features and complementary components available, but the fundamental principles and procedures stay unchanged over a hundred years [30].

Langmuir monolayers are considered a useful platform for precise studies on intermolecular interactions at the phase boundaries. The primary advantage of the Langmuir technique is accurate control of the surface pressure and area per molecule at the interface, which makes it a simple and versatile physical model [32,35]. Lipid monolayers are well-defined, homogenous, stable two-dimensional systems. The availability of numerous complementary techniques for the extensive characterization of thin films is also a significant advantage. Thanks to the controllable molecular organization within a 2D plane via the LB method, it becomes a promising and powerful tool in interfacial nanoarchitectonics [6,32,36].

Contradictory opinions about the LB technique have emerged recently. According to some researchers, the straightforward technique has already been exploited, and for others, it has some out-of-the-box applications, and only the researchers' imagination will tell which direction it will evolve [30]. The layer-by-layer (LbL) and self-assembled monolayer (SAM) methods are frequently mentioned as an alternative methodology to the LB technique [34].

1.4. Binary Langmuir monolayers and two-component systems

The two-component monolayers can be considered in two ways: (a) when both components can form monolayers and both are water-insoluble, or (b) as monolayers interacting at the interface with additional substances adsorbing from the water subphase, such as nanoparticles, pharmaceutically active substances, peptides, proteins, and other bioactive compounds (Figure 6A,C). The Langmuir monolayer technique is a universal tool for determining the interactions between components of mixed systems, which leads to a better understanding of their nature. Moreover, it allows for quantitative analysis of the thermodynamic stability of the systems [15].

Multi-component monolayers comprise two or more insoluble component that acts as a mixture in the thermodynamics approach. Mixed monolayers of this type are formed by the preparation of a spreading solution containing both substances in a particular ratio, dissolved in a volatile solvent (Figure 6A,B) [21,37]. For an ideal binary system (A^0_{12}), the average area per molecule at a given surface pressure (π) is calculated according to the straightforward principle:

$$A^0_{12} = X_1A_1 + X_2A_2, \quad (2)$$

where X_1 and X_2 correspond to the molar fractions of substances 1 and 2, and A_1 and A_2 are the molecular areas at the given surface pressure π in the pure substance monolayers.

When the calculated A_{12} shows a positive deviation from the ideal value, repulsive interactions between the molecules exist, while the negative deviation indicates the attraction between the molecules. Additionally, the thermodynamic parameters on mixed monolayers, like excess free energy of mixing (ΔG^{exc}), can be determined based on the data from the isotherms. Depending on the excess free energy

of mixing value, binary monolayers of amphiphilic substances can be miscible, immiscible or partially miscible over a range of surface pressures [14,21,37].

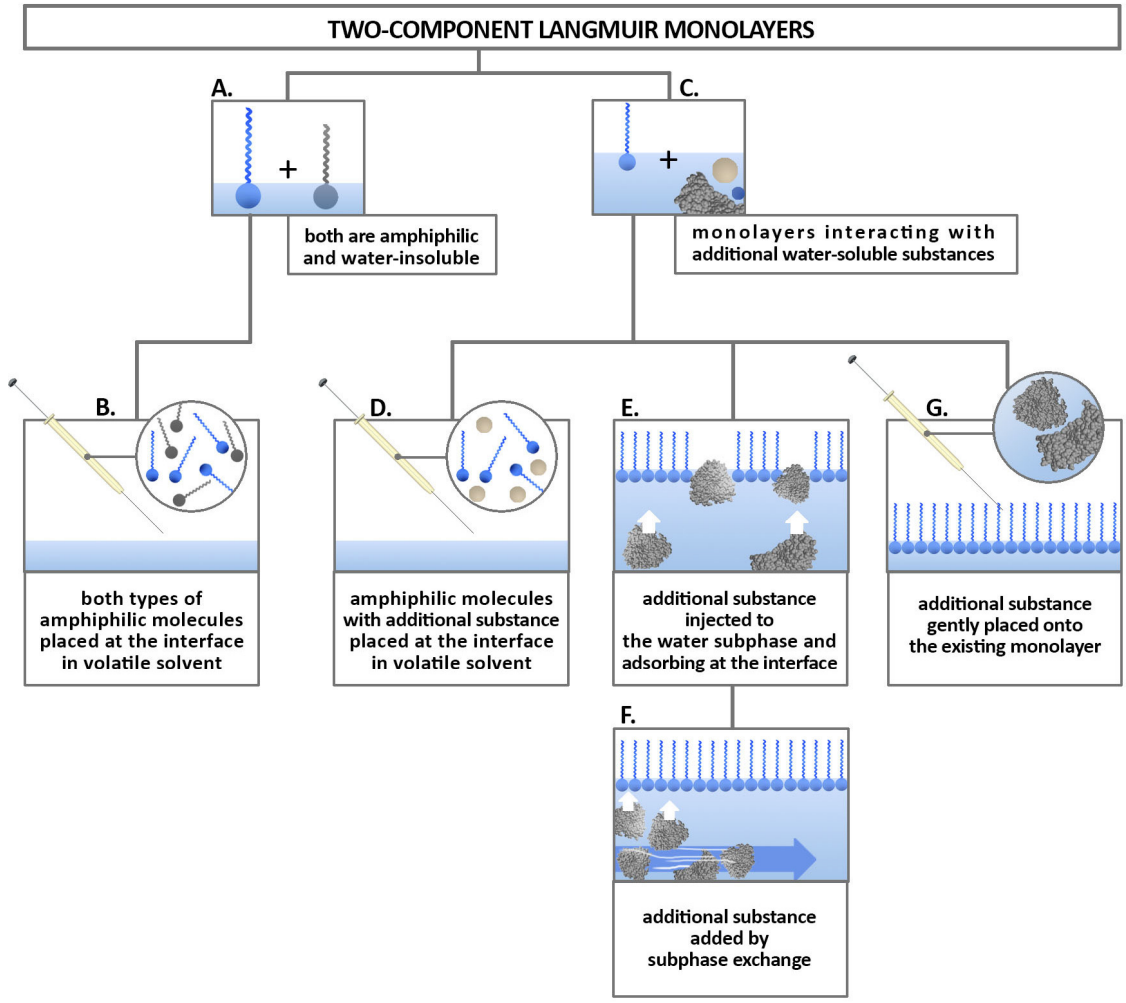

Figure 6. Different types of two-component monolayers with regard to possible formation methods

The Langmuir monolayer technique is suitable for the characterization of the interfacial interactions of lipids with water-soluble substances, additionally concerning the mechanism of interaction and stoichiometric relationships. Soluble amphiphilic molecules, adsorbing from the aqueous subphase, interact with the existing monolayer through their hydrophilic and hydrophobic moieties [14]. One of the possibilities to create a mixed monolayer of lipids and other substances is to mix them together in the stock solution [38,39]. Both components in the volatile solvent are spread at the water surface (Figure 6D). Other components of the mixed monolayers can also be injected into the aqueous subphase and adsorb at the interface interacting with the existing

monolayer (Figure 6E). A relatively new and not yet fully explored method of delivering additional substances to the system is employing a peristaltic pump exchanging the bulk phase underneath the existing monolayer with a new liquid already containing molecules of interest. Moreover, this methodology provides the possibility of changing the measurement conditions during an ongoing measurement (Figure 6F) [40–42]. A less common method of the formation of a two-component film involves the application of the soluble compounds onto the already spread monolayer in the region of the hydrophobic chains (Figure 6G) or, alternatively, monolayer spreading on the subphase already containing additional substances [36,43,44].

The incorporated compound affects the monolayer causing its expansion or condensation (Figure 7A). The monolayer expansion takes place when the incorporating compound shifts the isotherm to larger values of molecular areas. Adsorbing molecules interact with the monolayer by occupying space at the interface and increasing surface pressure, altering the molecule's orientation, or even replacing molecules at the interface. Shifting the isotherm towards lower molecular areas may indicate the loss of amphiphilic molecules from the surface or the adsorption of water-soluble compounds in the region of the monolayer's polar heads which minimizes lateral repulsion. The molecules removed from the interface can form aggregates dispersed in the subphase or aggregate near the surface [14]. Moreover, additional molecules affect the shape of the π-A isotherms, thus this phenomenon is helpful in the characterization of the interfacial interactions [36].

To get a better insight into the specificity of the compounds like peptide and protein binding to lipid monolayers, one can determine the maximum insertion pressure (MIP) using tensiometry (Figure 7B). The MIP reflects the maximal surface pressure up to which the soluble compound can insert into the monolayer. The lipids are spread onto the water subphase and compressed to the particular surface pressure marked as π_i. Then the compound is introduced to the water subphase and increases surface pressure when adsorbing onto the monolayer until the system reaches equilibrium surface pressure (π_e). The difference between π_e and π_i, denoted as $\Delta\pi$, depends on the π_i value and reflects the extent of interfacial interactions between monolayer and adsorbing

compound. From the plot of $\Delta\pi$ vs. π_i, the binding parameters like MIP, the synergy, and $\Delta\pi_0$ can be obtained. According to the graph (Figure 7B), the MIP and $\Delta\pi_0$ are determined by extrapolating the linear regression to the x-axis and y-axis, respectively. The synergy value can be calculated by adding 1 to the slope, as shown in Figure 7B. A positive value of the synergy demonstrates a favorable binding of the compounds to the monolayer, while a negative value indicates an unfavorable binding. $\Delta\pi_0$ signifies the tendency of the lipid to alter the compound's surface activity [14,36,45].

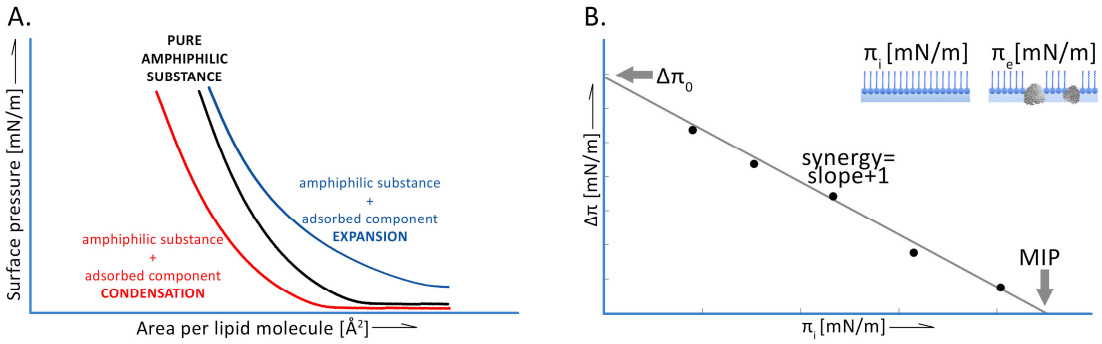

Figure 7. Effect of introducing additional substances to system A. condensation and expansion effect B. determination of the binding parameters

Many factors influence the interfacial interactions between the monolayer and additional components (Figure 8). The overall picture of the interactions is affected by the physical state of the monolayer as well as the characteristic of the additional substance, and the external conditions during the measurements. Aqueous subphase containing various salts or cations may modulate the kinetics of protein binding [45]. Temperature affects the thermodynamic properties of the monolayer and the protein conformation [45–47]. Lipid properties modify the monolayer behavior, oxidation level, and physical state. For mixed lipid-protein monolayers, the protein properties like orientation, conformation, intactness, or lateral distribution are of high importance. In monolayer interaction with nanoparticles, the crucial is type, size, shape, surface chemistry, presence of ligands, and charge [48]. Moreover, a significant factor concerning interfacial interactions is an experimental approach [36,45].

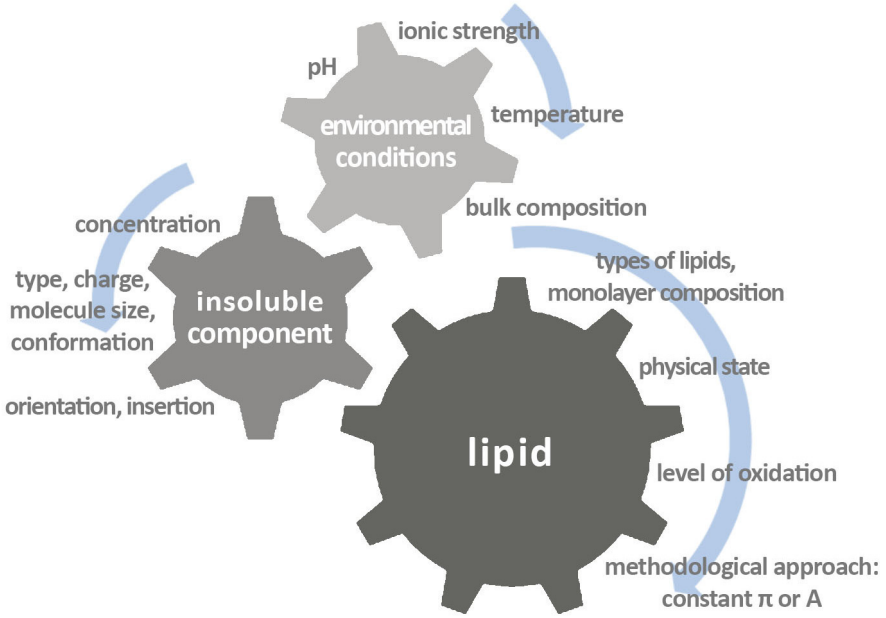

Figure 8. Parameters crucial when investigating interactions among multi-component monolayer

The behavior of the monolayer in time or the interactions of additional components with the monolayer can be studied experimentally by two different approaches - by relaxations at constant surface pressure or relaxations at the constant molecular area (Figure 9A, B). Although the results obtained with both approaches are complementary, each method serves slightly different purposes. While the surface pressure of the monolayer is kept constant, the mean area per lipid molecule is monitored as it changes due to the interaction with additional substances adsorbing at the interface. This approach allows measurements by compressing the monolayer to a specific surface pressure, such as 25-35 mN/m representing the surface pressure of biological membranes [49–51], and observing the monolayer penetration by additional molecules. Moreover, the constant surface pressure approach demonstrates effects resulting from the addition of non-lipid molecules to the system. On the other hand, when the area per molecule is held constant, surface pressure is allowed to fluctuate, then the changes in the monolayer phase-state arise either from the monolayer dissolution or the addition of the binding molecule. However, when desired surface pressure is relatively high and close to the monolayer collapse, there is a risk of the misinterpretation of the decrease in mean molecular area. After the monolayer collapse,

the area per molecule usually decreases, which may be taken as the lipid reorganization due to the presence of an additional substance. Furthermore, to hold the surface pressure constant, the monolayer is compressed or expanded by moveable barriers, which can affect the structural changes in the monolayer. Another disadvantage is that the constant surface pressure approach requires much more additional substance, which is significant for expensive or rare chemical substances. During the constant surface area experiment, after the additional component introducing, the surface pressure variations are registered. This experiment type is particularly useful to gain information about the surface activity of the components adsorbing towards the monolayer and to determine binding parameters (MIP, synergy, and $\Delta\pi_0$) [36].

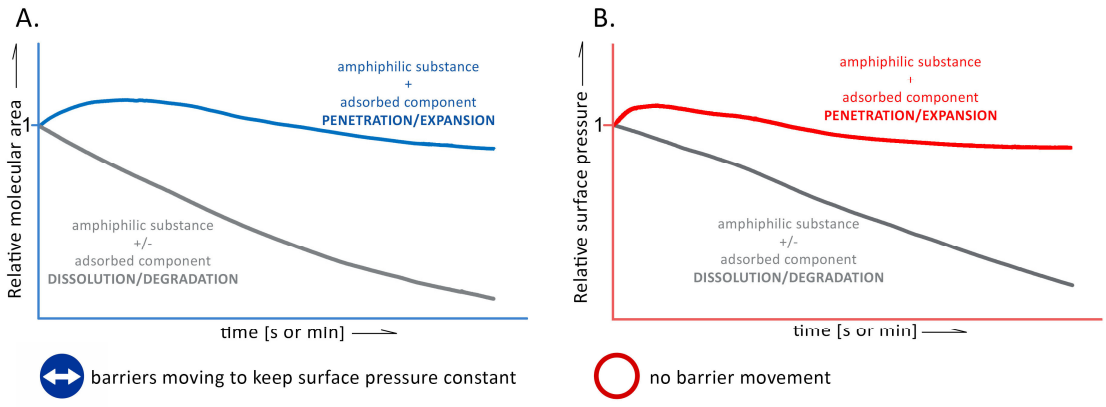

Figure 9. Two approaches to conduct relaxation experiments A. at constant surface pressure B. at constant molecular area

Since lipids at the interface can interact with other components through several mechanisms like hydrophobic interactions, electrostatic interactions, hydrogen bonds, or binding specific groups, the subtle differences in the methodology of experiments lead to the elucidation of various phenomena.

1.5. Langmuir monolayers of biomimetic character

The Langmuir technique is inseparable from amphiphilic lipids. These, on the other hand, are ubiquitous in the biological world. About 30 Å thick film acts as a margin between environments for every individual cell of living organisms. Eukaryotic cells generate thousands of types of lipids serving different functions. In addition to acting as

energy storage, lipids are generally utilized as messengers in molecular recognition or signal transduction and pose as a matrix of the membrane in cells [52–54].

Model cellular membranes

Cellular membranes comprise polar lipids built of hydrophobic and hydrophilic moieties. The unique ability of amphiphilic molecules to self-association, entropically driven by water, underlies the spontaneous formation of cell membranes. Thanks to the barrier function of lipids, first cells can segregate internal constituents from the outer environment and produce separated organelles. Lipids enable cells to divide and perform intracellular membrane trafficking and biological reproduction. Moreover, lipids can influence membrane proteins to disperse or aggregate [52]. Due to the complexity of the biological membrane's composition, model membranes are considered a useful and versatile tool to investigate membrane interactions via less complex mixtures or even individual lipids [45]. Among the numerous models of membrane systems, the Langmuir monolayer technique is distinguished by the simplicity of experimental methodology, the ability to adjust model composition, the ability to control the thermodynamic parameters, and a high level of molecule organization [20,36,45,53–57]. Although the natural cell membrane is a lipid bilayer (comprising two weakly coupled monolayers), there is a direct thermodynamic correspondence between the bi- and monolayer. Thus, experiments with the Langmuir technique by forming monolayers reflect the conditions of the bilayers. Furthermore, the membrane's outer and inner leaflets are compositionally diversified, which can be reflected in the Langmuir technique studies. In many cases, the Langmuir technique is combined with supporting techniques like surface-specific microscopies, spectroscopies, and/or rheological techniques [36,44,45,53].

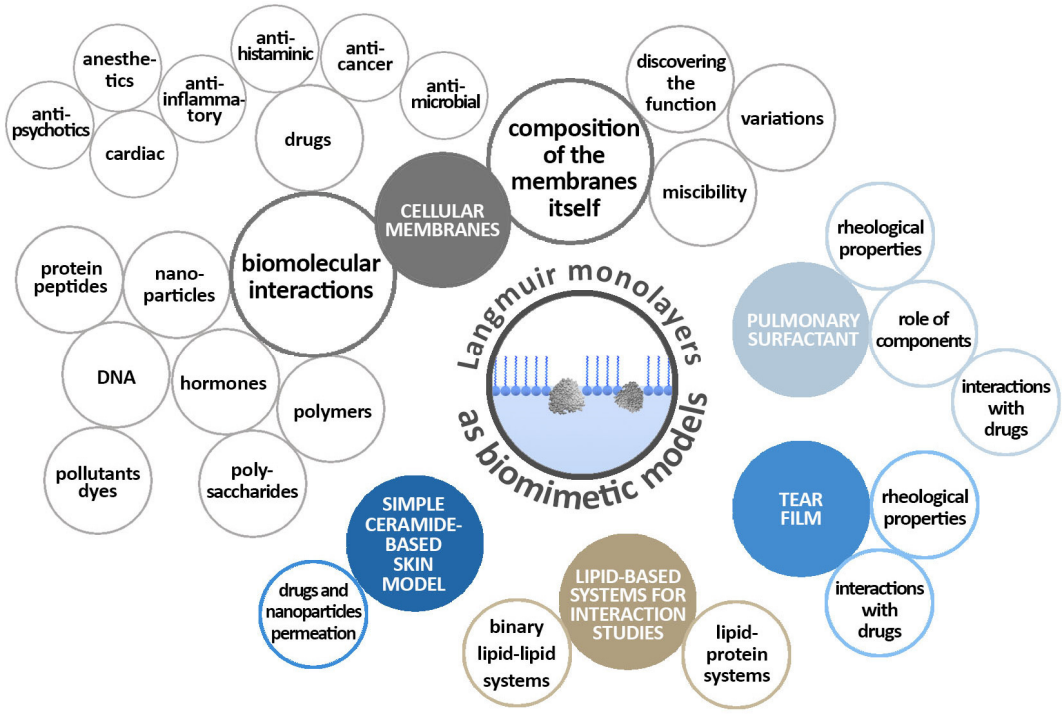

Figure 10. Biomimetic systems represented by the Langmuir monolayers

Membrane lipids include three major groups: glycerophospholipids, sphingolipids, and sterols [58]. Langmuir monolayers as model cellular membranes (not only mammalian) have been used successfully for many years for a variety of purposes (Figure 10). Some investigations focused on discovering the role of individual lipid components in functions performed by a particular membrane or changing the composition under the influence of various factors. The most extensive studies have been carried out regarding the condensing effect of cholesterol in lipid membranes [59–61]. The outcome of the interaction of biomolecules and nanomaterials with model membranes can be adsorption or coupling, penetration within a monolayer, and structural changes (like monolayer expansion, condensation, or even disruption) [44].

Abundant systems mimicking membrane environment have been developed to explore interactions with proteins. The explorations in this area run in two ways. The first line of investigation concerns enzymes, antibodies, and antigens immobilized in lipid films utilized in molecular recognition processes. The second area of research is focused on revealing the biological interface phenomena, mainly in cellular membranes [44].

The Langmuir monolayers serve as *in vitro* models, which help to decipher the mechanism of protein-membrane interactions and alterations in the lipid organization due to protein presence [47]. However, model biological membranes via Langmuir monolayers are not adequate for experiments on transport across the membranes. For such studies, other types of model systems, such as vesicles, are more relevant [44].

Moreover, Langmuir monolayers have been utilized as model systems for understanding the origins and mechanism of Alzheimer's disease by studying the interactions between proteins, peptides, and the lipid film [62,63]. Misfolding and aggregation of tau protein forming the amyloids in the brain are believed to be one of the causes of this neurodegenerative disease. Investigation of the amyloids at the air-water interface disrupted the structural integrity of the lipid membrane, thus suggesting the disease mechanism induced by protein aggregates [44,62].

Numerous studies have also been carried out on the interaction of phospholipid monolayers with various types of proteins. Since the mucus layer is the first barrier for mucoadhesive drug delivery systems to access the organism and it comprises mucin, the interactions between the mucin and model biological membranes under the physiological conditions (subphase pH and temperature) have been studied [64]. Phase transition and the properties of membrane model have become the subject of research on monolayer penetration by β-lactoglobulin as the main protein component of milk whey. The penetration kinetics of β-lactoglobulin were tested at different physical states of monolayer and increasing protein concentrations [65]. Human and bovine serum albumin have been widely studied and reported as a good example of an adsorption model with Langmuir monolayers [44,66].

An extended group of experiments concerns the coupling of DNA to the model membrane of cationic lipids thus the DNA-based pharmaceuticals in gene therapy, nano-devices and biosensors need verification of DNA-lipid interactions [47].

Recently, the design, synthesis, and functionalization of novel nanoparticles for biomedical applications have been the focus of academia and industry. However, detailed insight into their influence on the organism, toxicity, and interactions with the cell membrane is still lacking. Langmuir monolayers serve as useful models to investigate

the physicochemistry of these phenomena. Tens of studies focused on *in vivo* and *in vitro* biocompatibility of silver, gold, carbon, silica nanoparticles, fullerenes, polymers, and metal oxides, and numerous publications concern therapeutic applications [44]. Studies on nanocomponent's influence on model membranes aimed to understand the mode of action and indicate the type of interactions. However, it should be highlighted that the information obtained on the impact of nanocomponents on cell model membranes is insufficient, and systematic studies on the effect of nanoparticle size, concentration, and morphology on the monolayer behavior are still necessary [44].

Various membrane-active drugs have been examined via the Langmuir monolayers to verify their toxicity or indicate molecular targets. A particularly important advantage of this approach is the possibility to selectively reveal the affinity of a drug to a particular cellular component, which usually is impossible to perform on isolated membranes or living cells. The most extensive studies concern antimicrobial, anticancer, anti-inflammatory, anti-histaminic, cardiac drugs, as well as anesthetics and antipsychotics [44].

Model pulmonary surfactant

In addition, scientists investigated the interactions of drugs and pollutants with other biomimetic systems, such as the pulmonary surfactant layer (mentioned in Chapter 1.1). Lung surfactant in alveoli reduces the surface tension at the air-water interface and, thus, is crucial for the proper functioning of the respiratory system. Additionally, lung surfactant is the first barrier for pulmonary administrated drugs via inhalation or against airborne pathogens and particles. The influence of steroid drugs [39] and antibiotics for inhalation administration [38] on the thermodynamic properties of a model pulmonary surfactant was investigated as well. In light of growing air pollution, research on the impact of pollution, like benzo[a]pyrene, on the stability and the rheological properties of a model pulmonary surfactant is also more common [67].

Model tear film

Langmuir monolayers can also mimic the outermost layer of the tear film at the air-liquid interface. The tear film lipid layer containing phospholipids, ensures proper functioning of the eyelid onto the ocular surface and helps to re-spread when blinking

due to the reduction of the surface tension. Numerous publications discuss the interaction of pharmaceutically active substances on the properties of the tear film at the interface [68].

Simple ceramide-based skin model

The monolayer formed by the lipid mixture may also mimic the composition of the stratum corneum to reveal the mechanism of how nanoparticles permeate through the skin. The results on silver nanoparticles for instance could help design nanoparticle-based products for cosmetic applications [69].

Fatty acid-based systems for interaction studies

As has already been emphasized, the Langmuir technique is a valuable physicochemical tool to investigate mutual interactions at the phase boundaries on a molecular scale. This approach allowed obtaining molecular insight into the mechanism of lipid-protein complex formation. In the current trend of employing natural compounds in cancer therapies, a tumoricidal complex of oleic acid (OA) with α-lactalbumin (α -LA) was identified in the 1990s [70]. The complex was named 'HAMLET' from the acronym of the descriptive 'Human α-Lactalbumin Made LEthal to Tumor cells'. The components of the HAMLET are derived from human milk, and under natural conditions, the complex forms in the stomach of breastfed infants [71]. To create the HAMLET complex *in vitro* the infant's gastric conditions of pH and temperature are necessary. α-Lactalbumin is a two-domain (α and β) globular protein with a molecular mass of 14.2 kDa and an isoelectric point of 4.6. It binds metal ions, including Ca^{2+}, which stabilize the structure and is concerned as significant in the protein regeneration of the denatured to native form. Due to a low pH or by the removal of Ca^{2+} ions at a slightly denaturing environment, α-LA undergoes partial unfolding and transforms to a molten globule form. The α-LA in the molten globule form is of a similar radius of gyration to the size of the native protein. The partial unfolding of the protein structure leads to new functional properties. A molten globule state is an intermediate form between the native and fully denaturated protein. The HAMLET complex forms from the α-LA molten globule form (by removal of Ca^{2+}) in the presence of oleic acid as a cofactor (Figure 11). Molten globule state of α-LA reverts to the native form when the environmental

conditions are normalized (presence of Ca^{2+}, temperature, or pH) [71–74]. Calcium depleted form of α-LA is thermally denatured above 43°C, thus at 36.6°C this phenomenon is not observed [75].

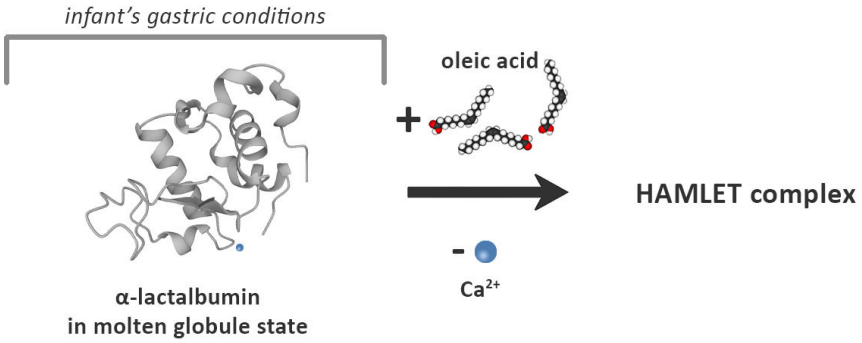

Figure 11. Scheme of HAMLET complex formation; α-lactalbumin structure from Protein Data Bank (1A4V) [76] and oleic acid structure from PubChem [77]

The HAMLET is the most recognizable example of a wider group of substances – the HAMLET-like complexes consisting of other unsaturated fatty acids and α-LA analogs proteins. The BAMLET, CAMLET, and GAMLET, respectively, are complexes of the bovine, camel, and goat milk counterparts of α-LA and oleic acid, and ELOA comprises equine lysozyme with oleic acid, which exhibits biological activity. Additionally, the tumoricidal activity had been confirmed for complexes containing other C18 unsaturated fatty acids like linoleic, *cis*-vaccenic, and palmitoleic acid. However, C16 and C20 *cis* unsaturated fatty acids or C18 *trans* fatty acids were found to be inefficient cofactors in biologically active complexes formation [71,78].

The cytotoxicity of HAMLET complex has been successfully confirmed in clinical trials for a few cancer types, like skin papillomas, glioblastoma, and bladder cancer and for intestinal cancer in animal models [71,79]. Nevertheless, despite numerous investigations, the biomedical mechanism of its formation is still insufficiently recognized. Moreover, scientists still try to determine the contribution of both protein and fatty acid in the cytotoxic activity of the complex. In tumor cells, after initial membrane interactions, HAMLET accumulates, targets organelles, and leads to cell death through several pathways. Surprisingly, normal differentiated cells are resistant

to HAMLET. Experiments proved, that α-LA unfolding and OA binding are both required for uptake into tumor cells [71,72,79].

Various approaches were used to prepare the HAMLET complex, like chromatography, alkaline method titration, heat treatment, and even simple mixing. However, since oleic acid is an amphiphilic film-forming substance, it was also possible to implement the interfacial pathway of HAMLET formation via Langmuir monolayers [72].

Triterpenic acid-based systems for interaction studies

The biomimetic systems based on other amphiphilic substances can also be investigated using the Langmuir technique. Surface activity is a significant feature of triterpenoids, previously mentioned as plant secondary metabolites of pronounced pharmacological activity. Like any drug in the bloodstream, triterpenoids interact with plasma proteins. Oleanolic acid (OLA), a representative of the terpenoid group, demonstrates beneficial activity for human health (antitumor, anti-inflammatory, antiviral, antioxidant, and cardioprotective). However, OLA is poorly soluble, which limits its bioavailability and absorption properties. On the other hand, serum albumin, abundant in the blood, is frequently used as a model protein in drug binding due to its well-known structure. Human serum albumin (HSA) affects the bioavailability of active substances and decreases their toxicity. Due to the poor solubility of triterpenoids, human serum albumin can be used as a drug carrier to improve its properties [26]. Thanks to the amphiphilic structure of OLA, the Langmuir technique may be helpful to assess interactions between a drug and a carrier at the phase boundaries [80].

Oleanolic acid is ubiquitous in the plant kingdom as a component of epicuticular waxes covering the foliage and is also present in olive oil. In these substances, OLA occurs naturally together with oleic acid, and both of them are distinguished by their therapeutic potential. In addition to using the protein drug carriers, another approach to improve OLA bioavailability is the implementation of nanotechnology-based drug delivery systems composed, for instance, of OA. The Langmuir monolayer technique may be utilized for the physicochemical analysis of the mixed fatty acid-triterpenoid system.

The Langmuir technique has found application in a wide range of biomimetic systems, from lipid membranes interacting with drugs, through enzymes, pollutants, and dyes, to biomimetic multicomponent systems, and only a few of them were mentioned here. This simple physicochemical methodology for studying biomimetic systems is still of interest to many researchers. On the other hand, physiological processes usually depend on multi-stage, complex molecular mechanisms, which cannot be reflected by simple models, like the Langmuir technique. The exact cellular membrane cannot be replicated either [44]. However, the Langmuir monolayer models of biomimetic systems contribute significantly to understanding the biophysics of some fundamental phenomena occurring at the molecular level [53].

Motivation and aim

Observing nature and drawing inspiration from it results in implementing its solutions in various fields of technology. The recent progress of science and medicine has shifted this process to the nanoscale. The development of medicine and technology concerns not only the search for new active substances, but also novel and more effective routes of drug administration to the body, innovative diagnostic methods, or improving the biocompatibility of implants. The application of the Langmuir methodology may be a way to address these challenges. Mixed monolayers formed on the aqueous subphase can be transferred onto the solid as the Langmuir-Blodgett films. This technique, besides expanding knowledge, enables using the research results in pharmaceutical design (such as designing novel drug delivery systems), technology (such as surface modification and biosensors), or the industry (such as the food and cosmetic industry). The utilization of the Langmuir and Langmuir-Blodgett technique for the characterization of the monolayers of natural, potentially therapeutic substances is a current subject and provides scientific novelty.

The main scientific aim of the presented doctoral thesis is to conduct detailed qualitative and quantitative characterization of the biomimetic systems with therapeutic potential and assess the suitability of the systems as modern pharmaceutical formulations. A hypothesis has been put forward that studies on monolayers containing substances with therapeutic potential bring physicochemical insight into designing novel pharmaceutical dosage forms.

Many various experiments were planned and conducted to:

1) enable extensive physicochemical studies on the systems composed of natural-origin amphiphilic substances, such as fatty acids and triterpenoids with proteins, and the fatty acid-triterpenoid system,

2) determine the structure of mixed lipid-protein monolayers and two-component lipid monolayers on the aqueous subphase,

3) analyze the morphology and discuss the application perspectives for films transferred onto a solid substrate,

4) specify the type of interactions between the components of binary monolayers, asses the influence of measurement conditions,

5) examine the effect of film composition on the physicochemical and viscoelastic properties and stability of the monolayers.

To achieve the assumed objective of the thesis, the tasks were divided into detailed stages, each with individual research goals:

- P1 – to investigate the possibility of the HAMLET-like complex formation at the air/water interface in simulated gastric conditions and evaluate the influence of temperature, pH, and molecular packing on its formation;
- P2 – to investigate the role of calcium ions and dynamically varied temperature and pH on the lipid-protein complex of α-lactalbumin with oleic acid at the interface by using the subphase-exchange method;
- P3 – to explore the molecular mechanism of interaction between oleic acid monolayer and α-lactalbumin and to unravel the role of protein concentration on the interactions;
- P4 – to investigate the penetration of linoleic acid monolayer by α-lactalbumin at various pH values;
- P5 – to investigate the properties of the oleanolic acid monolayer in the presence of human serum albumin in terms of its thermodynamics, morphology, and viscoelasticity;
- P6 – to explore the physicochemical properties of the mixed system of oleanolic acid and oleic acid at the air/water interface in various molar ratios.

An extensive introduction of this thesis demonstrates the significance of complex surface phenomena. Thus, this dissertation concerns research within the fascinating field of the phase boundary, which is of an indiscernible volume, however determining the properties of entire systems. The mentioned subject includes the Langmuir technique as well. Despite the opinions occasionally occurring about the overexploitation of the relatively straightforward Langmuir technique, this work confirms that monolayers of amphiphilic substances on the aqueous subphase can still bring novelty to science and constitute the basis for modern research techniques.

The presented research falls within the scope of application of pharmaceutical sciences, but results can only provide physicochemical insights as guidelines for designing pharmaceutical formulations.

CHAPTER 2. Factors governing the formation of the HAMLET-like complexes at the interface

Summary of the publications

PUBLICATION P1.	
Title	Temperature, pH, and molecular packing effects on the penetration of oleic acid monolayer by α-lactalbumin
Authors	Martyna Krajewska, Katarzyna Dopierała, Marek Weiss, Krystyna Prochaska
Journal	Langmuir
Details	2019, 35, 8, 3183–3193
DOI	https://doi.org/10.1021/acs.langmuir.8b04153
PUBLICATION P2.	
Title	Lipid–protein interactions in Langmuir monolayers under dynamically varied conditions
Authors	Martyna Krajewska, Katarzyna Dopierała, Krystyna Prochaska
Journal	The Journal of Physical Chemistry B
Details	2020, 124, 1, 302–311
DOI	https://doi.org/10.1021/acs.jpcb.9b10351
PUBLICATION P3.	
Title	Study on pH-dependent interactions of linoleic acid with α-lactalbumin
Authors	Katarzyna Dopierała, Martyna Krajewska, Krystyna Prochaska
Journal	Food Hydrocolloids
Details	2021, 111, 106217
DOI	https://doi.org/10.1016/j.foodhyd.2020.106217
PUBLICATION P4.	
Title	Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study
Authors	Martyna Krajewska, Katarzyna Dopierała, Paweł Wydro, Marcin Broniatowski, Krystyna Prochaska
Journal	Journal of Molecular Liquids
Details	2020, 319, 114089
DOI	https://doi.org/10.1016/j.molliq.2020.114089

2.1. Introduction

Preliminary examinations performed by Prof. Prochaska's research team (including the author of this dissertation) proved that the α-lactalbumin (α-LA) derived from human milk together with oleic acid forms a fatty acid-protein complex at the interface, which can be considered a HAMLET complex [72]. Initial studies were performed also for the bovine α-LA and lysozyme forming the BAMLET and the ELOA complex, respectively. So far, in the preliminary experiments of our research team, only conditions simulating the environment of the infant's stomach have been applied without systematic evaluation of the impact of environmental conditions on the interactions between the components of the HAMLET-like complex in the form of a monolayer. Since the monolayer stability, phase transitions, and collapse phenomenon occurrence depend on the amphiphilic substance (degree and position of unsaturation) as well as the environment conditions (temperature, aqueous subphase pH, and cations present), in the π-A isotherm studies, the limiting area occupied at the interface by oleic acid molecule (OA) is significantly larger than for saturated fatty acid of the same alkyl chain length. It can be explained by the tilted orientation towards the interface and enhanced molecular collisions at elevated temperatures, which increase the distances between molecules in the monolayer. Due to the hysteresis experiments (successive compression and expansion of the monolayer), higher temperature intensifies the oleic acid monolayer degradation. Relaxation studies explained the mechanism of the systematic degradation by the dissolution of the molecules in the subphase dependent on the applied surface pressure, where higher surface pressures result in faster loss of molecules from the monolayer. α-LA molecules adsorb and bind to the monolayer of oleic acid, forming a stable structure at the interface [72].

Based on the above findings and extensive research undertaken on the HAMLET-like complexes at the interface, which are the subject of this work, the general features and dependencies of these complexes under changing conditions have been identified. There are multiple factors affecting the possibility of the HAMLET-like complexes formation or determining its features at the interface (Figure 12). First of all, interfacial behavior depends on the type of complex-forming substances, both fatty acid, and

protein. Temperature and pH affect the structure of fatty acid monolayer as well as the physical state of the protein, thus it is crucial for the protein adsorption and unfolding process. Moreover, the α-LA unfolding is also moderated by the calcium ions presence. Monolayer physical state reflected by the molecular packing density controls the possibility of the film penetration by the protein and enables further interactions. The method of protein introduction to the system and protein concentration significantly influence the kinetics of the interaction with the fatty acid monolayer. All the mentioned factors will be described in detail in the following sections. The selected results from the presented publications will be summarized to support the conclusions drawn.

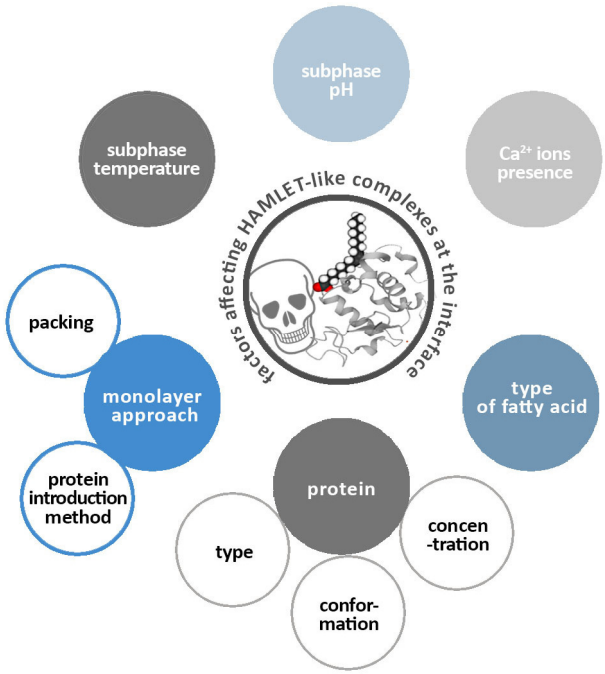

Figure 12. Factors affecting the HAMLET-like complexes studied by the Langmuir monolayers

2.2. Temperature dependence

Since the temperature was previously recognized as one of the main factors determining the HAMLET-like complexes formation at the air-water interface, the role of temperature in binding OA by the α-lactalbumin was investigated within the paper **P1** entitled *Temperature, pH, and molecular packing effects on the penetration of oleic acid monolayer by α-lactalbumin*. The temperature dependence was studied through the relaxation/penetration studies at the constant surface pressure for the system of oleic

acid with BLA III (bovine α-lactalbumin) in *apo* form (calcium depleted). The issue was further developed in **P2** entitled *Lipid–protein interactions in Langmuir monolayers under dynamically varied conditions*, where the stability of the complex at the interface was examined using an upgraded methodology for the same system.

Langmuir monolayers are prone to the temperature changes of the water subphase onto which they are spread. Typically, when the Langmuir monolayer is exposed to the increased subphase temperature, a single molecule covers a larger area at the interface due to the higher flexibility of the apolar chain at elevated temperatures. However, some of the PUFAs perform anomalous temperature dependence. Moreover, the effect of area contraction intensifies with the increasing degree of unsaturation of the hydrophobic chain. A possible explanation for such a phenomenon lies in the increased solubility of PUFAs molecule according to elevated temperature [20].

As already mentioned in Chapter 2.1. temperature affects the behavior of the OA monolayer. The relaxation experiments have been performed at pH 2, corresponding to the conditions of the infant's stomach. After monolayer compression to the surface pressure of 5 mN/m, the relative area per molecule (A/A_0) changes were recorded in time. The dashed lines in Figure P1.1 clearly demonstrate the temperature-dependent interfacial behavior of the OA monolayer. The higher the temperature of the subphase during the experiment, the faster the degradation of the unsaturated fatty acid monolayer due to the increased solubility in elevated temperature. The behavior of the OA monolayer at various temperatures is governed by a straightforward mechanism, but the OA-BLA III system is of a complex background. The increase of the mean area per OA molecule takes place due to the introduction of the protein to the subphase underneath the monolayer. The higher the temperature, the more significant the increase in molecular area, corresponding with more intensive monolayer penetration. Although the molecular area increase is attributed to the film penetration by the BLA III molecules, the reason for the relative area increase is not only the protein adsorption at the interface. As previously mentioned, the elevated temperature is one of the factors causing conformational changes of α-LA and the transition to the molten globule state. The subphase temperature variations significantly affect the protein adsorption rate as

well as trigger structural changes. The differences in the maximum A/A_0 value achieved in time due to various temperatures kept, prove the different stoichiometry of protein binding to the OA monolayer. As shown in Figure P1.1, despite the significant instability of the pure OA monolayer at the highest temperature tested, corresponding to the human body temperature, the most stable interfacial structure was obtained. Surprisingly, even at 21°C, over a long measurement time, the value of the relative molecular area stabilized, which suggests the formation of a stable two-component structure at the interface.

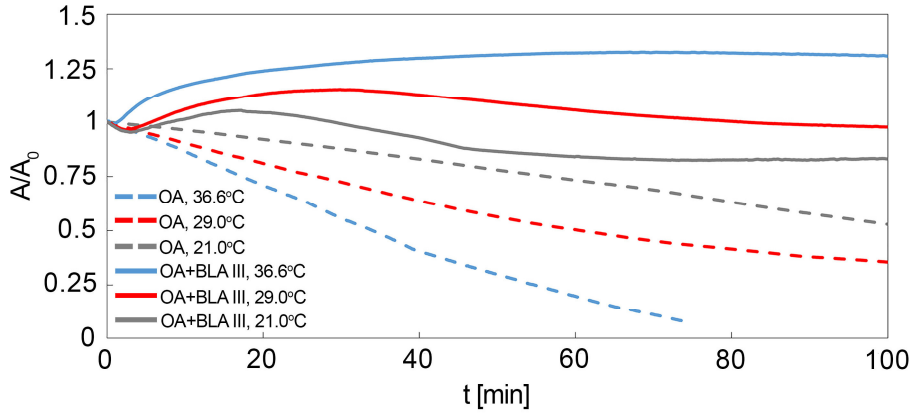

Figure P1.1. The relative area changing in time for the monolayer of OA and OA penetrated by the BLA III at constant surface pressure π=5 mN/m for various temperatures; the subphase pH=2; the time t = 0 [min] corresponds to the moment of protein injection to the subphase. The protein concentration was equal to 1 mg/L

In the next step of the research, the influence of temperature on the OA-BLA III complex at the interface was studied. Here, the novel methodology utilizing the peristaltic pump has been applied (Figure 6F). The dosing pump was employed not only to introduce the protein molecules under the monolayer but also to exchange the bulk phase with preheated or precooled liquid to investigate the effect of altering conditions on the stability of the mixed monolayer. The HAMLET complex composed of OA and BLA III gives a meaningful example for studies on thermodynamic parameters stabilization over time.

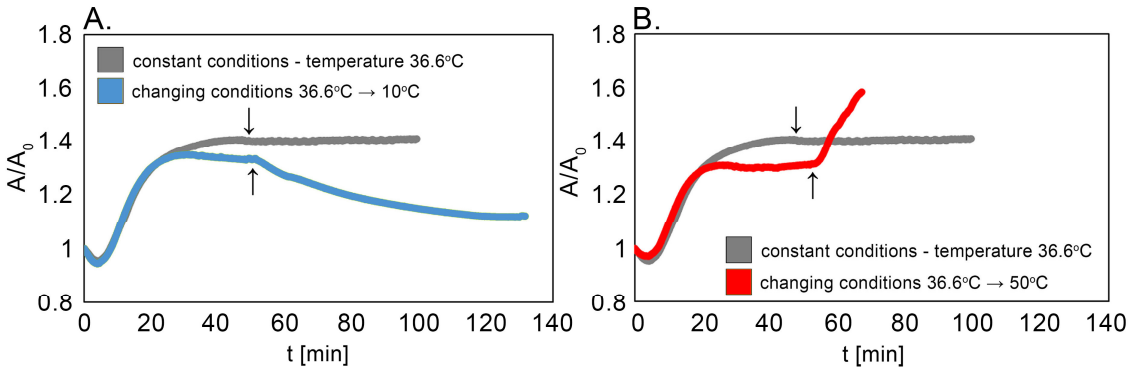

Figure P2.1. The relative area changing in time for OA-BLA III at constant pH = 2.0 and (A) changing temperature from 36.6 to 10°C, (B) changing temperature from 36.6 to 50°C; the gray curve is at a constant temperature of 36.6°C for reference; arrows correspond to the beginning of the subphase exchange

As shown in Figure P2.1, the bulk phase exchange with a precooled (A) and a preheated (B) liquid for the quick and efficient altering of the experimental conditions caused the equilibrium shift. As a result of reducing the system temperature to 10°C, the relative area dropped significantly and remained stable. This phenomenon was attributed to the structural transformation of a protein that is susceptible to temperature variations. Proteins in a molten globule state may partly return to the ternary form when exposed to low temperatures and occupy a reduced area at the interface. Nevertheless, the results have demonstrated that temperature reduction does not induce degradation of the complex. On the other hand, an increase in the temperature to 50°C caused an increase in the A/A_0 value. Unfortunately, equipment limitations did not allow the measurement to be continued until obtaining a stable value. From the results of our investigation, it can be concluded that initially stable OA-α-LA monolayer at the biomimetic conditions of the human stomach undergoes conformational alterations when exposed to dynamically varied conditions. Although the relative area per molecule is shifted to lower or higher values, the complex remains stable, and the interfacial structure is not disturbed.

2.3. pH dependence

The experiments on the impact of pH on the HAMLET-like complexes were described in the paper **P1** entitled *Temperature, pH, and molecular packing effects on the penetration of oleic acid monolayer by α-lactalbumin* and **P2** entitled *Lipid–protein*

interactions in Langmuir monolayers under dynamically varied conditions. The analysis of the π-A isotherms conducted at pH 2.0 and at the aqueous subphase proves significant structural differences even for the pure oleic acid monolayer (Figure P1.2A, B). On the aqueous subphase, OA molecules occupy a smaller area than in an acidic environment. The phenomenon of monolayer loosening at pH 2.0 is related to the interfacial pK_a of fatty acid, which is 6.6 for OA. Thus, at pH 2.0, OA molecules are unionized. But, at the water subphase of pH 6.8, which is close to the pK_a value, there are ionized and nonionized head groups of OA molecules at the interface, and ion-dipole interactions appear between them, enhancing close packing. Moreover, the transition of BLA III to the molten globule state seems to be pH-dependent, so the lipid-protein mixed monolayers at various pH were also examined.

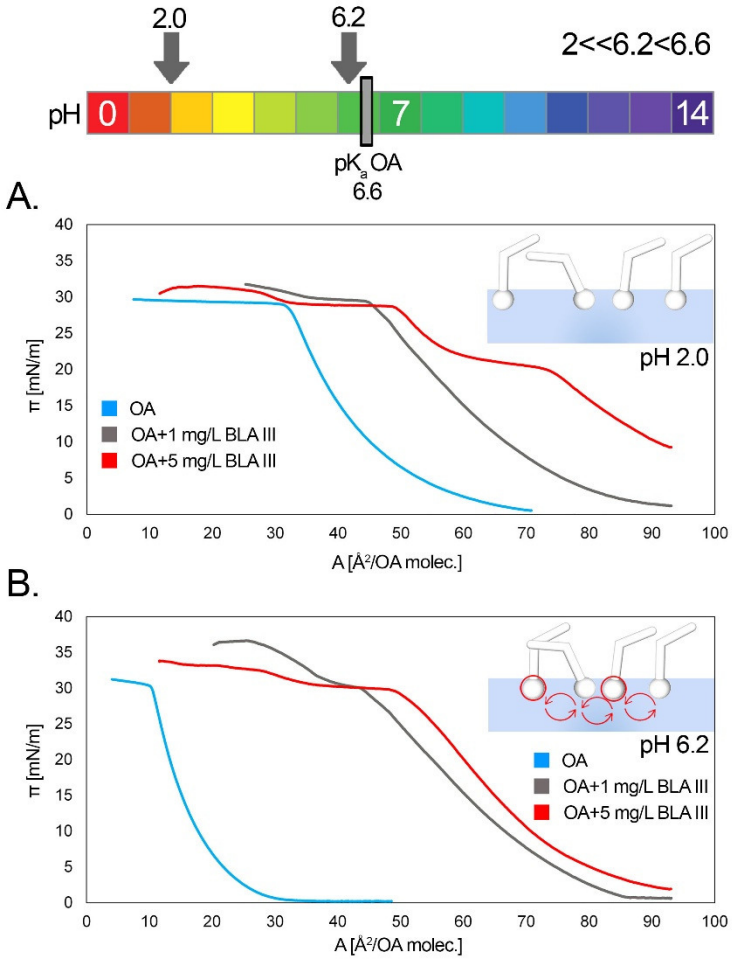

Figure P1.2. The π–A isotherms of OA and OA with BLA III at 36.6°C onto A. subphase of pH 2.0 and B. aqueous subphase. The protein was introduced to the subphase before the compression; inset: pH scale with OA pK_a indicated

Because of the presence of the protein in the subphase, OA isotherms are shifted towards larger mean areas per molecule, indicating the expansion due to the interaction between OA and BLA III. The BLA III concentration effect is more pronounced at pH 2.0 because the same amount of protein caused more intense monolayer expansion than on the water subphase. Since the isoelectric point (IEP) of BLA III is equal to 4.6, it is apparent that, within the tested range, in addition to affecting the fatty acid monolayer, pH influences the interfacial behavior of the protein as well. In highly acidic conditions, BLA III is positively charged, opposite to the behavior on the aqueous subphase. Thus, at pH 2.0, intermolecular interactions are hydrophobic in nature, supported by hydrogen bonds acting between the amino acid residues of protein and the carboxyl groups of the fatty acid, while at pH 6.2 the interactions are of electrostatic origin.

The pH-dependent behavior of HAMLET-like complexes was also evaluated using relaxation/penetration experiments for the OA-BLA III system. The BLA III solution was injected under the OA monolayer previously compressed to 5, 10, 15, and 25 mN/m to record the A/A_0 changes in time. As follows from Figure P1.3A, the mechanism and kinetics of the protein incorporation in the OA monolayer are strongly pH-induced. When pH is equal to 2.0 in the $A/A_0(t)$ curve, several distinct steps can be distinguished for 5 and 10 mN/m. After the initial growth of the A/A_0 value and reaching the maximum, the curve stabilizes at a value ≥1. Such behavior results from subsequent diffusion and adsorption of the protein at the interface covered with the fatty acid monolayer. The stabilization is an effect of the interaction of OA with BLA III leading to the protein incorporation into the monolayer. The issue concerning subsequent steps of interaction between protein and fatty acid at the interface will be developed further in the next part of this work. The interaction between OA and BLA III is related to the molten globule state adopted by the protein in low pH. Therefore, when the protein tertiary structure is loose, the hydrophobic residues are exposed, contributing to the interactions with the alkyl chain of OA. The previously proposed mechanism of interaction, based on hydrophobic forces complemented with hydrogen bonding, was confirmed by this study. For 15 and 25 mN/m, a stepwise degradation was noted instead of an A/A_0 increase, which proves that in this case, the monolayer is not penetrated by the protein.

This phenomenon is attributed to the molecular packing effect, which is described in Chapter 2.5.

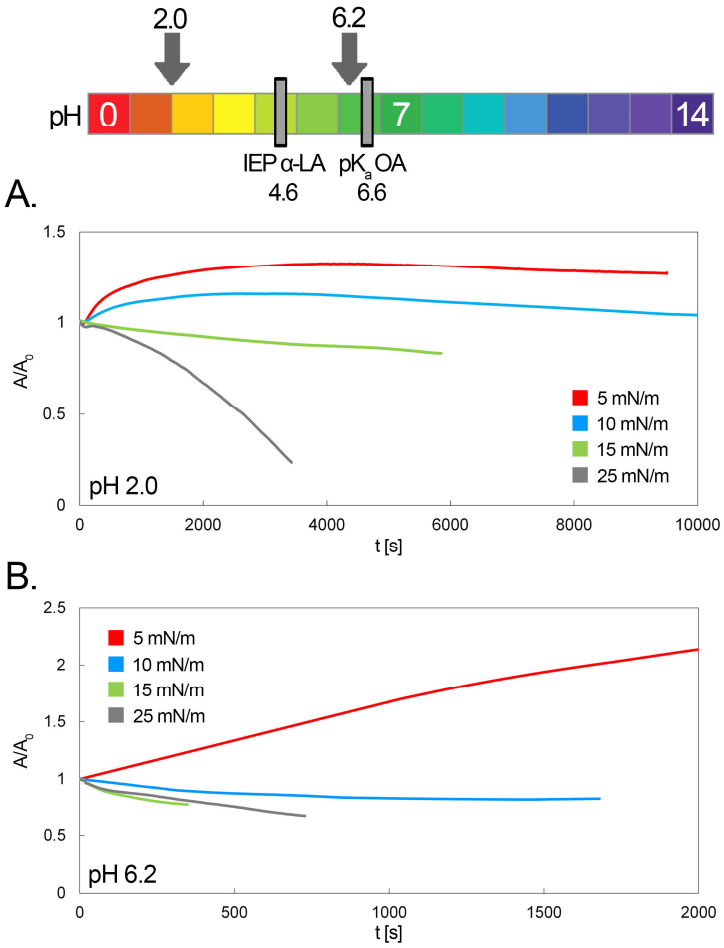

Figure P1.3. The relative area changing in time for the OA monolayer penetrated by BLA III at 36.6°C for various surface pressures and spread onto the A. subphase of pH 2.0 and B. water surface; time t = 0 [min] corresponds to the moment of protein injection to the subphase. The protein concentration was equal to 1 mg/L. Inset: pH scale with OA pK_a and IEP of α-LA indicated

The behavior of the OA monolayer after the introduction of BLA III to the aqueous subphase of pH 6.2 is quite different. For the surface pressure of 5 mN/m, the mean molecular area was continuously expanding without reaching the stable value during the measurement. Since at pH 6.2, the OA is close to surface pK_a and is ionized, and BLA III is below the IEP and is negatively charged, the forces governing the interfacial interactions in this system are mainly electrostatic (Figure P1.4). Therefore, a phenomenon observed in Figure P1.3B is a result of the competition between OA and

BLA III for the area at the interface via electrostatic repulsion. In this case, the surface active BLA III displaced the unsaturated fatty acid from the interface.

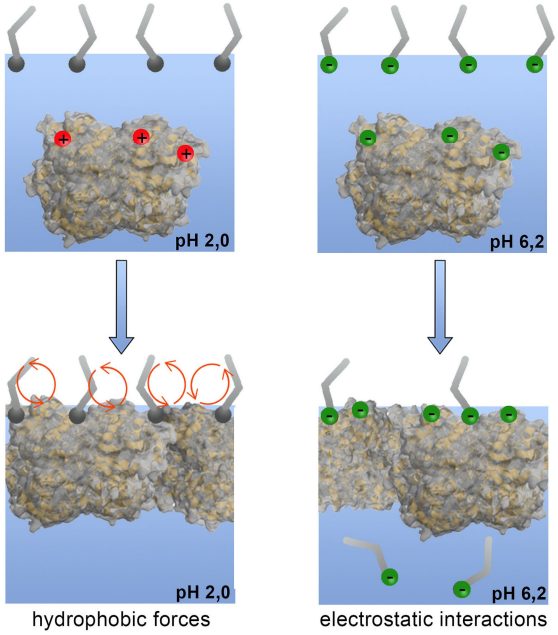

Figure P1.4. A schematic depiction of the pH-dependent behavior of the OA-BLA III system

As in the case of the temperature dependency of the already formed and stable OA-BLA III complex, the effect of pH was also investigated using a subphase exchange approach (Figure P2.2). Analogously to the temperature decrease, increasing the pH resulted in an A/A_0 reduction and stabilization at the lower value. However, the result was achieved much faster due to the immediate effect of the pH change on the protein conformation.

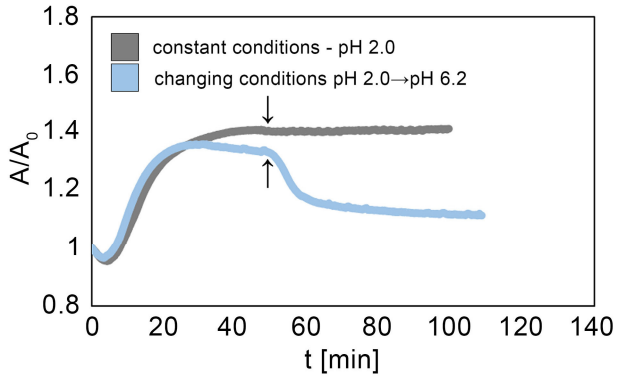

Figure P2.2. The relative area changing in time for OA-BLA III at temperature of 36.6 °C and pH 2.0 (gray curve for reference) and changing pH from 2.0 to 6.2; arrows correspond to the beginning of the subphase exchange

Since various long-chained fatty acids can modify the biological properties of BLA III, extensive studies have been conducted on the interaction of linoleic acid (a *cis*-unsaturated fatty acid with two double bonds in the alkyl chain, LA) with BLA III. The pH-dependent interfacial properties of LA-BLA III monolayer mimicking HAMLET-like complex were explored within the paper **P3** entitled *Study on pH-dependent interactions of linoleic acid with α-lactalbumin*. Despite the obvious analogies between the mixed monolayers of OA-BLA III and LA-BLA III, each system shows specific features resulting, in this case, from the morphology of the LA film and the structure of the molecule itself. Thus, each of the complexes belonging to the group of HAMLET-like complexes should be considered separately. However, some general trends in HAMLET-like complexes formation can be formulated.

Characterization of the linoleic acid monolayers was based on the π-A isotherms conducted at pH 2.0 and 6.2 (Figure P3.1). As follows from the isotherms, the $A_{lift\text{-}off}$ value of pure LA is independent of pH, while the A_{lim} of the LA monolayer at pH 2.0 is 30Å^2/molecule and at pH 6.2 is 28 Å^2/molecule. The LA monolayer is less sensitive to pH than OA (monounsaturated fatty acid of the same alkyl chain length), as reported in **P1**. For the LA film at pH 2.0, the collapse was recorded at 28 mN/m, while at the aqueous subphase, it was 2 mN/m lower. Still, lower pH favors the formation of slightly more stable LA monolayers when collapsing at higher surface pressure. The isotherms of LA monolayers, spread at the subphase containing BLA III, are shifted towards larger values of area per LA molecule for both pH values investigated, which confirms the surface activity of BLA III reported previously. Due to the presence of the protein, the A_{lim} increased to 80 and 66 Å^2/LA molecule at pH 2.0 and 6.2, respectively, proving the protein adsorption at the interface in both cases. At pH 6.2, the monolayer composed of LA and BLA III collapses at lower surface pressure than pure LA monolayer but at a larger molecular area. On the other hand, at pH 2.0 monolayer of LA-BLA III collapses at higher surface pressure than LA only.

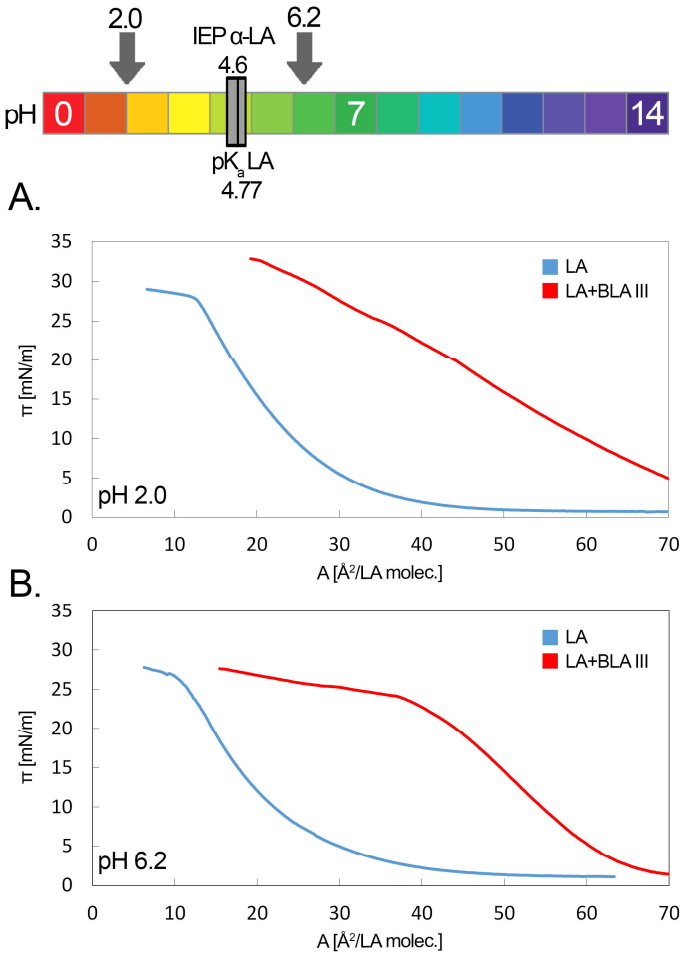

Figure P3.1. The π–A isotherms of LA and LA with BLA III at 36.6°C onto A. subphase of pH 2.0 and B. aqueous subphase. The protein was introduced to the subphase before the compression; inset: pH scale with LA pK_a and IEP of α-LA indicated

As can be seen from Figure P3.2, the change in the behavior of the LA monolayer pre-compressed to 5 and 10 mN/m at different pH is significant. The decrease in the A/A_0 value is observed in time due to the desorption of the fatty acid molecules to the subphase. However, the effect of molecule loss is faster at pH 2.0. The monolayer degradation is more pronounced for the monolayer at higher surface pressure, which is discussed in a further chapter.

The response of the LA monolayer to the presence of the protein is clearly dependent on pH and surface pressure. At the acidic pH, there was a significant increase in the A/A_0 value, followed by the stabilization on the higher level for the lower surface pressure from the two examined. In contrast, at pH 6.2, the linear increase of the

molecular area took place due to the monolayer expansion. The effect was almost the same for the monolayers pre-compressed to 5 and 10 mN/m.

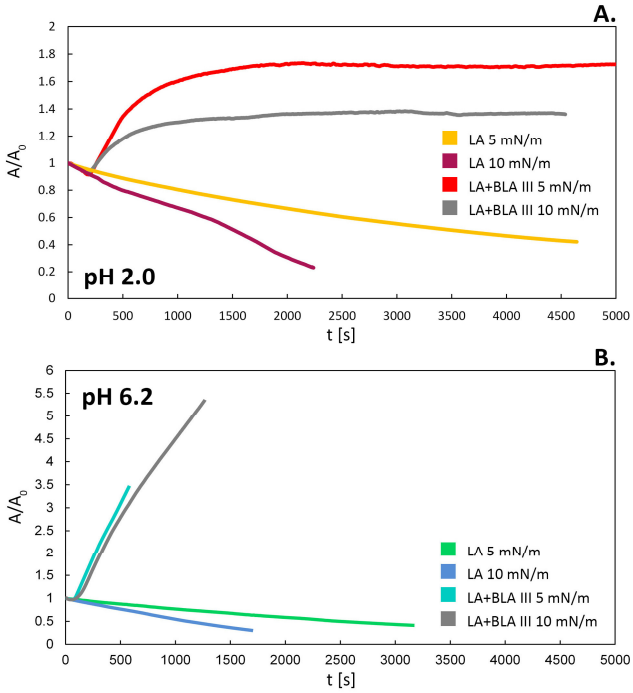

Figure P3.2. The relative area changing in time for the pure LA monolayer and LA monolayer penetrated by BLA III pre-compressed to the surface pressures of 5 and 10 mN/m and spread onto the A. subphase of pH 2.0 and B. water surface; temperature 36.6°C; time t = 0 [s] corresponds to the moment when the desired surface pressure is reached or to the protein injection to the subphase. The protein concentration was equal to 1 mg/L

To get a better insight into the kinetics of LA penetration by the BLA III, the relaxation/penetration experiments were performed at various surface pressures within the range of the π-A isotherm for pH 2.0 and 6.2 (Figure P3.3). At pH 2.0, depending on the degree of film pre-compression, two mechanisms acting on the monolayer were distinguished: monolayer expansion due to the adsorbing protein at the interface or the monolayer degradation. The significant increase in the molecular area, described in the previous paragraph, is characteristic only for a relatively loose monolayer. It should be emphasized that the system obtained an almost constant value of the relative area per molecule, which is associated with the equilibrium reached at the interface. Nevertheless, when the monolayer is compressed to a surface pressure >15 mN/m, the incorporation of the protein into the film seems to compete with the ongoing monolayer degradation, which is more and more pronounced at higher surface pressures.

Surprisingly, when the same experiment was performed at 6.2 pH, the kinetics of the lipid-protein interactions appeared different. Apart from the linear increase in the relative molecular area for the lowest surface pressure tested, the A/A_0 stabilization is achieved for surface pressures of 15-20 mN/m. Only the monolayer pre-compressed close to the collapse does not show either an A/A_0 increase or stabilization.

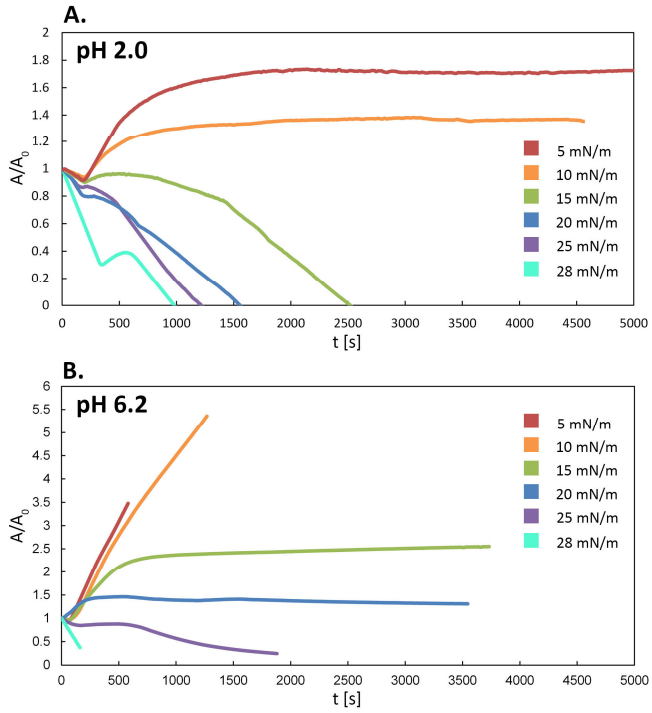

Figure P3.3. The relative area changing in time for the LA monolayer penetrated by BLA III pre-compressed to the various surface pressures between 5-28 mN/m and spread onto the A. subphase of pH 2.0 and B. water surface; temperature 36.6°C; time t = 0 [s] corresponds to the moment when the desired surface pressure is reached or to the protein injection to the subphase. The protein concentration was equal to 1 mg/L

The surface pK_a of the LA is 4.77, while for the OA it is significantly higher (6.6). Therefore, at pH 6.2, the number of the negatively charged molecules within the monolayer would be higher for LA than OA. These findings concur with the results obtained at the same conditions for both systems, which have shown that for the OA-BLA III (Figure P1.3), there was no A/A_0 increase for the system at 10 mN/m and no stabilization for even more compressed monolayers. In the case of pH 6.2, the driving force of the interactions is the electrostatic force. The interactions between LA and BLA III on the aqueous solution result from the competition of highly surface-active protein with the fatty acid monolayer at relatively low surface pressure. The continuous

expansion of the relative molecular area is due to the dynamic exchange of molecules between the interface and the bulk. Thus, at pH 6.2 and low surface coverage, the formation of stable LA-BLA III film is unachievable due to the electrostatic repulsion. For more condensed monolayers, the equilibrium is reached because of the stronger interactions, such as hydrophobic forces supported by hydrogen bonds. On the other hand, for pH 2.0, analogously like for the OA-BLA III system, the BLA III bears the positive net charge, while LA is unprotonated. Thus, the electrostatic forces are not involved in the interactions of fatty acid and protein but are governed by hydrophobic forces between the alkyl chain of LA and residues of BLA III exposed in a molten globule state. The results of the relaxation experiment confirm the formation of stable LA monolayers with α-LA adsorbed at both tested pH levels but with various thermodynamic characteristics, and governed by different forces. However, changes in the relative molecular area with protein adsorption suggest that protein reaches the surface and occupies the area at the interface. Analysis of the kinetics of protein incorporation into the fatty acid monolayers implies that the protein concentration and lipid/protein ratio at the interface should be taken into account while studying the interactions.

It should be noted that the mechanisms of the HAMLET-like complexes formation discussed previously result from the synergistic effect of the temperature (discussed in Chapter 2.2) and the pH. The experiments on the temperature influence were performed at pH 2.0, while the studies on the pH effect were conducted at 36.6^{o}C. Both pH and temperature affect the fatty acid monolayer and the conformation of the protein. Thus, the simultaneous temperature reduction and pH increase lead to the A/A_0 stabilization at a lower level. Figure P2.3 shows the response of a stable monolayer at pH 2.0 and 36.6^{o}C to a simultaneous decrease in temperature and increase in pH by subphase exchange. The graph outlines that the relative area decrease after subphase exchange runs into two kinetic stages, which may be explained by two overlapping effects. The initial sudden A/A_0 drop is attributed to the pH shift, while, the gentle reduction of the mean molecular area is due to the temperature reduction, which triggered the more time-consuming conformational changes at the interface. Simultaneous lowering of the temperature and increasing the pH resulted in a drop of

the relative molecular area value to the lowest level achieved in the experiments on a dynamic change of conditions. That suggests that both factors significantly affect the physical state of the lipid-protein system at the interface. Based on this, it was determined that HAMLET-like complexes at the interface are stable over time, and varying conditions do not cause their degradation but rather conformational modifications.

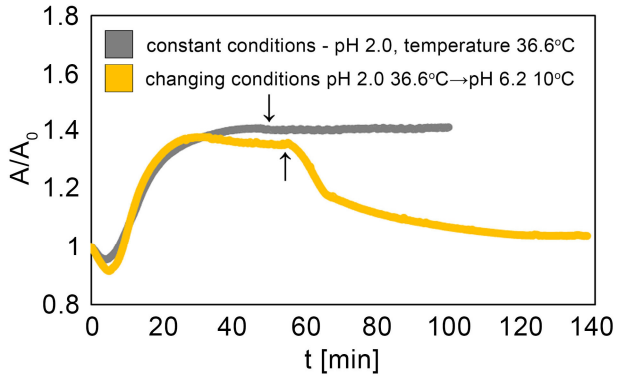

Figure P2.3. The relative area changing in time for OA+BLA III at a temperature of 36.6 °C and pH 2.0 (gray curve for reference) and simultaneously changing pH from 2.0 to 6.2 and temperature from 36.6°C to 10°C; arrows correspond to the beginning of the subphase exchange

2.4. Molecular-packing effect

The role of the physical state of the fatty acid monolayer was systematically studied by the relaxation/penetration experiments at the constant molecular area in **P1** entitled *Temperature, pH, and molecular packing effects on the penetration of oleic acid monolayer by α-lactalbumin*. An intriguing correlation between the molecular packing effect and protein concentration effect was revealed in **P4** titled *Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study*. However, the behavior of the fatty acid-α-LA mixed monolayer pre-compressed to various surface pressures was presented and interpreted previously (Figure P1.3, Figure P3.2, Figure P3.3). Here, at pH 2.0 and 36.6°C, the BLA III was injected under the OA monolayer compressed to 60, 40, or 20 Å^2/OA molecule, and the surface pressure varying in time was recorded. The relaxation experiments of pure OA were conducted for comparison. From Figure P1.5, it can be seen that the incorporation of the protein into the OA monolayer demonstrated by the surface pressure increase is enhanced for

the expanded films. Additionally, the kinetics of the protein binding and the equilibrium reached by the system compressed to 60 Å^2 per molecule indicate the interface saturation and formation of the stable mixed films. For a highly condensed monolayer, the relative surface pressure was constantly decreasing. However, the relaxation in the OA-BLA III system was significantly slower than for pure OA monolayer.

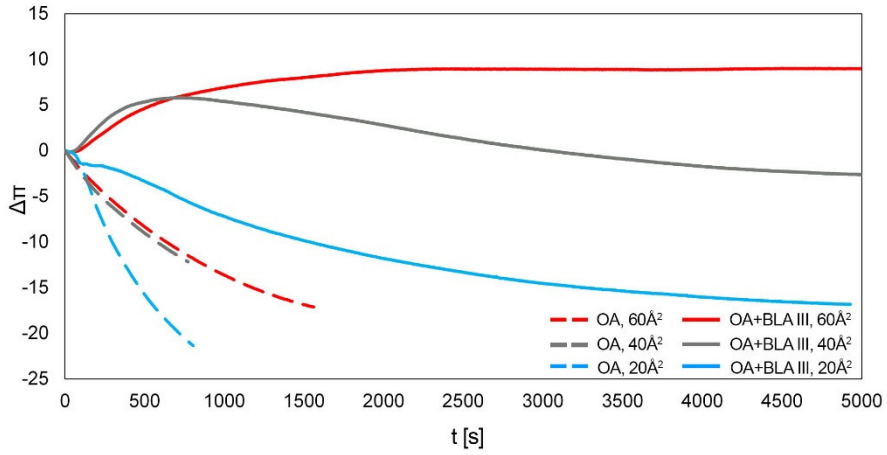

Figure P1.5. The relative surface pressure changing in time for the monolayer of OA and OA penetrated by the BLA III at different values of constant molecular area; temperature 36.6°C, the subphase pH=2.0; time t = 0 [min] corresponds to the moment of protein injection to the subphase. The protein concentration was equal to 1 mg/L

Since in such conditions, the interactions between OA and BLA III are governed by the hydrophobic forces acting between alkyl chains of OA and amino acid residues of BLA III, at more loose monolayers, fatty acid chains are more accessible for binding. Due to the monolayer compression, the orientation of the OA becomes more perpendicular to the surface at a liquid-condensed state, and OA head groups occupy the entire free interface hindering protein access to the hydrophobic chains of fatty acid (Figure P1.6). Thus, the binding of BLA III to the highly compressed OA monolayers is limited.

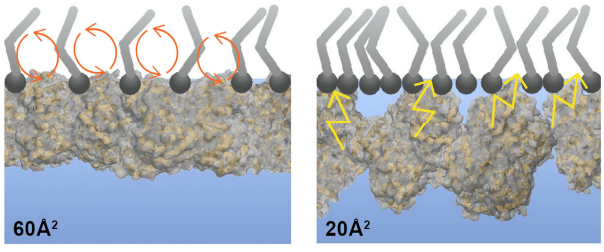

Figure P1.6. Schematic depiction of the effect of the monolayer's physical state on HAMLET-like complex formation

The influence of the OA molecular packing on the HAMLET-like complexes formation was additionally tested by AFM onto a mica substrate after the transfer via the Langmuir-Blodgett (LB) technique (Figure P1.7). Since the binding possibility and complex stability depend on the monolayer surface pressure, the LB transfer and AFM experiments were conducted for pure OA monolayers and mixed OLA-BLA III monolayers at 10 and 25 mN/m. Moreover, despite the solubility in the aqueous subphase, the adsorbed layer of BLA III was transferred onto a solid substrate at a surface pressure of 10 mN/m. Although the transfer of monolayers from the aqueous subphase to the solid support at low surface pressure is unrecommended, in this case, a surface pressure of 10 mN/m was applied because the relatively loose monolayer ensures protein penetration. The AFM studies revealed that the OA monolayer under given conditions of pH 2.0 and 36.6^{o}C, transferred at 10 mN/m is continuous and homogenous. When transferred at a higher surface pressure (25 mN/m), the film was fragmented and comprised of small separated islands, likely due to the low stability of the condensed monolayer close to the collapse.

To obtain a complete picture of the OA-BLA III system, a successful attempt was made to transfer BLA III adsorbed at the interface (at a surface pressure of 10 mN/m) onto a solid. It was possible due to the attraction between the negatively charged mica and the positively charged protein. The partial unfolding of the protein favored the hydrophobic groups exposition. Due to the surface imaging by AFM, the BLA III covered the entire surface randomly, but there were also visible depressions creating characteristic nanochannels and hills. The location and size of the depressions and hills correspond with properties of the molten globule state, which structure is composed of α-domains and disordered β-sheets.

The AFM topography of the OA-BLA III system at 10 mN/m revealed the number of aggregated structures and relatively flat surface profile. Thus, it may be concluded that the OA molecules bound to the protein and filled the depressions created by the protein reducing their depth. Moreover, with the OA in the system, the structure of the protein is different from that of the protein alone, suggesting that the protein is inhibited from folding back to the native conformation. The results also strengthened

the hypothesis about OA and BLA III binding and confirmed the presence of both components at the interface.

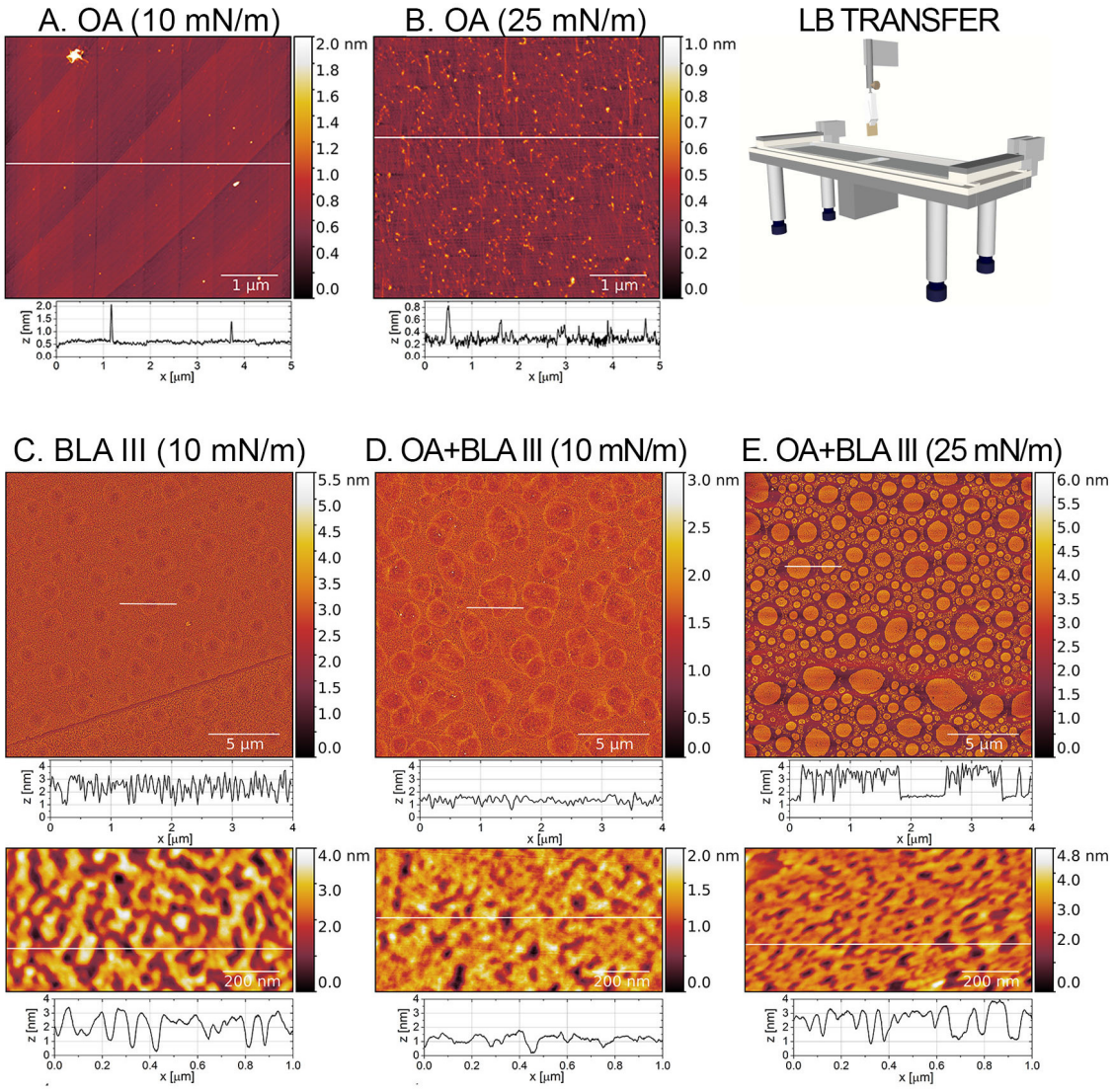

Figure P1.7. The AFM topographical images and associated cross-sections of the LB films of OA (A,B), BLA III (C) and OA-BLA III (D,E) deposited on cleaved mica substrate. The image size is (5 × 5) µm and the scale is 1 µm for A and B, and (20 × 20) µm whereas the scale is 5 µm. Inset: schematic representation of the Langmuir-Blodgett film transfer

The film composed of OA and BLA III at an elevated surface pressure of 25 mN/m under AFM tests revealed the discontinuity as large depressions understood as uncovered mica. The LB layer contained two types of morphologically various objects: a large, discovered to be pure unfolded BLA III, and a small but higher, being bilayer composed of OA and BLA III.

Considering Figure P4.1, a striking conclusion is that the molecular packing of the film (correlated with the initial surface pressure) is a key factor ensuring the ability of the protein to reach the surface when it adsorbs. It is worth emphasizing that the same monolayer response cannot be obtained by adjusting the protein concentration and surface packing. Thus, even a relatively high protein concentration is unable to raise the π above the π_i if the monolayer is densely packed.

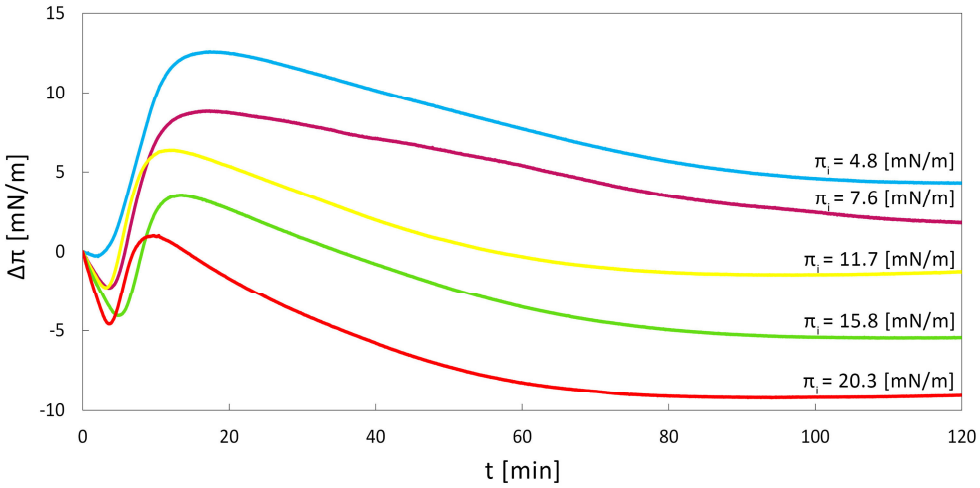

Figure P4.1. Surface pressure changes in time for the OA monolayers at a constant molecular area corresponding to the various surface pressure (π_i, initial surface pressure) after the introduction of the same protein concentration of 5 mg/L. The presented results were used to determine the protein binding parameters in section 2.5.

2.5. Protein-concentration effect

The protein concentration influence on the kinetics of the BLA III binding to OA monolayer in gastric conditions (pH 2.0 and 36.6°C) was the aim of further studies. The interfacial interactions between OA and BLA III molecules were discussed in terms of maximum insertion pressure (MIP) and mixed monolayers reorganization via spectroscopic techniques in paper **P4** entitled *Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study*. The structural changes of the protein and fatty acid molecules, likely underlying the OA-BLA III complex formation, were investigated.

Bearing in mind that the OA monolayer itself undergoes rapid degradation in the environment mimicking gastric conditions, the grey curve in Figure P4.2 is a reference for OA-BLA III systems. In this graph, for every experiment, the same number of OA

molecules but various protein concentrations were used. After protein adsorption, which rate depends on its concentration in the subphase, the protein molecules penetrate the fatty acid film until the surface saturation or depletion of protein from the subphase. Next, the reduction in surface pressure takes place due to the film rearrangements until finally stable OA-BLA III complex is formed at the interface. As was already mentioned, under the model gastric conditions, the BLA III in the system with fatty acid induces the relative surface pressure growth due to its surface activity and affinity to OA. The magnitude of the maximum increase depends to some extent on the protein concentration. The addition of protein in low concentration, after a relatively long stage of π/π_0 decrease, induces only a slight surface pressure growth and subsequent stabilization. As the BLA III concentration in the subphase grows, the monolayer response is more pronounced in the π/π_0 peak height and the π/π_0 value when stabilized. The trend of the relative surface pressure increase for higher protein concentration occurs until the maximal value is reached. Thus, above a particular BLA III concentration, the progressive increase in the protein amount does not generate more surface pressure changes. Due to this, in further experiments, the protein concentration was kept at 5 mg/L.

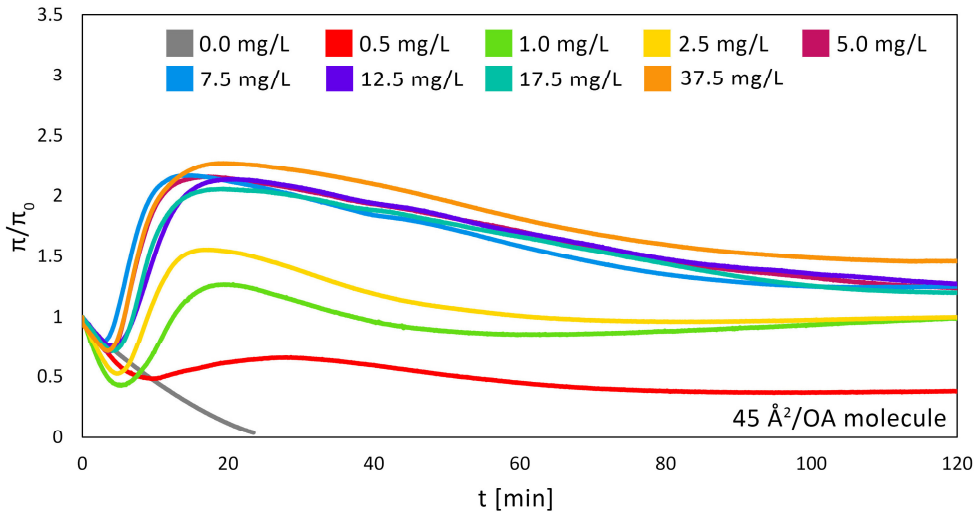

Figure P4.2. The relative surface pressure changes in time for the OA monolayer precompressed to 45Å^2 per OA molecule and penetrated by various concentrations of BLA III

Based on the experiments from Figure P4.1, the binding parameters were determined and presented in Figure P4.3. The MIP, synergy, and $\Delta\pi_0$ were described in

this work previously in Chapter 1.4 and Figure 7B. The calculated MIP value was 22.5±2 mN/m. Thus, it is lower than the collapse surface pressure of the pure oleic acid monolayer, which is 28 mN/m under the corresponding conditions. That is in line with previous results obtained via relaxations at constant surface pressure (Figure P3.3A) when no monolayer expansion was noted for the film pre-compressed to π_i>20 mN/m.

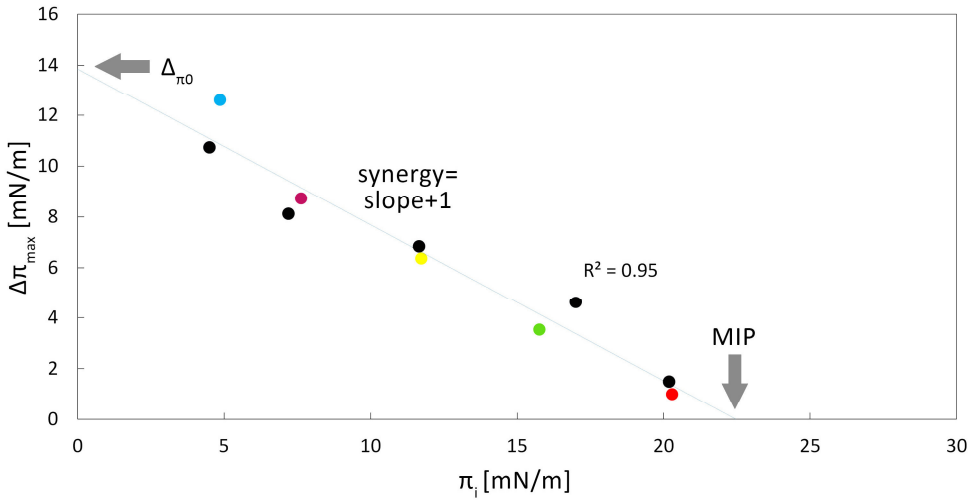

Figure P4.3. Determination of the binding parameters in the system of OA-BLA III - the plot of $\Delta\pi_{max}$ vs. π_i to determine the MIP based on Figure P4.1

Furthermore, under model gastric conditions, the positive value of the synergy proves the BLA III favorable binding to the monolayer of OA. The $\Delta\pi_0$ was determined as 13.9±0.7 mN/m and the maximal value of the surface pressure recorded when pure BLA III was adsorbed onto the water surface was about 12 mN/m for the protein concentration of 17.5 mg/L. It means that $\Delta\pi_0$ is higher or similar to the surface pressure value obtained by the protein itself. Thus, OA monolayer can be considered as a factor enhancing BLA III adsorption.

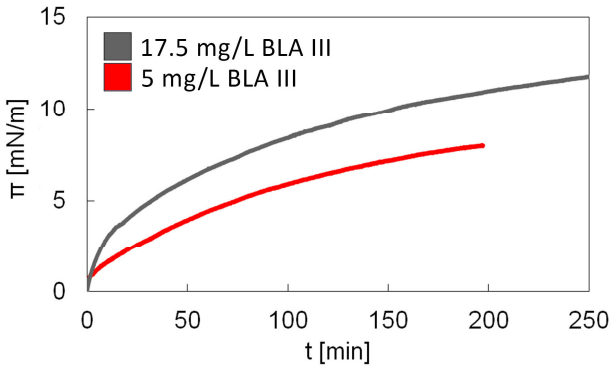

Figure P4.4. The surface pressure changes due to the injection of various concentrations of BLA III into the subphase recorded in time

On the visual representation of the surface pressure increase in time due to the adsorption of the BLA III from the aqueous subphase (Figure P4.4), there are no surface pressure drops. Thus, it was excluded that the surface pressure reduction of the mixed OA-BLA III relaxation/penetration studies corresponding to the conformational changes is due to the protein denaturation when in contact with air. Such behavior has been attributed to the interaction between OA and BLA III, resulting in lipid-protein complex formation. Moreover, the surface pressure decrease before stabilization does not mean a loss of the material from the interface but is an effect of the conformational changes of the BLA III molecules embedded in the OA monolayer or positional shifts of the OA because of the BLA III presence. To finally confirm the presence of both lipid and protein components on the surface, the polarization modulation-infrared reflection-adsorption spectroscopy (PM-IRRAS) for the mixed OA-BLA III monolayer was used (Figure P4.5). Before the BLA III injection to the subphase, the OA monolayer was compressed to 60 $Å^2$/molecule, which corresponds to the surface pressure of ca. 5 mN/m.

The PM-IRRAS measurements were conducted every 20 minutes during the relaxation/penetration experiments at constant surface pressure to gather information on the conformational properties of film components in time. Usually, for ordered monolayers of carboxylic acid, in the PM-IRRAS spectra, a C – O stretching band is observed in the region of 1210-1320 cm^{-1}. However, during the initial 15 min of the experiment, it is not visible because if the OA monolayer is relatively loose, it is also completely disordered, and the COOH group can take any orientation. Over time more

protein molecules adsorbed at the interface forcing the fixed orientation of the carboxyl groups, which is reflected by the appearance of the band with a maximum at 1240 cm^{-1} increasing in intensity. Moreover, bands characteristic for an antiparallel β-sheet for protein and a hydrated α-helix induced by surrounding water molecules were observed. The intensity of mentioned bands increases with an increasing number of protein molecules at the interface, and what is more, the peak assigned to the hydrated α-helix becomes more distinct, suggesting protein conformational changes as it interacts with OA.

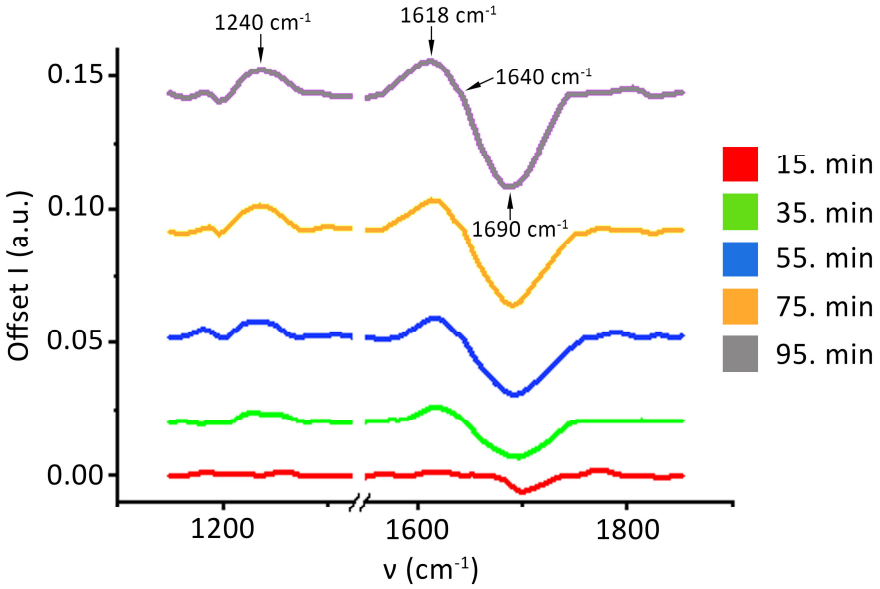

Figure P4.5. The PM-IRRAS spectra of the OA-BLA III system when protein incorporates into the OA monolayer at 29°C and pH 2.0; t = 0 [min] corresponds with the protein introduction

2.6. The influence of calcium ions

The role of the Ca^{2+} ions on the interactions between OA monolayer and BLA III and the stability of the complex was investigated in publication **P2** entitled *Lipid–protein interactions in Langmuir monolayers under dynamically varied conditions.*

Most of the studies on HAMLET-like complexes in this thesis were performed on the interactions between fatty acids and bovine α-lactalbumin in the calcium-depleted form (BLA III). Ca^{2+} is known to stabilize the structure of α-LA. As was mentioned in Chapter 1.5 and depicted in Figure 11, the removal of Ca^{2+} ions enhances the protein transformation from the native to molten globule form when OA is a cofactor. However,

what is particularly important in this part of the study is that the molten globule state of α-LA may return to the native form if the environmental conditions, like the presence of the calcium ions, temperature, or pH, are normalized.

To confirm the significance of the calcium ions within the α-LA structure on the interaction with the OA monolayer, the relaxation/penetration experiment was utilized using the native form (BLA I – containing calcium ion) and the calcium-depleted form (BLA III). As follows from Figure P2.4, the response of the OA monolayer on the presence of BLA III in the subphase is significantly more pronounced than for BLA I. Moreover, the equilibrium surface area was achieved at a much lower level and after a considerably longer time. This experiment demonstrates that calcium-free α-LA promotes the tertiary structure loss by the protein and facilitates the OA molecules binding, supporting the HAMLET-like complex formation. The relative molecular area expansion due to the protein molecules present at the interface is significantly more intensive for BLA type III than I, which proves the weaker unfolding of the protein in the presence of Ca^{2+}.

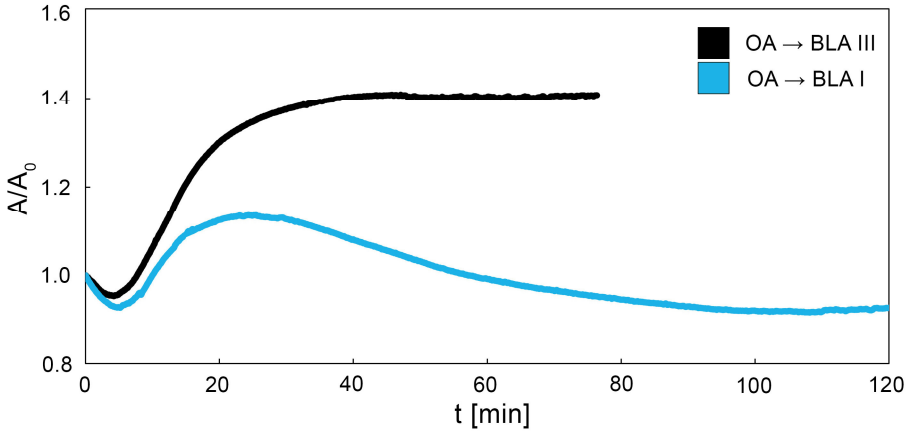

Figure P2.4. Relative area changing in time for OA monolayer at a temperature of 36.6 °C and pH 2.0, penetrated by the same concentration of α-LA type I and III

However, to unravel the impact of the calcium ions on the OA-α-LA system, complex studies were needed on the behavior of the pure OA monolayer and the mixed OA-BLA III monolayer, in the presence of different concentrations of calcium ions. The pure OA and OA-BLA III systems were added as a reference. Both, BLA III and $CaCl_2$ were introduced under the existing monolayer compressed to 5 mN/m via the peristaltic pump (the methodology is explained in Figure 13). First, the effect of calcium ions on

the OA film was investigated. Preliminary studies in **P2** on OA monolayers spread at the calcium-containing subphase proved that calcium ions cause the OA monolayer condensation.

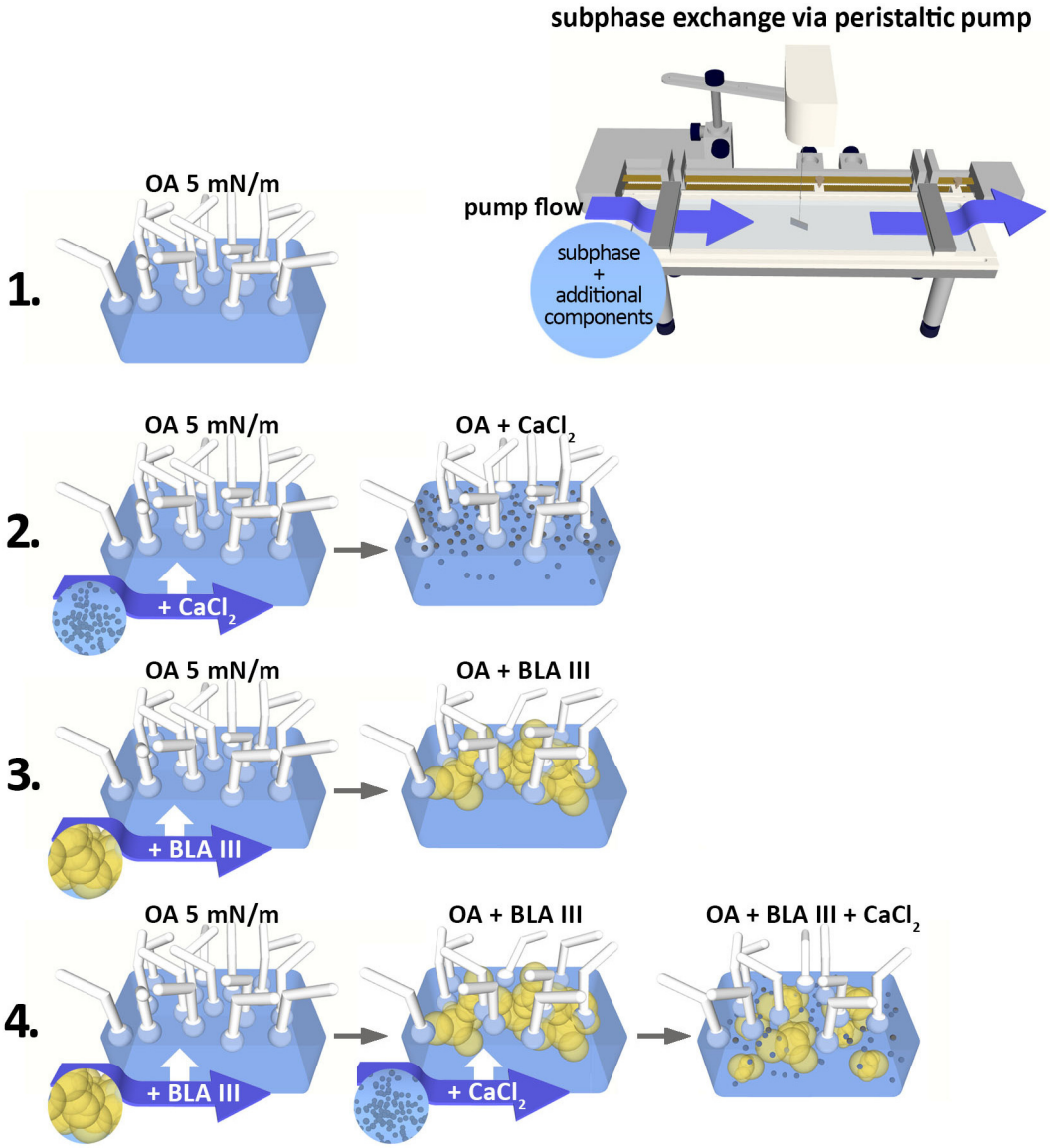

Figure 13. The scheme of procedure for conducting relaxation/penetration experiments using a peristaltic pump to introduce additional components into the system. The numbering is consistent with the legend in Figure P2.5

According to Figure P2.5 A, with the smallest calcium ions amount tested, the decline in the relative molecular area was slightly slower than for pure OA film. With increasing calcium ions concentration, the effect on the monolayer stability is enhanced due to the ions adsorption at the interface (Figure P2.5 B, C, D). As mentioned before,

as BLA III is delivered into the subphase, the OA monolayer expansion occurs when the protein penetrates the film. The equilibrium achieved by OA-BLA III allowed the investigation of the effect of Ca^{2+} on the system stability. For this purpose, after reaching the maximum area expansion in the OA-BLA III system, the peristaltic pump was reused to deliver the calcium ions. Even a small concentration of ions present in the subphase led to a decrease in the value of the relative area and this effect is more pronounced for a higher amount of Ca^{2+} (Figure P2.5 A and B). Considering the behavior of the OA film in the presence of $CaCl_2$, the decrease in relative molecular area is attributed to conformational changes of α-LA. The presence of calcium ions induces protein refolding, which is demonstrated as the A/A_0 decrease. However, a large excess of the Ca^{2+} ions in the system of OA-BLA III initially caused the slight A/A_0 reduction due to the protein conformational changes, but then a significant increase was noted (Figure P2.5 D). Here, the excess calcium ions conceal the effect of conformational alterations within the protein structure because of the adsorption of ions at the interface.

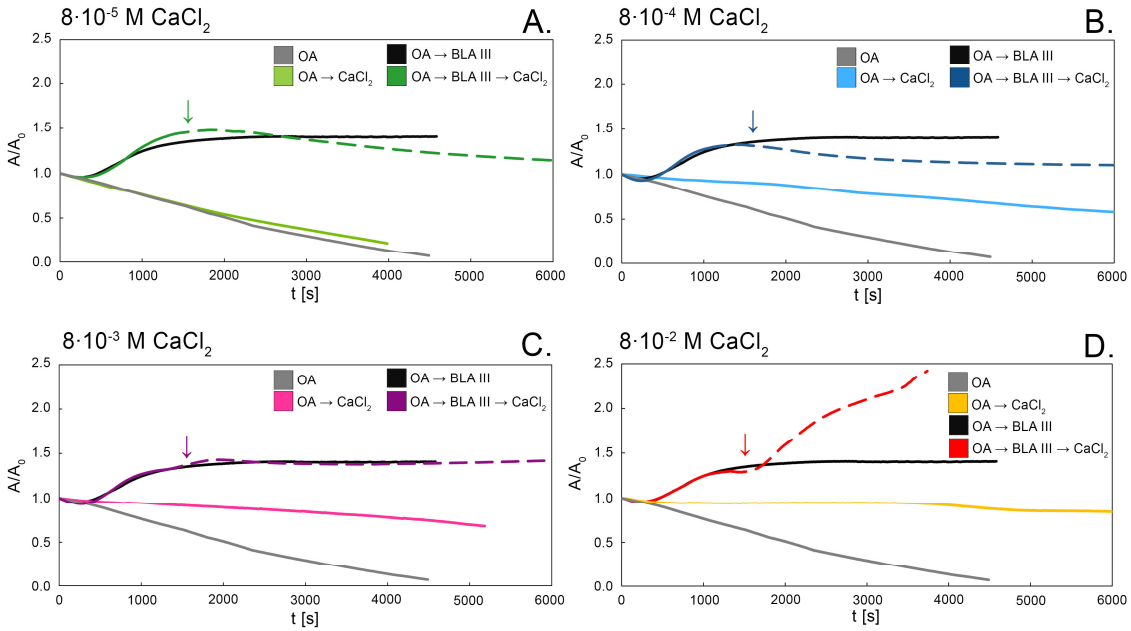

Figure P2.5. The relative area changing in time during relaxation/penetration at a temperature of 36.6°C and pH 2.0 for OA and OA-BLA III monolayers in the presence of various concentrations of $CaCl_2$. All additional components were added to the OA monolayer using the peristaltic pump – in single subphase exchange (solid line) or double subphase exchange (dashed line); arrows indicate the start of subphase exchange

It has been demonstrated that the presence of calcium ions, both introduced with the protein in the calcium-loaded form (*holo*-α-lactalbumin) or added to an already stable system, strongly affects the structure of α-LA and its activity at the interface.

2.7. Conclusions

From the results of the extensive investigations involving numerous research techniques, it is possible to conclude that various factors have a synergistic effect on the possibility of forming HAMLET-like complexes at the interface.

In the model gastric conditions, the fatty acid monolayer response to the α-lactalbumin adsorption at the interface is divided into a few individual steps of significantly different kinetics. Upon protein introduction to the system, after a short delay for the protein molecules to reach the interface, the OA monolayer answers with the increase of the relative surface pressure, then it reaches the maximal value, decreases to a certain level due to the structural rearrangements, and stabilizes. The plateau region of the π/π_0 occurs due to the formation of a stable mixed monolayer after the surface saturation by protein and conformational rearrangements. The step of stabilization is identified with the thermodynamic equilibrium achieved by the system. Generally, the more protein molecules adsorbed at the interface, the higher the relative surface pressure increase until complete surface coverage. Due to the following further stages, the relaxation/penetration curves have a distinctive bell-like shape (Figure 14).

Based on the obtained results, general rules governing the formation of HAMLET-like complexes at the interface can be determined. In the tested temperature range (10-36.6°C), the higher the temperature, the more enhanced the interaction between fatty acid and protein. The most important effect of the temperature is that it affects the ternary structure of the α-lactalbumin, promoting transformation into a molten-globule state. Moreover, at elevated temperatures the fatty acid monolayer undergoes faster degradation because of the increased solubility, enabling the more intensive monolayer penetration by the protein. Depending on the pH affecting the molecules' charge, the interaction between protein and fatty acid in the monolayer is governed by forces of different nature. At pH 2.0, the α-LA is positively charged, while the oleic and linoleic acid molecules are unionized. Thus the interactions are considered hydrophobic,

complemented by hydrogen bonds. At pH 6.2, the interactions in the system are mainly electrostatic because the oleic acid is ionized, and BLA III is negatively charged. The kinetics of the HAMLET-like complexes formation is an effect of the synergistic influence of the temperature and the pH. The monolayer penetration by the protein is enhanced for expanded films. The interactions between OA and BLA III act between alkyl chains of OA and amino acid residues of BLA III, but for the monolayers pre-compressed to high surface pressure or small area per molecule, the fatty acid chains are less accessible for binding. Protein concentration determines the rate of adsorption at the interface, and the magnitude of surface pressure growth, as protein molecules reach the surface. The surface pressure increases with increasing concentration of BLA III in the subphase until the surface saturation. However, it should be noted that other factors (like monolayer compression, temperature, pH, and calcium ions presence) affect the stoichiometry and kinetics of interactions between fatty acids and α-LA at the interface.

The absence of calcium ions enhances the α-LA transformation into molten globule form. But on the other hand, the protein in the molten globule state returns to the native conformation when the calcium ions are back in the system. The presence of Ca^{2+} induces protein refolding, lowering the position of the equilibrium state of the interfacial system.

Each of the factors described in this chapter affects the structural changes of the components, the state of the monolayer, and the possibility of interaction, and acting synergistically, they determine the formation of a stable structure at the interface.

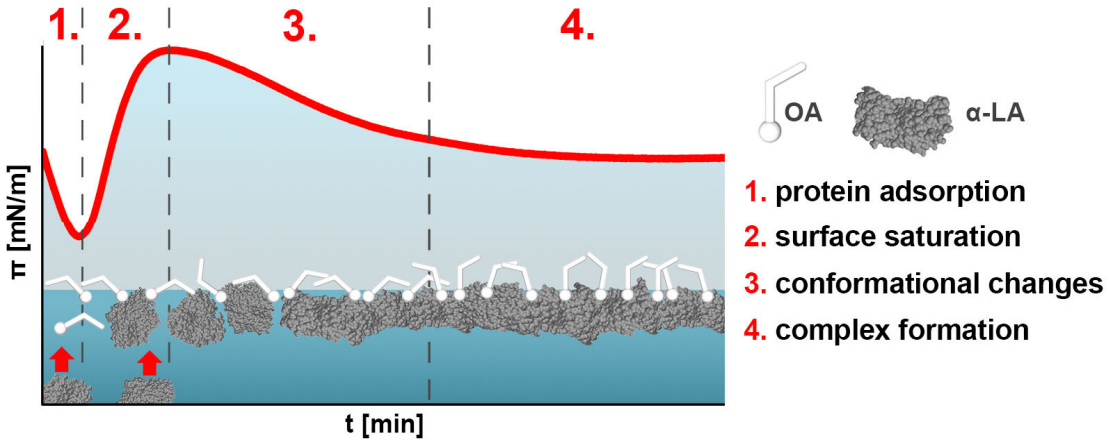

Figure 14. The successive stages of the HAMLET-like complex formation at the interface, which were determined based on the experiments in the Langmuir technique

CHAPTER 3. Interactions between OLA and HSA at the interface

Summary of the publication

PUBLICATION P5.	
Title	Physicochemical characterization of oleanolic acid–human serum albumin complexes for pharmaceutical and biosensing applications
Authors	Katarzyna Dopierała, Martyna Krajewska, Marek Weiss
Journal	Langmuir
Details	2020, 36, 13, 3611–3623
DOI	https://doi.org/10.1021/acs.langmuir.0c00087

3.1. Introduction

As was mentioned in Chapters 1.2 and 1.5, triterpenes have a beneficial effect on health, but their bioavailability is limited because of low solubility in water and instability. Since addressing this issue is the goal of the innovative drug delivery systems, the publication **P5** entitled *Physicochemical characterization of oleanolic acid–human serum albumin complexes for pharmaceutical and biosensing applications* provides the physicochemical insight into the interaction between a triterpenoid and a possible carrier. The studies presented there were performed on oleanolic acid (OLA) as a representative of triterpenoids. Effective pharmaceutical formulations developed for OLA include a variety of carriers such as liposomes, nanoparticles, and nanoemulsions. However, numerous limitations like insufficient stability, drug loading, and the need for biocompatible excipients still have to be overcome until scaled-up production. Among substances considered to be advantageous carriers, human and bovine serum albumin (HSA and BSA) have been widely studied as proteins frequently used as models. The HSA as a carrier has been shown to reduce the toxicity of the drugs, alter the distribution and bioavailability of the drugs, and control the final therapeutic efficiency. The aim of the **P5** paper was the investigation of the interfacial properties of the OLA in the presence of HSA in terms of morphology and thermodynamics. The Langmuir monolayer approach was utilized here to introduce a molecular-scale insight into studies for designing better and innovative drug delivery systems.

The paper discusses the behavior of OLA and HSA at the air-water interface and explores the effect of protein concentration and monolayer packing in detail through π-A isotherms and compressibility modulus, relaxations, interfacial shear rheometry, and Brewster angle microscopy. Moreover, monolayers formed on the aqueous subphase were transferred onto a solid substrate vertically via the Langmuir-Blodgett and horizontally via the Langmuir-Schaefer protocol. The film was examined for wettability, surface free energy (SFE), and topography studies performed by atomic force microscopy (AFM).

It is worth mentioning that only a part of the results from the comprehensive physicochemical characterization of the OLA-HSA complex is reported here to support the hypotheses about the possibility of using this system in novel pharmaceutical formulations.

3.2. Interfacial behavior of OLA-HSA monolayer

Due to the bolaamphiphilic nature of oleanolic acid's molecule (already mentioned in Chapter 1.2) and the presence of the rigid pentacyclic hydrophobic part separating the polar groups, OLA monolayers are inhomogenous. Within the film, there are OLA molecules oriented both, with the –COOH group facing the aqueous subphase and OLA molecules with the –OH group directed towards the aqueous subphase. Within the tightly packed monolayer, due to the higher polarity of the COOH group, the orientation with the carboxyl group facing the water dominates. The issue of OLA monolayer morphology is developed in **P5** and **P6**. As follows from Figure P5.1A, the presence of HSA in the system pronouncedly alters the shape of the OLA isotherm and moves the curves toward the larger values of the molecular area. Moreover, the protein-concentration effect is distinct here – with growing protein concentration in the subphase, the initial surface pressure increases. However, above the concentration of 3.2 mg/L, the shifts in the molecular area are inhibited.

It is broadly known that HSA forms adsorbed and spread films at the air-water interface [81]. Thus, HSA is considered to incorporate into the OLA film. According to the graph of compression modulus vs. surface pressure (Figure P5.1B), the LC state for pure OLA shifts to LE-LC or even LE state in the presence of HSA at the interface. In this

case, protein leads to monolayer fluidization. The Cs^{-1} curves minima prove that OLA-HSA monolayers are of a more complex organization than OLA film. Since the IEP of HSA at 25°C is 5.1, at the aqueous subphase of pH 6.25, the protein is of slightly negative charge due to the ionized R–COO^- groups. Furthermore, the pK_a value of OLA is 5.11. Thereby, the explanation of this phenomenon cannot be the interaction between OLA and HSA by electrostatic attraction. The shift in an area occupied by OLA molecule as an effect of HSA introduction to the system, along with the molecular dimensions of OLA and HSA, leads to the conclusion that HSA reaches the interface. However, it occupies less space than it would result from the cross-sectional area. That demonstrates that OLA and HSA compete for the interfacial area striving to obtain the most thermodynamically favorable orientation.

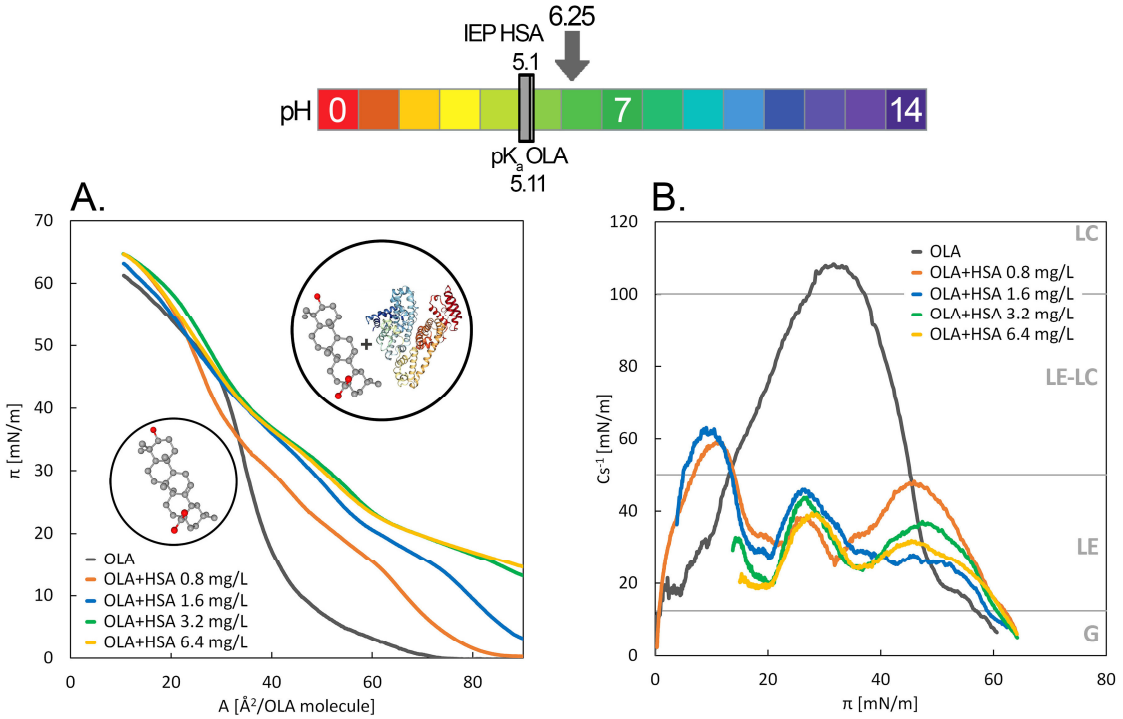

Figure P5.1. The π–A isotherms (A) and compression modulus Cs^{-1} vs. surface pressures (B) for the OLA monolayer spread at the subphase containing various concentrations of HSA; temperature 25°C, the subphase pH=6.25; upper inset: pH scale with OLA pK_a and HSA IEP indicated; lower inset: the structure of OLA from PubChem [82] and OLA+HSA from Protein Data Bank (1AO6) [83]

The kinetics of HSA adsorption at the interface already covered with OLA monolayer was investigated through relaxation/penetration experiments (Figure P5.2). The relaxation of OLA compressed to various surface pressure over time were presented

as a comparison (Figure P5.2A). The graph of OLA monolayer relaxation indicates the progressive loss of the material from the interface for each compression level. However, it reveals also that for monolayers at π>10 mN/m, the surface pressure starts to fluctuate (the relative molecular area fluctuations corresponding to this are shown in inset A in Figure P5.2). This phenomenon is attributed to the specific behavior of oleanolic acid in monolayer due to the bolaamphiphilic structure and pentacyclic hydrophobic part. As an effect, the OLA monolayer is extremely stiff, and the artifact-free relaxation curves can be obtained only for relatively loose monolayers. When the HSA molecules are injected under the pre-compressed monolayer, the effect of protein in the system is pronounced since no material loss was recorded (Figure P5.2B). For the π=10 mN/m, the molecular area expansion signifies that the surface-active HSA penetrates the loosely-packed film and occupies space at the interface. The presented data, together with BAM images (not shown here), suggest that protein molecules are expelled from the monolayer into the upper regions of the film when compressed, creating a stable bilayer-like structure. However, the protein can adsorb only in the vacant interfacial area, in the vicinity of nonionized carboxylic moieties of oleanolic acid. When the OLA monolayer is more tightly compressed, the interactions with HSA are limited only to the region of the polar headgroups. The shape of relaxation curves and the stable value of A/A_0 of about 1 indicates the formation of a bilayer composed of HSA molecules located just below the OLA monolayer, without film penetration by the protein molecules. The possible explanation of the adsorption and binding of OLA at high surface pressure is the exposition of domains to the interface and forming hydrogen bonds between the –NH group within amino acids and the oxygen atom of a carbonyl group in OLA. The conclusion can be drawn that depending on the monolayer compression, HSA can bind OLA by different binding sites.

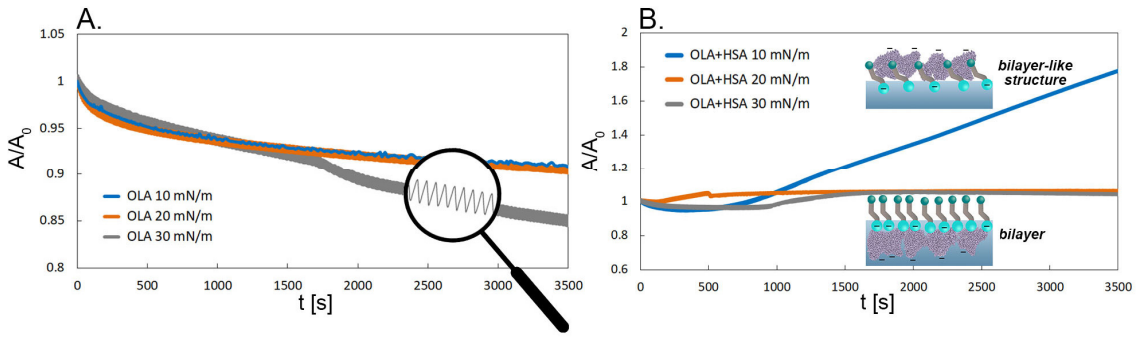

Figure P5.2. The relative area changing in time during relaxation/penetration for OLA (A) and the OLA-HSA (B) at various surface pressures; the HSA concentration is 0.8 mg/L; temperature 25°C, the subphase pH=6.25; inset at A: close up of fluctuations during relaxation; inset at B: scheme of the mixed monolayer at 10 mN/m and bilayer formed at higher surface pressures

3.3. OLA-HSA films transferred onto a solid substrate

Since the deposition of OLA-HSA monolayers onto a solid substrate enables further detailed characterization, the monolayers were transferred from the aqueous subphase onto a solid via Langmuir-Blodgett and Langmuir-Schaefer approach. Then, the wettability measurements were performed to investigate the surface character. The values of contact angle measured with three liquids (polar: water, formamide, and non-polar diiodomethane) were used to calculate the surface free energy (data not shown here). However, even the analysis of the contact angle (CA) values in the context of various systems, conditions, and transfer methods, can provide valuable conclusions. As follows from Figure P5.3, the contact angle values for films of pure OLA at 10 mN/m transferred via Langmuir-Blodgett and Langmuir-Schaefer methodology vary significantly due to the characteristics of the particular procedure (Figure 5, Chapter 1.3) and bolaamphiphilic structure of triterpenoid. Larger contact angles for polar liquid measured for the LS film suggest different structures and transfer quality. Moreover, the pronounced differences of contact angle values for OLA films deposited at 10 mN/m and 20 mN/m (grey frame in Figure P5.3A) reflect the alterations of OLA monolayer structure when compressed to π>10 mN/m. The presence of HSA within the film of OLA at 10 mN/m affects the CA values considerably, revealing several effects. The water CA for pure OLA deposited onto a solid is significantly lower than 90° indicating that the surface is of hydrophilic character, but for the mixed OLA-HSA film, it is twice as high and

exceeds 90°. According to the proposed explanation about bilayer-like structure formed for OLA-HSA film at 10 mN/m, as depicted in Figure P5.3D, after transfer via the LB method, the protein is exposed to interact with measurement liquid. The water contact angle is high due to the hydrophobic groups of the protein facing the air phase. The relationship between the contact angle values for the used liquids is different in the case of the OLA-HSA system at 20 mN/m (blue frame in Figure P5.3B and D).

As it was proposed, for a compressed OLA monolayer, when HSA is present in the subphase, it adsorbs beneath, forming a bilayer structure. Thus, after transfer via the LB procedure, the OLA is mainly exposed to the measuring liquid. When the LS procedure, acting as a stamp, is implemented, the values of the contact angles for all measuring liquids are almost identical for OLA and OLA-HSA (yellow frames in Figure P5.3 A, B, D). That confirms the OLA-HSA monolayers organization scenario proposed earlier. Both LB and LS transfer provided similar values of contact angles for HAS films. Small differences may be the result of exposure of individual residues to air (Figure P5.3C).

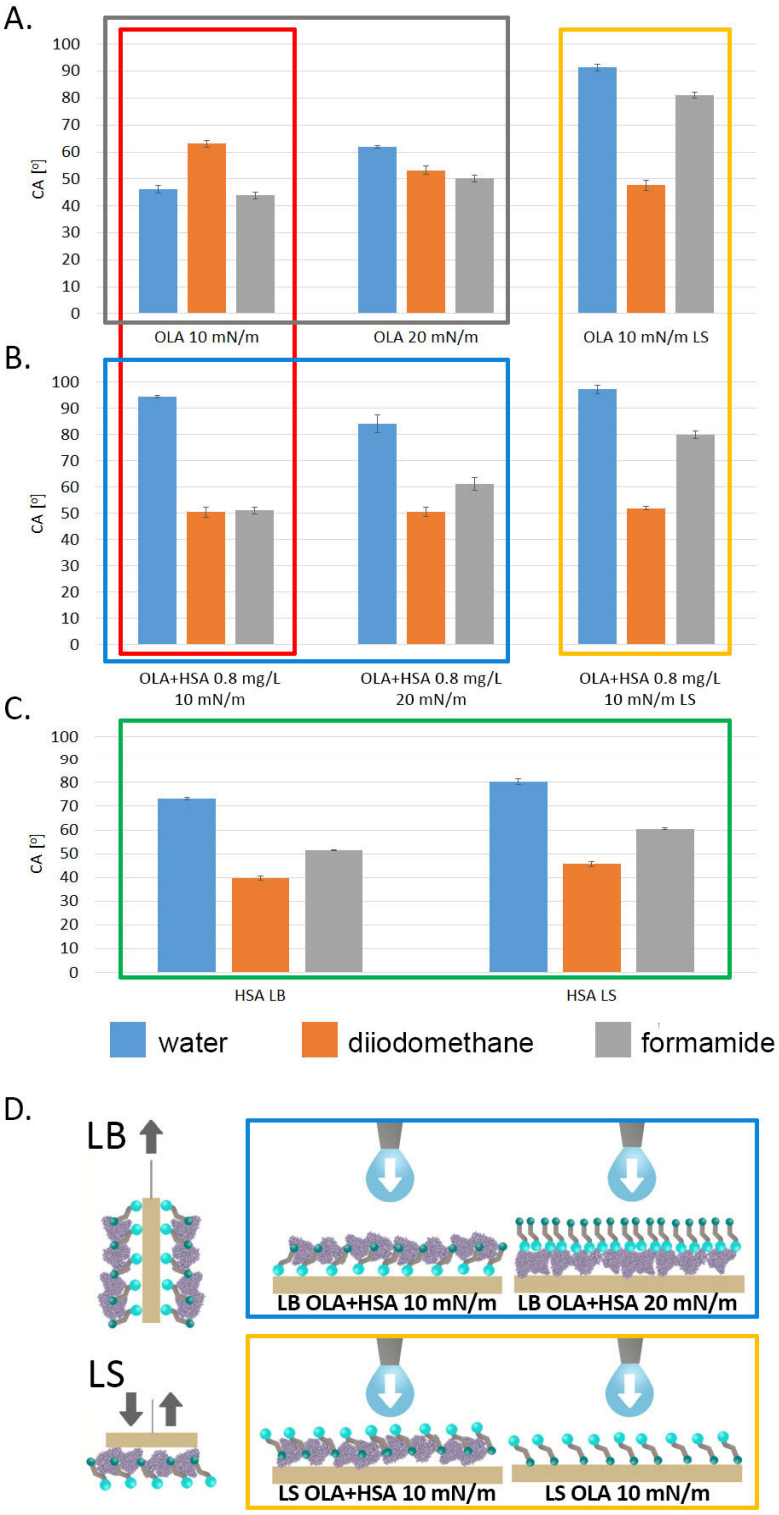

Figure P5.3. The values of contact angles (measured with water, diiodomethane, and formamide) for films of OLA (A), OLA+HSA (B), and HSA (C) at various surface pressures for the LB and LS transfer protocol and schematic representation of molecules organization when administered to contact angles measurements (D); colored frames indicate the systems compared in the discussion

The OLA, OLA-HSA, and HSA layers deposited at various surface pressures, via LB and LS methodology were studied by AFM to investigate the film topography. Here, only the results from the Langmuir-Blodgett approach experiments are presented. From Figure P5.4A, it can be seen that the pure HSA monolayer consists of small islands and seems to be fragmented. However, due to the uniform phase-contrast signal, there is a possibility that the protein layer is continuous, but it comprises molecules in different conformational states. In contrast, the pure OLA monolayer is well-organized and continuous regardless of the surface pressure tested. Since the surface pressure affects the molecular organization, it alters the film thickness, which is higher for π=20 mN/m. The OLA-HSA monolayers are continuous, but there are some visible nanometer-scale defects present. What is worth noting, the thickness of two-component monolayers, transferred at both 10 and 20 mN/m, is lower than for pure OLA monolayer. Thus, it proves that the presence of HSA alters the arrangements of OLA molecules, which leads to tilted orientation. The horizontal transfer via the Langmuir-Schaefer procedure promotes the formation of multilayers, while the molecular reorganization is limited during deposition. As a result, it is incomparable with a film obtained by the LB technique.

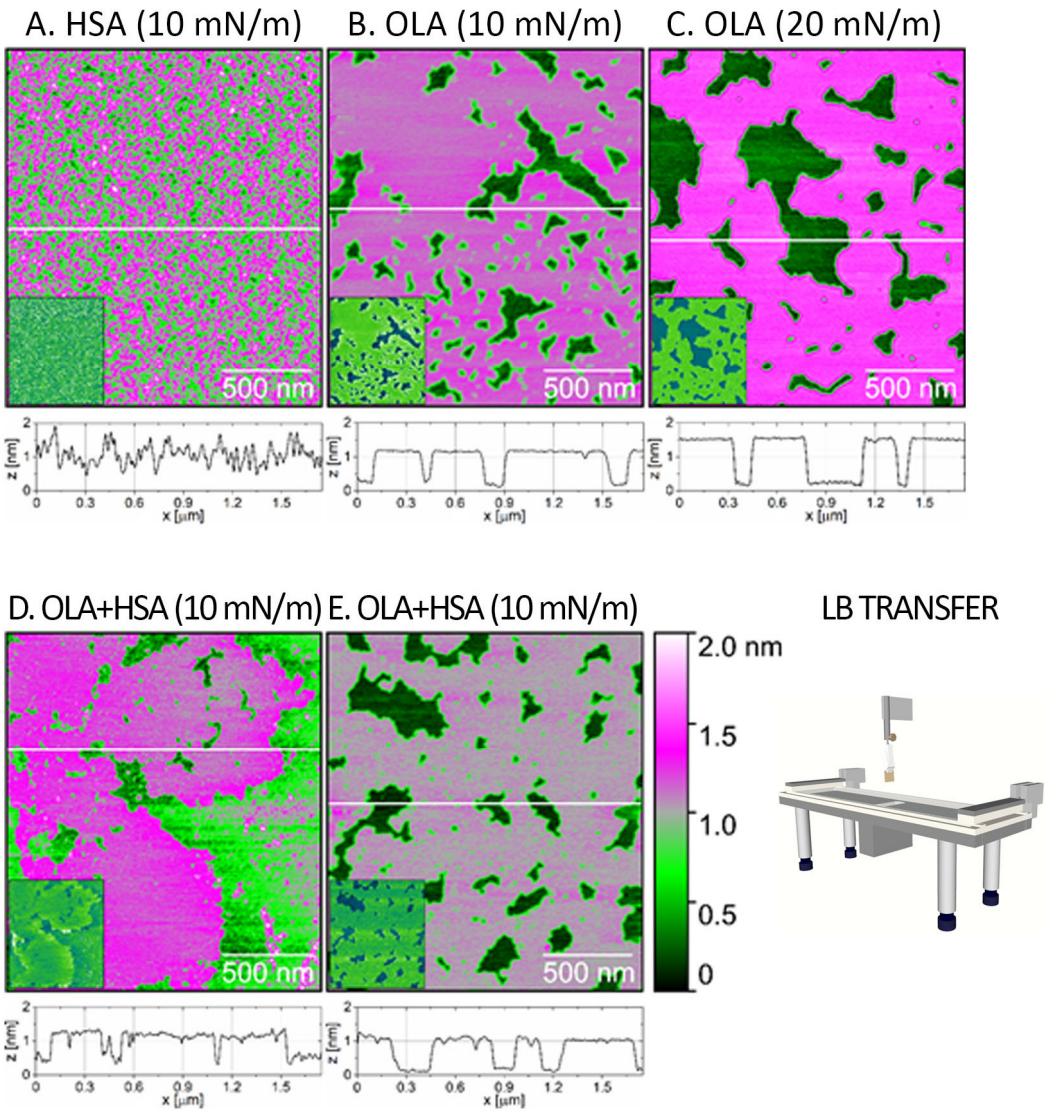

Figure P5.4. The AFM topographical images with typical cross sections of pure HSA (A), OLA (B, C), and mixed OLA-HSA films (D, E) deposited via LB approach; each inset represents the particular phase contrast signal; the vertical scale bar is common for all topographical images and the horizontal scale is 500 nm

3.4. Conclusions

The OLA-HSA system has been thoroughly tested both at the air-water interface and after transfer onto a solid. The main conclusion from the physicochemical characterization of this system is that OLA is bound by the HSA adsorbed at the interface. The HSA incorporation into the OLA film pronouncedly affects the organization of triterpenoid molecules. It was also found that the binding of oleanolic acid molecules by human serum albumin is irreversible at the considered conditions of pH, temperature,

and ionic strength and leads to the formation of thermodynamically stable bilayer film. Therefore, it can be suggested that serum albumin has a significant impact on the oleanolic acid distribution within the human body. Moreover, the physicochemical properties of the OLA-HSA system are of great importance when designing stable pharmaceutical formulations with OLA as API (active pharmaceutical ingredient).

CHAPTER 4. Physicochemical characterization of the binary monolayers composed of oleic and oleanolic acid

Summary of the publication

PUBLICATION P6.	
Title	The biomimetic system of oleanolic acid and oleic acid at the air-water interface–interactions in terms of nanotechnology-based drug delivery systems
Authors	Martyna Krajewska, Katarzyna Dopierała, Krystyna Prochaska
Journal	Membranes
Details	2022, 12, 12, 1215-1-1215-15
DOI	https://doi.org/10.3390/membranes12121215

4.1. Introduction

In **P6,** entitled *The biomimetic system of oleanolic acid and oleic acid at the air-water interface–interactions in terms of nanotechnology-based drug delivery systems*, an approach employing novel nanotechnology-based drug delivery systems was adopted to solve the problem of the limited solubility of triterpenoids as active therapeutic compounds. As oleic acid is a widely used pharmaceutical excipient, acting as a solubility enhancer and contributing to obtaining product stability, its application potential in the mixture with triterpenoid was inspired by nature and utilized in pharmaceutical formulations. Oleanolic acid (OLA) as triterpenoid, together with oleic acid (OA), is ubiquitous in the plant kingdom - in olive oil or epicuticular waxes covering leaves. Moreover, the biomimetic system of OLA-OA is considered the bioavailability-improving agent for other APIs. Hence the extensive analysis of the OLA-OA system at the air-water interface was conducted. It provided a physicochemical insight into the properties, miscibility, and rheological behavior of this system, which is of interest in pharmaceutical design. Since the properties of mixed monolayers depend on their composition, the OLA-OA system at various molar ratios was examined for morphology, miscibility, the excess free energy of mixing, and rheological characteristics. The differences in the structure of OLA and OA molecules are crucial for the stability of mixed Langmuir monolayer, which can be associated with pharmaceutical products.

4.2. The structure of OLA-OA binary monolayers

The molecular structures of OLA as triterpenoid and OA as fatty acid are significantly distinct, which translates into the various shape of isotherms and the behavior of monolayers of pure substances at the interface (Figure P6.1A). Each of the individual OLA-OA mixtures has features firmly depending on the monolayer composition, and the isotherm curves of mixed monolayers are located between the isotherms of pure OLA and OA. The increasing content of OLA shifts the curves of binary systems towards the higher molecular areas, corresponding to the values typical for oleanolic acid monolayers. However, for systems of OLA-OA 2:1, 1:1, and 1:2, there are some indicators of instability, such as disrupted isotherm shape and double collapse regions.

The conformation of molecules at the interface associated with the compressibility is depicted in Figure P6.1B. Due to the presence of an unsaturated bond in the middle of the hydrocarbon chain of fatty acid, the compression modulus of the OA monolayer is lower than 50 mN/m, therefore, the monolayer remains in a liquid-expanded state (already presented in Figure 4C). Apparently, the unsaturation enhances film fluidity and prevents tight compression. On the other hand, the OLA monolayer at the Cs^{-1} maximum is in the liquid-condensed phase. Since the compressibility is strongly related to the isotherm shape, the Cs^{-1} values of mixed OLA-OA systems depend on the composition. Particular attention should be paid to the systems with excess OLA concentration, which reaches even higher Cs^{-1} values than pure triterpenoid. On the other hand, such a low amount of OLA, as in the OLA-OA 1:5 system, enhances the compressibility of binary monolayers.

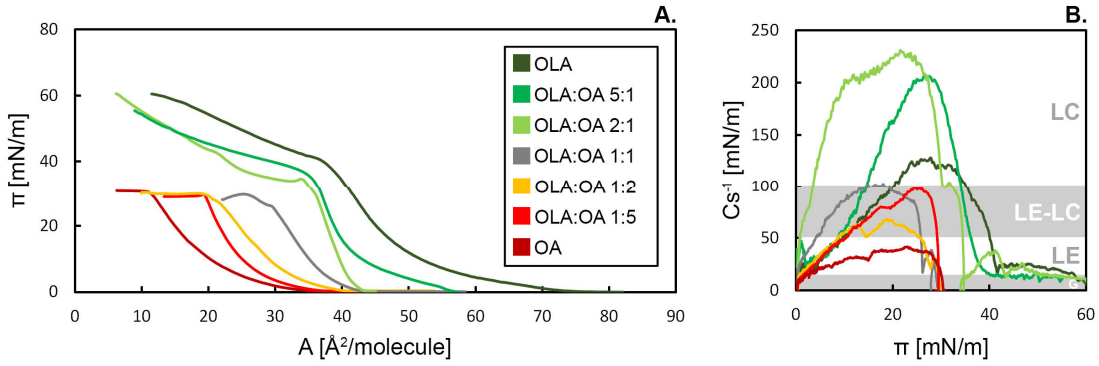

Figure P6.1. The π–A isotherms of pure substances and mixed systems (A) and the compression modulus vs. surface pressure graph (B) based on the isotherms; the ranges of the Cs^{-1} states according to Davies and Rideal classification [5] are highlighted

The Brewster Angle Microscopy (BAM) images were taken during the monolayer compression to evidence the phase separation. Figure P6.2 illustrates tile-like, angular domains of various brightness and size within the OLA monolayer, corresponding with two orientations of OLA molecules (dark green frame). The inhomogeneity of this film is visible even upon monolayer compression, close to the monolayer collapse. Since the molecular structure of OA is significantly distinct from OLA, the BAM images revealed different monolayer morphology. Oleic acid forms at the interface characteristic round-shaped microdomains, fusing due to the compression and finally creating a homogenous monolayer (dark red frame in Figure P6.2). For OLA-OA 5:1 at low π, the mixed monolayer morphology is analogous to pure OLA, with slightly smoother tile edges, but, what is particularly important, when compressed, it remains homogenous and devoid of domains or aggregates (green frame in Figure P6.2). The other systems of higher OA molar ratio induce the formation of bright elongated aggregates (yellow and red frames) attributed to the phase separation.

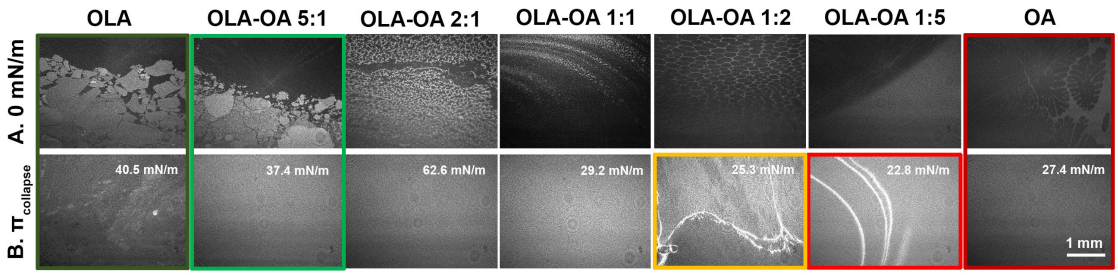

Figure P6.2. The BAM images of OLA and OA monolayers and binary OLA-OA systems of various molar ratios A. at 0 mN/m just before compression and B. at π close to collapse, specific for each system; colored frames indicate the systems compared in the discussion

4.3. The miscibility and stability of OLA-OA binary monolayers

The miscibility of multi-component Langmuir monolayers results from the interaction between molecules of both substances. If the film components are fully immiscible or ideally miscible, the dependence of mean molecular area (A_{12}) on the composition (X_{OLA}) is linear (Figure P6.3A). However, due to the interactions between components, mixed monolayers usually exhibit non-ideal behavior, and then the negative deviations evidence attractive interactions, and positive ones indicate repulsive interactions or phase separation. Analogously, in the graph of excess free energy of mixing (Figure P6.3B), for a monolayer of perfectly miscible components, the ΔG^{exc} value is zero. The negative ΔG^{exc} value is related to the stronger attraction between molecules than in a single-component film. Moreover, the lower the value, the more pronounced the film stability is. It has been found that for the system of OLA-OA 1:2, a phase separation occurs. Positive values of excess free energy of mixing for this composition in the whole range of surface pressures prove the presence of strong repulsive interactions between OLA and OA molecules. The positive ΔG^{exc} values were obtained for OLA-OA 1:5, 1:1, and 2:1 as well, but only when compressed to the π>10 mN/m. However, the OLA-OA 5:1 system exhibits negative ΔG^{exc} within the entire range of surface pressures examined. That signifies the occurrence of attractive interactions, which results in good miscibility of the components and stability of the system over time.

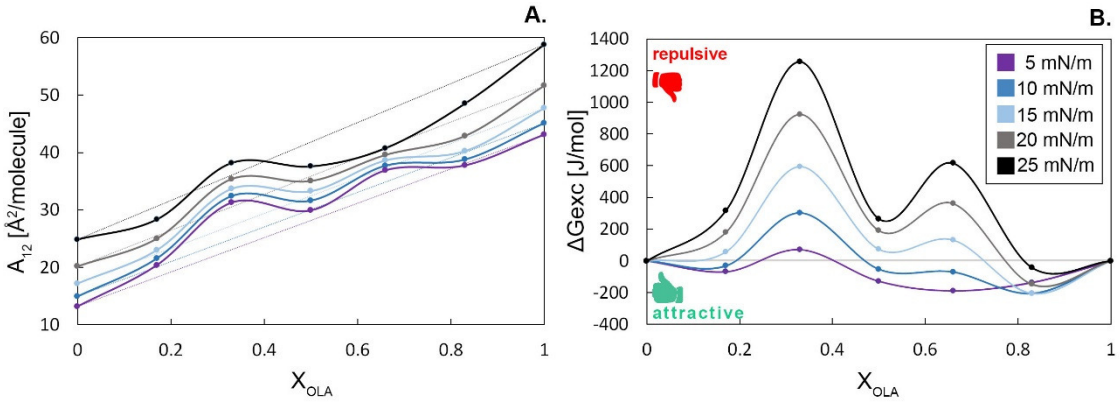

Figure P6.3. The mean area per molecule (A_{12}) vs. monolayer composition (X_{OLA}) for OLA-OA mixed monolayers (A) and the excess free energy of mixing (ΔG^{exc}) vs. monolayer composition (X_{OLA}) for OLA-OA mixed monolayers (B); experiments were conducted at constant surface pressures ranging from 5 to 25 mN/m; the gray lines in A represent the behavior of the ideal binary system

Because enhanced stability of binary systems is related to good miscibility, relaxation studies were performed (Figure P6.4). In the relaxation experiments at a constant surface pressure of 5 and 10 mN/m, the relative area changes were monitored over time for both pure substances and mixed systems. The rates of disruption for single-component OLA and OA film for both examined levels of surface pressure differ considerably. The OLA monolayer, after the initial relative area decrease, remains almost stable over time, while the OA film disrupts quickly due to the unsaturated bond. As with isotherms and miscibility, the stability over time of mixed OLA-OA systems evidently depends on the composition of the monolayer. The relaxation curve of the OLA-OA 5:1 system stabilizes at the A/A_0 level even higher than pure OLA. On the other hand, the OLA-OA 1:5 system disruption is more pronounced than pure OA. These results concur with the miscibility studies. Moreover, the enhanced stability of the OLA system after the addition of a small amount of OA is related to the attractive interactions between the components. Thus, the accelerated destruction of the OA monolayer with an admixture of OLA is the result of repulsive interactions in this system.

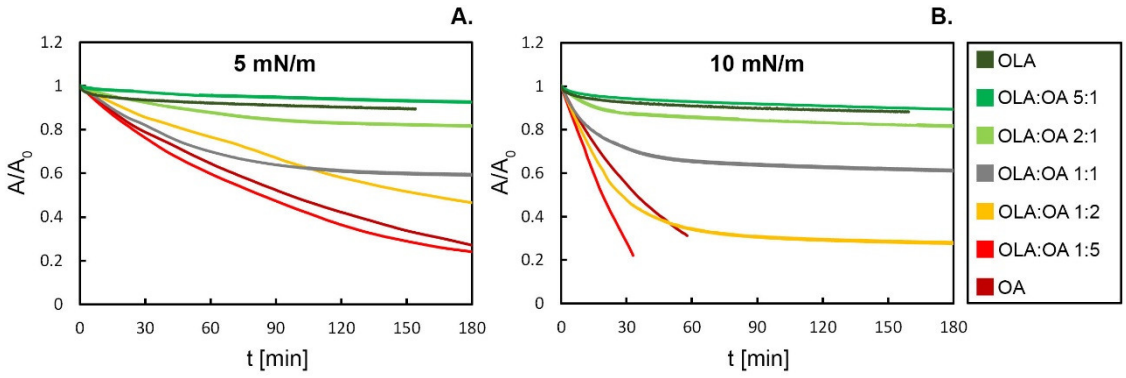

Figure P6.4. The relative area changes over time for pure monolayers (OLA and OA), as well as, for binary monolayers at various mole fractions at the surface pressure 5 mN/m (A) and 10 mN/m (B)

4.4. Conclusions

As a result of extensive research via the Langmuir technique on the OLA-OA system at various molar ratios, it was found that the monolayer composition determines the morphology, miscibility, and stability of the binary systems at the air-water interface. It was demonstrated that only in a system containing a small addition of OA (OLA-OA 5:1), in the whole range of surface pressure tested, the interactions between molecules are energetically more favorable than for pure substances. That phenomenon is attributed to the incorporation of fatty acid molecules into the two-dimensional OLA lattice at the interface and the occurrence of attractive intermolecular interactions. Fatty acid molecules penetrate terpenoid structure, affecting the orientation of OLA molecules and improving the monolayer stability. On the other hand, the miscibility gaps between the film components in different molar proportions are due to the OA exclusion from the film, leading to phase separation, which limits the applicability.

The presented results provide information on the interaction within the system of OLA-OA. Moreover, since the selection of appropriate excipients and carriers is crucial for the stability and maintaining biological activity, the physicochemical guidelines help to design novel pharmaceutical formulations.

General conclusions

The research in the presented dissertation is focused on the formation of biomimetic systems of therapeutic potential based on the Langmuir and Langmuir-Blodgett techniques. In the thesis, three types of systems are characterized: fatty acid-protein complexes, triterpenic acid-protein complex, and fatty acid-triterpenic acid system. The conducted experiments regarding two-component, two-dimensional systems based on amphiphilic substances allow confirming the applicability of the Langmuir technique in assessing the physicochemical properties of the potential therapeutic systems.

The scope of research in this doctoral thesis was divided into stages for each system. The first step involved the evaluation of the structure of two-component monolayers on the aqueous subphase using the Langmuir technique and complementary techniques. Some already characterized mixed monolayers have been transferred onto a solid, which opened the way to use other techniques. Since the structure of films apart from the aqueous subphase could have changed during the deposition process, further research strived to determine the film morphology on a solid substrate. The experiments carried out in the next stage allow for determining the surface properties and morphology of the films formed on a solid. Undertaken activities aimed at assessing the usefulness of the biomimetic films for use in biosensors, as drug carriers, or for surface modification.

The conducted research and conclusions drawn have expanded the knowledge about the mechanisms of interaction between various substances at the air-water interface. The results presented in this thesis help in more effective designing novel drug formulation based on biomimetic systems, including the selection of carriers and excipients improving stability and preventing nonspecific binding. The successful transfer of lipid-protein films via the Langmuir-Blodgett technique poses a significant step forward to immobilizing such systems and developing novel pharmaceutical nanoformulations.

A meaningful achievement of this thesis is the finding that the subphase exchange, in addition to delivering additional substances to the system (previously

developed and used by other scientists), is also a suitable tool for changing the measurement conditions (temperature and pH) during the experiments. This approach has been investigated in this work for lipid-protein systems, but it opens the possibility of new applications of the Langmuir technique to systems that require a dynamic change of conditions during the measurement.

The obtained results were in detail discussed in publications P1-P6. It should be firmly emphasized that the conducted investigations confirm the hypothesis about the usefulness of the monolayer studies in the presence of substances of therapeutic potential, bringing physicochemical insight into designing novel pharmaceutical dosage forms.

Key findings

1. Based on the obtained results, general rules governing the formation of HAMLET-like complexes (complexes of unsaturated fatty acids with α-lactalbumin) at the air-water interface were determined. The role of temperature, pH, molecular packing, protein concentration, and calcium ions presence on the formation of the HAMLET-like complexes at the air-water interface was insightfully evaluated:

- The higher the subphase temperature (in the measured range), the more intensive the monolayer penetration by the protein. The temperature affects not only the properties of the fatty acid monolayer but also induces conformational changes of α-LA (transition to the molten globule state) and influences the protein adsorption rate.
- In a strongly acidic environment imitating the conditions of the human stomach, the interactions between fatty acid and α-LA are hydrophobic in nature, supported by hydrogen bonds, while at neutral pH the intermolecular interactions are mostly electrostatic.
- The molecular packing of the monolayer is the main factor ensuring the ability of the protein to reach the interface. In model gastric conditions, where interactions are governed by hydrophobic forces, fatty acid chains are more accessible for binding in expanded films. Moreover, the same monolayer

response cannot be induced only by increasing the protein concentration for a more tightly compressed monolayer.

- In the relaxation experiment, the relative surface pressure increases more pronouncedly when the higher concentration of α-LA is present in the subphase, but only until the maximal value is reached. Thus, above a particular α-LA concentration, the progressive increasing the protein amount does not induce more changes in the surface pressure of fatty acid monolayer.
- Since calcium ions are known to stabilize the structure of α-LA, and its removal facilitates the α-LA transformation to the molten globule conformation, it was proven that even a low concentration of Ca^{2+} decreases the relative molecular area in fatty acid-α-LA monolayer. Moreover, this effect is even more pronounced for a higher concentration of calcium ions. The decrease of the relative molecular area is assigned to the α-LA refolding due to the presence of calcium ions.
- Each of the factors mentioned above affects the structural changes of the HAMLET-like complex components, the state of the monolayer, and the intermolecular interaction and synergistically determine the formation of a stable structure at the interface.

2. It has been elucidated that despite the similarities between monolayers of various long-chained fatty acids, each of the tested fatty acids differing in molecular structure modifies the binding ability of α-LA, and each of the HAMLET-like complexes at the interface shows specific features.

3. It has been confirmed that the binding of the α-lactalbumin to the fatty acid monolayer occurs in several steps of significantly varying kinetics: upon α-LA introduction underneath the fatty acid monolayer, protein molecules strive to the interface, penetrate the monolayer, saturate the surface, undergo conformational rearrangements, and finally reach the stabilization step identified with the thermodynamic equilibrium achieved by the system.

4. The oleanolic acid monolayer bound the human serum albumin adsorbed at the air-water interface and, in effect, the organization of triterpenoid molecules is

pronouncedly altered. At the considered conditions, the binding of OLA molecules by HSA is irreversible and leads to the formation of a thermodynamically stable bilayer structure.

5. Fatty acid molecules incorporate into the two-dimensional triterpenoid lattice at the air-water interface, affecting the orientation of OLA molecules. Due to the attractive intermolecular interactions between OA and OLA molecules in this molar ratio, the monolayer stability increases. The miscibility gaps between the binary monolayer components are due to the OA exclusion from the interface, leading to phase separation.

CHAPTER 5. Future application fields of the Langmuir methodology

Lipid monomolecular structures have been under the interest of scientists for over a hundred years. However, in 2004, McCullough and Regen [84] indicated that despite notable efforts at exploiting the Langmuir-Blodgett technique, some unresolved difficulties (low stability and poor quality) hampered lipid film applicability on a larger scale. The LB method was successively losing importance in the fabrication of monolayers and multilayer structures on a solid substrate in favor of the self-assembly techniques due to their greater robustness and ease of preparation [84]. Although the self-assembled monolayers (SAM) and preparation of functional thin films via layer-by-layer (LbL) assembly are highly versatile, the LB films should not be neglected [6].

Recently we witnessed a significant breakthrough in surface science driven by the development of microscopic techniques and characterization tools. Moreover, lipid monolayers created via the Langmuir technique to mimic biological membranes successfully proved the convergence of the obtained data with the results of studies on actual cell membranes. Despite the dynamic progress of other model studies, the single-layer lipid models remained meaningful and indispensable in interfacial science, providing basic information about the structure, mechanical properties of membranes, and phase transitions [53]. Since more potential fields of application in terms of interfacial science and technology exist, the fact that the Langmuir-Blodgett technique is a well-developed and straightforward methodology for molecular film formation should be its advantage [6].

Currently, besides acting as model biological membranes and mimicking existing systems, numerous fields of application of the Langmuir technique rely on films transferred onto solid substrates. Furthermore, nanoscience and nanotechnology are rapidly developing in material science and technology, creating a need for observation and understanding of nanoscale systems and phenomena. Since the significance of surfaces is well recognized (Chapter 1.1 and Figure 1), the progress of basic sciences actively contributes to completing the applications.

Films of nontypical amphiphilic structure

Although most of the studies in Langmuir and Langmuir-Blodgett were performed with typical amphiphilic molecules, the current applications require properties that cannot be obtained by small amphiphiles or molecules incorporated into the films. Thus, LB films of small amphiphilic molecules are often unattractive for novel application fields due to their lack of mechanical resistance and insufficient electrical and optical properties. To address this issue, the LB technique has been extended to the nontypical amphiphiles, including polymers, carbon nanotubes, graphene, carbon-based particles, silica, or inorganic and organic-inorganic hybrid materials, like macrocyclic molecules, dendrimers, black phosphorus, magnetic soft spheres, clay nanosheets, ionic liquids, gold nanoparticles, nanoparticles/quantum dots, and inorganic nanowires. However, for nontypical amphiphiles, several fundamental obstacles have to be overcome when preparing Langmuir monolayers. The most important of them concerns the spreading ability, which may be limited in the case of nontypical amphiphiles, and the difficulties with dissolution in most organic solvents suitable for the Langmuir methodology. Moreover, some amphiphiles of complex architecture form 3D structures as an effect of the aggregation driven by cohesive forces. Achieving a uniform deposition and a high transfer ratio is challenging as well. The deposition of nontypical amphiphilic onto solid substrates has been used since the 1980s to produce stable films used in devices and sensors [14].

Recently, forming of LB films with nontypical amphiphiles strives to the development of nanomaterials, such as metal–organic frameworks (MOFs), black phosphorus nanosheets (BPNS), metallic nanoparticles, covalent organic frameworks (COFs), ZnO nanowires, carbon nanotubes and LB films with other carbon-based nanomaterials (such as graphene and fullerenes), which potentially may be used in solar cells, touch pads, light-emitting diodes, and sensors [6,14].

Nanoarchitectonics

The concept of *nanoarchitectonics* emerged as the combination of nanotechnology with other fields of science, such as organic chemistry, supramolecular chemistry, material science, nano- and micro-fabrication, and bio-related technology.

According to the nanoarchitectonics strategy, in the functional materials design, there are various processes combined, like atomic/molecular manipulation, self-assembly/organization, nano/micro-fabrication, chemical conversion, and bio-related treatments to meet the requirements of novel materials production, sensing, catalysis or biomedical applications. The Langmuir-Blodgett technique is a promising and powerful tool in the nanoarchitectonics strategy due to the controllable molecular organization within a 2D array [6,14].

Since sensing and biosensing phenomena involve interface effects, the surface functionalization of nanomaterials is highly relevant. Thus, the LB films are widely exploited in various categories of sensors, including chemical sensors in liquid samples (such as electronic tongues), gas sensors (such as electronic noses), and flexible/wearable sensors (such as human respiration sensors, strain sensors, and electronic skin). The molecular architecture of LB films is adapted to preserve the enzymatic activity, which is utilized in biosensors. The enzyme-containing film (pure or mixed with lipid components) interacts specifically with the analytes present in a sample. The enzyme-based biosensors tailored with the LB technique usually contain lipid mono- or multi-layers, serving as a beneficial matrix to enhance enzyme activity [14].

Improvements and modifications of the technique

The competition of various methods of producing thin films and nanostructures forces the LB technique for continuous development, not only in terms of the instrument but also in the fields of application. Usually, the Langmuir technique experiments require conditions free from vibrations and disturbances, but a novel breakthrough approach has been proposed recently to associate the LB technique with vortexing (the vortex LB method) [14,30]. That methodology was successfully used to produce the carbon nanofilm from the nanoring molecules. A uniform assembly of the nanoring molecules was achieved by spreading them on water that was then stirred. Next, that structure was transferred onto a solid substrate and subjected to carbonization, which significantly expands the scope of the application.

Furthermore, the Langmuir technique methodology is susceptible to elevated temperatures, and it is generally unfavorable to conduct experiments at a temperature exceeding 40^{o}C due to the inevitable interference by the evaporating aqueous subphase. That limitation was overcome by the utilization of ethylene glycol (which is of a broad liquid temperature range) as a subphase instead of water. The LB experiments performed at unusually high temperature at non-aqueous subphase is called the high-temp LB. That is how the Langmuir technique was successfully applied at a temperature close to 100^{o}C to obtain uniform films of a highly oriented polymer and transfer it via LS approach onto a solid substrate.

Molecular machines

The LB technique can act as a link between macro-scale phenomena and molecular interactions. Since the air-water interface is a dynamic environment, a molecular organization can be controlled via mechanic actions at the macro scale. The monolayer may undergo visible size alterations associated with changes in a molecular organization. Therefore, the Langmuir monolayers can operate molecular machines by mechanical actions done at the macro-scale. Recently, the Langmuir methodology has been harnessed to manually control the system of steroid cyclophanes composed of the ring structure and steroidal moieties connected via flexible spacers. Thanks to this specific interfacial structure, when the pressure is applied by the monolayer compression, a 3D cavity is formed. As an effect of the structural transformation, some guest molecules present in the aqueous subphase can be captured or released due to the monolayer expansion. Consequently, moving the barrier by tens of centimeters at the macro scale results in capturing and releasing molecules at the nano-scale [14,30]. The concept of controlling the molecular machines by macroscopic actions can find application in many fields, such as control of fluorescence resonance energy transfer, manipulating of molecular pliers, rotation of molecular rotors, flapping of molecular wings, and nano-cars deformations [14].

Control of cell development

Since the tailored nanostructures are able to regulate living cell behavior, the LB technique can be used to achieve this goal as well. The aligned arrays of fullerene

whisker structures from one-dimensional C_{60} assembly acted as functional scaffolds for cultures of the human osteoblast cell lines in human stem-cell-based therapies. Cells were bound preferentially to the nanowhiskers, which led to cell growth along with the fullerene array. A large-area scaffold of C_{60} nanowhiskers formed using the LB approach could modulate cell−extracellular matrix interactions and control cell differentiation and proliferation, and thus are expected to contribute to tissue engineering development [14].

Since the air-water interface environment is of high surface tension in nature, it is unfavorable for biomacromolecules such as proteins (due to the denaturation induced and changes in secondary structure), as well as for living cells. Therefore, the liquid-liquid system was employed instead of the air-water interface to develop a culture of living cells and transfer it onto a solid substrate via the LB approach. The perfluorocarbon solvent was applied to create an interface with water because usual organic solvents destroy cellular membranes. The liquid-liquid interface of perfluorocarbon and water acts as an ultimately mechanobiologically-adaptable and soft medium. The LB films demonstrate the mechanical strength required to become a novel culture substrate [30].

The role of the Langmuir and Langmuir-Blodgett methodology is not only limited to fabrications of ordered thin films of nanoscale thickness and defined layer sequences. The purpose of this dissertation and other relative publications is to make the scientific world aware that the air–water interface act as a playground for the LB technique working as a medium for solving fundamental scientific issues. Moreover, the development of novel experimental methods or improvements in addition to already existing ones is essential to increase the scientific relevance of the Langmuir methodology. It has been stressed that interdisciplinarity is a key to understanding basic phenomena, and experiments or reconstructions might be performed *in vitro*, as well as *in silico,* using computer simulations [58]. The employing of molecular dynamics simulations provides detailed information on interactions at a molecular level, complementary to the Langmuir technique experiments [14]. Thus, development in computational modeling shifts the Langmuir technology to more advanced levels if

combined with molecular dynamics simulations. Numerous examples of novel applications for the Langmuir and Langmuir-Blodgett techniques presented in this chapter and described in the recent literature (Figure 15) prove that experiments in this methodology are needed and extensively exploited. However, the future applications and the Langmuir technique's utility still depend on the scientists' imagination and out-of-the-box thinking [30,53].

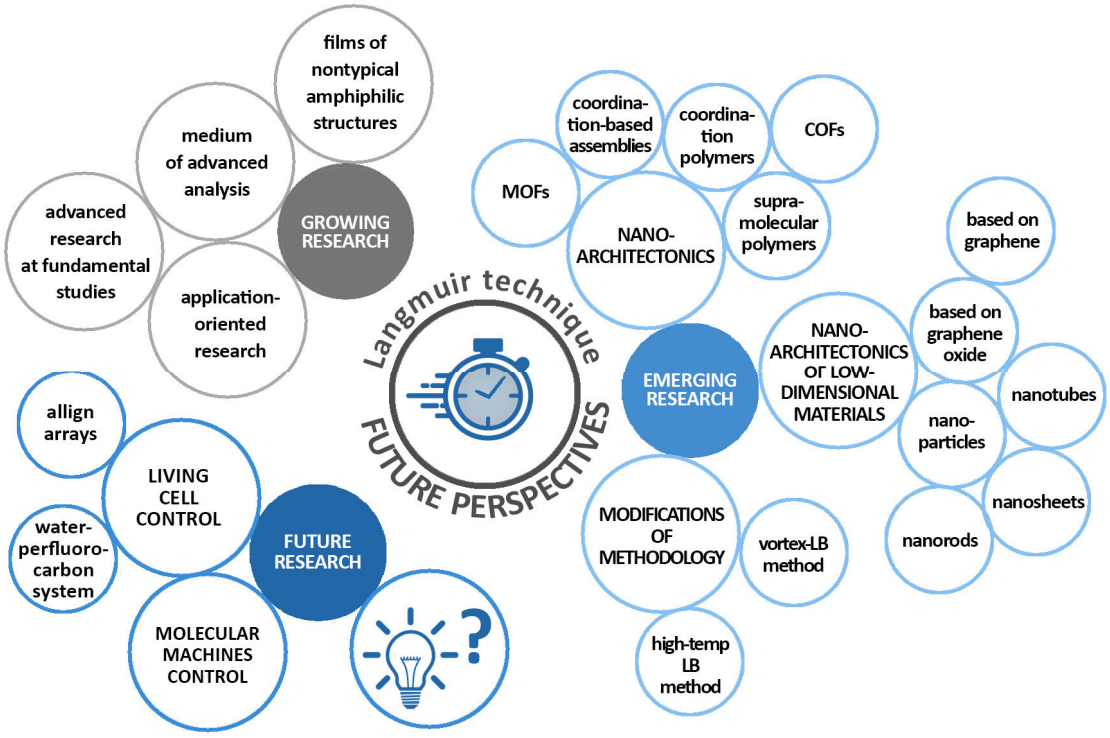

Figure 15. The future perspectives of the Langmuir technique divided into growing, emerging and future research; based on [6,14,30]

Symbols and abbreviations

A_{12}	mean molecular area in two-component monolayer
A/A_0	relative area per molecule
AFM	atomic force microscopy
α-LA	α-lactalbumin
$A_{lift\text{-}off}$	molecular area at which the π of a compressed monolayer increase >0 mN/m
A_{lim}	minimal area occupied by molecule within a monolayer
API	active pharmaceutical ingredient
BAM	Brewster angle microscopy
BAMLET	bovine α-Lactalbumin Made LEthal to Tumor cells
BLA I	bovine α-lactalbumin in *holo* form (calcium loaded)
BLA III	bovine α-lactalbumin in *apo* form (calcium depleted)
BPNS	black phosphorus nanosheets
BSA	bovine serum albumin
CA	contact angle
CAMLET	camel α-Lactalbumin Made LEthal to Tumor cells
COFs	covalent organic frameworks
Cs^{-1}	compression modulus
ΔG^{exc}	excess free energy of mixing
ELOA	equine lysozyme with oleic acid complex
G state	gaseous state
GAMLET	goat α-Lactalbumin Made LEthal to Tumor cells
HAMLET	human α-Lactalbumin Made LEthal to Tumor cells
HSA	human serum albumin
IEP	isoelectric point
LA	linoleic acid
LB	Langmuir-Blodgett
LbL	layer-by-layer
LC state	liquid condensed state
LE state	liquid expanded state
LE-LC state	liquid expanded-liquid condensed intermediate state
LS	Langmuir- Schaefer
MIP	maximum insertion pressure
MOFs	metal−organic frameworks
MUFAs	monounsaturated fatty acids
OA	oleic acid
OLA	oleanolic acid
π-A isotherms	surface pressure – area per molecule isotherms
π_{coll}	surface pressure during the monolayer collapse
$\pi_{Cs\text{-}1max}$	surface pressure at maximum compression modulus
π_e	equilibrium surface pressure
π_i	initial surface pressure
π/π_0	relative surface pressure
PM-IRRAS	Polarization Modulation Infrared Reflection Absorption Spectrometer
PUFAs	polyunsaturated fatty acids
S state	solid state
SA	stearic acid
SAM	self-assembled monolayer
SFE	surface free energy
X_{OLA}	two-component OLA-OA monolayer composition

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DOI: 10.1021/acs.langmuir.8b04153

P2. Martyna Krajewska, Katarzyna Dopierała, **Krystyna Prochaska**, Lipid–protein interactions in Langmuir monolayers under dynamically varied conditions, The Journal of Physical Chemistry B - 2020, vol. 124, iss. 1, p. 302-311

DOI: 10.1021/acs.jpcb.9b10351

P3. Katarzyna Dopierała, Martyna Krajewska, **Krystyna Prochaska**, Study on pH-dependent interactions of linoleic acid with α-lactalbumin, Food Hydrocolloids - 2021, vol. 111, p. 106217-1-106217-9

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P4. Martyna Krajewska, Katarzyna Dopierała, Paweł Wydro, Marcin Broniatowski, **Krystyna Prochaska**, Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study, Journal of Molecular Liquids - 2020, vol. 319, p. 114089-1-114089-9

DOI: 10.1016/j.molliq.2020.114089

P6. Martyna Krajewska, Katarzyna Dopierała, **Krystyna Prochaska**, The biomimetic system of oleanolic acid and oleic acid at the air-water interface–interactions in terms of nanotechnology-based drug delivery systems, Membranes - 2022, vol. 12, iss. 12, p. 1215-1-1215-15

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P4. Martyna Krajewska, **Katarzyna Dopierała**, Paweł Wydro, Marcin Broniatowski, Krystyna Prochaska, Interfacial complex of α-lactalbumin with oleic acid: effect of protein concentration and PM-IRRAS study, Journal of Molecular Liquids - 2020, vol. 319, p. 114089-1-114089-9

DOI: 10.1016/j.molliq.2020.114089

P5. Katarzyna Dopierała, Martyna Krajewska, Marek Weiss, Physicochemical characterization of oleanolic acid–human serum albumin complexes for pharmaceutical and biosensing applications, Langmuir - 2020, vol. 36, iss. 13, p. 3611-3623

DOI: 10.1021/acs.langmuir.0c00087

P6. Martyna Krajewska, **Katarzyna Dopierała**, Krystyna Prochaska, The biomimetic system of oleanolic acid and oleic acid at the air-water interface–interactions in terms of nanotechnology-based drug delivery systems, Membranes - 2022, vol. 12, iss. 12, p. 1215-1-1215-15

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PUBLICATIONS CHOSEN FOR THE BASIS OF THE Ph.D PROCEDURE