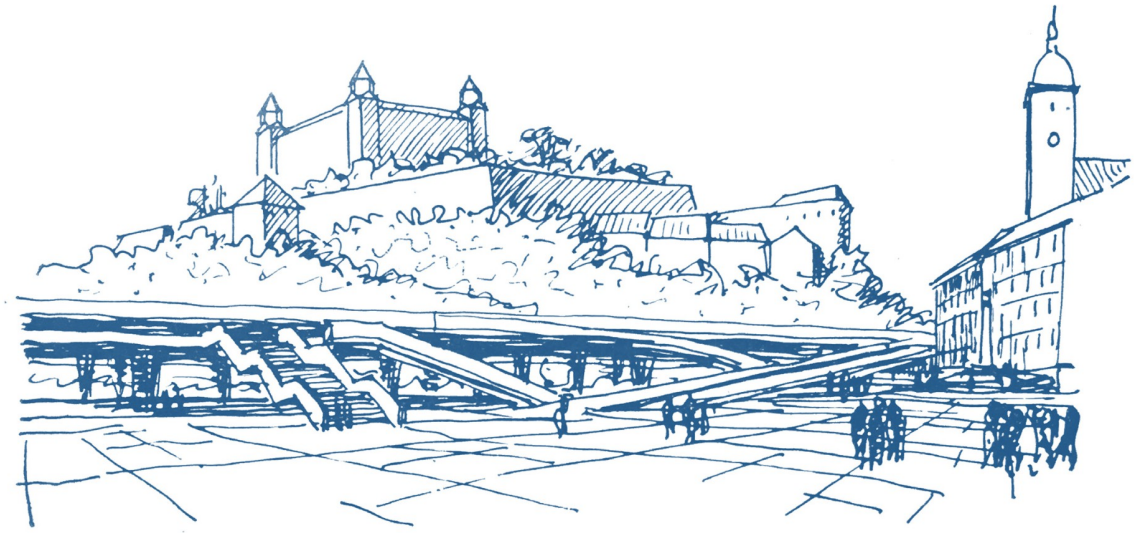
Maria Sklodowska Curie Actions - Research and Innovation
Staff Exchange

H2020-MSCA-RISE-2015 - FORMILK

International Workshop

Novel approaches for detection proteases in milk

Program and Abstracts



Bratislava, February 12, 2017

Picture at the cover page: View on the Bratislava castle and St. Martin Cathedral.
Author: prof. Emanuel Hruška

International workshop

Novel approaches for detection proteases in milk

Bratislava, Slovakia
February 12, 2017

Program and Abstracts

Organized by Faculty of Mathematics, Physics and Informatics, Comenius University in Bratislava
http://www.fmph.uniba.sk

in framework of

Marie Sklodowska-Curie Actions (MSCA)
Research and Innovation Staff Exchange (RISE)
H2020-MSCA-RISE-2015
FORMILK, Project No. 690898
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The goal of the workshop is to provide a venue for advancing characterization of the proteases activity in milk using various physical and analytical chemistry methods through a series of lectures and discussions. The workshop brings together experts in milk analysis, fundamental and applied aspects of acoustic and electrochemical methods. Workshop is organized in framework of the project FORMILK funded by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 690898.

ORGANIZING COMMITTEE

Tibor Hianik - chairman
Zuzana Garaiová
Maria Hianiková
Alexandra Poturnayová
Veronika Šubjaková
Marek Tatarko

PROGRAM

12:30-14:00 **Registration and coffee**

14:00-14:05 Welcome and Introduction (T. Hianik)

14:05-14:35 Curent analytical methods of milk analysis (A. Hucker, HDRI Mosonmagyaróvár, Hungary)

14:35-15:05 Milk microbiology and its importance for milk industry (A. Hucker, HDRI, Mosonmagyaróvár, Hungary)

15:05-15:35 Plasmin protease and importance of this detection for milk industry (Z. Keresztes, RCNS, Budapest, Hngary)

15:35-16:30 Coffee Break and Posters

16:30-17:00 Ultrasonic monitoring of biocatalysis in solutions and complex dispersions (V. Buckin, UCD Dublin, Ireland)

17:00-17:30 Development and testing of different acoustic biosensors for proteolytic activity detection on immobilised casein layer (M. Tatarko, Comenius University, Bratislava, Slovakia and I. Ivanov, CNMS, Oak Ridge, USA)

17:30-18:00 Casein assembly for gravimetric and optical detection of protease activity (V. Šubjakova, Comenius University, Bratislava, Slovakia and I. Ivanov, CNMS Oak Ridge, USA)

18:00-18:20 Progress on development of a portable potentiostat and related activities (M. Jagelka, Powertec, Ltd, Bratislava, Slovakia, Slovakia)

18:20-18:30 Concluding remarks

18:30 - 20:00 **Dinner**

Abstracts of oral presentations

O1
Current analytical methods of milk analysis

Katalin Szabó, Attila Hucker, Réka Sarok, Gábor Szafner, Gábor Császár, Róbert Kocsis

Hungarian Dairy Research Institute, H-9200 Mosonmagyaróvár, Lucsony u.24., Hungary.

Milk and milk products are widely consumed foods that are essential for the diet of several millions of people worldwide because they provide important macro- and micronutrients. Accurate food compositional analysis has been a key factor in helping the food industry to improve and standardize both processes and products.

In dairy products protein and fat are two of most important compositional elements that must be determined. Nowadays, lactose-free and low-lactose products in a functional foods markets are occupying to satisfy the needs of consumers with lactose intolerance. Laboratories have depended on a wide range of chemical analysis techniques to measure the level of these components that usually describe several International Standards. In addition, the dairy industry expects some different analytical methods to determine several parameters of milk and milk products, namely: density, freezing point, the total solids, total mineral, pH value, titratable acidity, aflatoxin M1 content. Many of these reference methods are time-, chemical-consuming (also dangerous), and required well-equipped laboratory and trained personnel, too. More recently, a number of rapid and accurate instrumental methods have been gradually introduced in dairy industry, which many advantages have compared to older techniques. The proper selection of analysis method requires a wide professional practice and knowledge.

O2
Milk microbiology and its importance for milk industry

Attila Hucker, János Nagy, Zsuzsanna Steinerné Smajda, Zoltán Kerényi, Judit Süle, Gábor Császár, Róbert Kocsis

Hungarian Dairy Research Institute, H-9200 Mosonmagyaróvár, Lucsony u.24., Hungary.

The hygienic condition of milk is important for the knowledge of the composition of milk microbiology. The presentation summarizes the key of the most important microbiological characteristics for the dairy industry, and their test methods and the aspects of the results' evaluation. Determining the basic direction of microbiological properties is primarily governed by regulations. In addition, customers' expectations, needs or product development have generated the tests.

Milk is an excellent medium for desired and undesired microorganism's growth. To reach the appropriate quality of products for dairy industry, a large amount of testing is needed. These analyses can change in a wide range starting from micro-organisms count (total aerobic count) of raw milk, through the finished product pathogen-free (*Listeria monocytogenes*, *Salmonella* spp.) status, the corresponding indicator organism numbers (Yeasts, Moulds, *Enterobaceriaceae* spp., Coliform bacteria, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, Sulfite-reducing *Clostridium* spp.) and also to the detection of culture origin microorganisms in fermented products (*Lactobacillus* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Lactococcus* spp.). The proper selection of analysis method and accurate evaluation of the test results require a wide professional practice and knowledge.

O3

Plasmin protease and importance of this detection for milk industry

Zsófia Keresztes

Research Centre for Natural Sciences, Hungarian Academy of Sciences, Hungary.

Protein degrading enzymes in raw milk can be divided into groups of enzymes with endogenous or exogenous origin, i.e. blood originated enzymes or extracellular protease enzymes related to bacterial contamination. The detection of these enzymes is important in respect of milk product development, shelf-life and health effects.

Plasmin enzyme is the most important among milk endogenous enzymes. In the blood it is responsible for the hydrolytic process to degrade several blood proteins, such as to dissolve fibrin blood clots.

Plasmin is filtered in raw milk as a complex enzyme system, containing the enzyme, its precursor and several inhibitors. The enzyme activation can proceed due to endogenous blood or tissue originated activators or bacterial cofactors. Post-harvest plasmin activation can take place as a result of dairy technological processes, such as different heat-treatment methods, altering the balance of the enzyme and inhibitor system.

Once present in milk, plasmin can hydrolyse casein, the major milk protein. The adverse effect of the slow enzymatic hydrolysis is more emphasised in long-shelf life dairy products. The enzymatic degradation products can manifest unwanted sensory properties, off-flavour or bitterness. The altered gelation processes can influence curd properties, thus the result of cheese ripening, such as cheese stretchability, melting ability.

For the above reasons, plasmin activity detection in post-harvest state and at line process analysis can have significance in quality assurance of several dairy products.

This work was financially supported by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No. 690898

O4

Ultrasonic monitoring of biocatalysis in solutions and complex dispersions

Vitaly Buckin

School of Chemistry, University College Dublin, Belfield, Dublin 4, Ireland

Being efficient and environmentally friendly catalysts of molecular transformations, enzymes are employed in a broad range of industrial sectors. The rapidly growing field of enzyme-based technologies is dependent on analytical tools available for non-destructive real-time assessment of various aspects of enzyme performance in complex media such as emulsions, suspensions, and gels, where most of the traditional analytical techniques are limited in their applicability or have high analytical cost. This presentation describes the fundamentals of application of high-resolution ultrasonic spectroscopy (HR-US) for real-time non-destructive monitoring of molecular transformations catalyzed by enzymes and for comprehensive analysis of performance of enzymes in complex formulations. The discussed examples of application of this technique focus on hydrolytic reactions (oligosaccharides and proteins) in aqueous solutions and milks.

05
Development and testing of different acoustic biosensors for proteolytic activity detection on immobilised casein layer

Marek Tatarko[1], Michael Thompson[2], Ilia N. Ivanov[3], Tibor Hianik[1]

[1] Faculty of Mathematics, Physics and Informatics, Department of Nuclear Physics and Biophysics, Comenius University in Bratislava, Slovakia.
[2] Department of Chemistry, Lash Miller Laboratories, University of Toronto, Canada.
[3] Center for Nanophase Materials Sciences, Oak Ridge National Laboratory, USA.

In the recent years there is a demand for dairy products of outstanding quality with proper flavour and texture. This demand is fulfilled by sufficient control of the proteolysis in the milk, especially degradation of the micelle-like particles composed of functionally unique casein types: alpha, beta and kappa casein [1]. Degradation is predominantly caused by activity of plasmin, protease present in bovine milk. Our research concentrated on immobilisation and self-assembly of the these molecules into casein layer and its reaction on changed conditions or addition of the protease. This behaviour was monitored by different acoustic sensors based on QCM (quartz-crystal microbalance) method. EMPAS (Electromagnetic piezoelectric acoustic sensor) [2], QCM utilizing electromagnetic field of the coil, was used to improve limit of detection for the proteolysis. Using extremely thin quartz discs, β-casein was deposited on immobilised amino-active layers and it was monitored as addition of protease was cleaving it. Different immobilisation technique was developed and tested by using QCM-D (quartz-crystal microbalance with dissipation), method registering fundamental and multiple higher harmonic frequencies [3]. Direct deposition of all casein types on hydrophilic SiO_2 surface [4] proved their different properties. κ-casein created dense layers, while layer composed of α-casein was mostly washed away. These layers were further tested for their stability by wash of solution with different pH or by their resistance against proteolytic activity of trypsin and plasmin [5]. Acquired results helped us to better understand behaviour of differently immobilised layers and to compare results from different QCM biosensors.

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06
Casein assembly for gravimetric and optical detection of protease activity

Marek Tatarko[1], Veronika Šubjaková[1], Eric S. Muckley[2], Tibor Hianik[1], Ilia N. Ivanov[2]

[1]Faculty of Mathematics, Physics and Informatics, Department of Nuclear Physics and Biophysics, Comenius University in Bratislava, Slovakia
[2]Center for Nanophase Materials Sciences, Oak Ridge National Laboratory, USA.

We investigated gravimetric and optical approaches to detection of protease activity. First approach requires on understanding of dynamics of assembly of milk proteins and stability of their layers [1]. We used 5MHz quartz crystal microbalance with the dissipation mode (QCM-D) [2]. The kinetics of protein assembly and removal of casein on hydrophilic SiO_2 were monitored by following changes in value of fundamental frequency, 3^d, 5^{th} 7^{th} and 11^{th} harmonics at 5, 15, 35 and 55 MHz respectively. Stabilization of fundamental frequency signalling the completion of the casein self-assembly was used as a marker for switching to the second step of procedure, to remove loosely held casein molecules/micelles. In final sequence we investigated stability of protein layer by following changes of fundamental and overtones for different pH-stabilized buffer solutions. In the first part of the presentation we will discuss the results of these experiments and compare them with the protease-based removal of protein.

In the second part of the presentation we will discuss optical protease activity sensor Raman using a substrate coated with casein]. Surface-enhanced Raman scattering (SERS) is a powerful technique where the Raman signal of the probe molecule is amplified through the excitation of localized surface plasmon resonances of the substrate. Localized plasmons are realized using gold nanoclusters, and by tuning their dimensionality using high surface area nonstoichiometric Si_xO_y. SERS could allow detection of analyte on sub-nanomolar level. We will discuss the effect of excitation wavelength on the efficiency of SERS substrates and concept of non-resonant Raman detection of protease activity based on β-casein film.

Acknowledgement: This research was conducted through user proposal CNMS2016-031 at the Center for Nanophase Materials Sciences through, which is a DOE Office of Science User Facility. MT, TH acknowledge support from European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 690898.

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[2] M.C. Dixon, *J. of Biomolecular Techniques*, 2008, **19(3)**, 151-158.

07
Progress on development of a portable potentiostat and related activities

Martin Jagelka[1,2], Peter Juhász[2], Peter Šimek[1], Martin Weis[2], Martin Donoval[1]

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In recent decade there are new researches based on plasmin detection in milk, which can clarify the impact of different methods of breeding cows [1]. Several methods of measuring this plasmin activity exist [1-3], but all of that are using rather large, expensive and not a user friendly equipments. The objective however is to provide these methods to ordinary day at farms. In this paper a potential design of user friendly potentiostat is described. The core of this potentiostat is the LMP91000 analogue front end (Texas Instruments) for low power chemical sensing applications. This component has integrated transimpedance amplifier with programable gain which lead to achieve down to 5 µA full scale current measuring range. To the reference pin an external voltage from digital-to-analog converter (DAC) of any waveform can be applied. Current response is provided to the output pin which can be connected to analog-to-digital converter (ADC) for data logging and analysing.

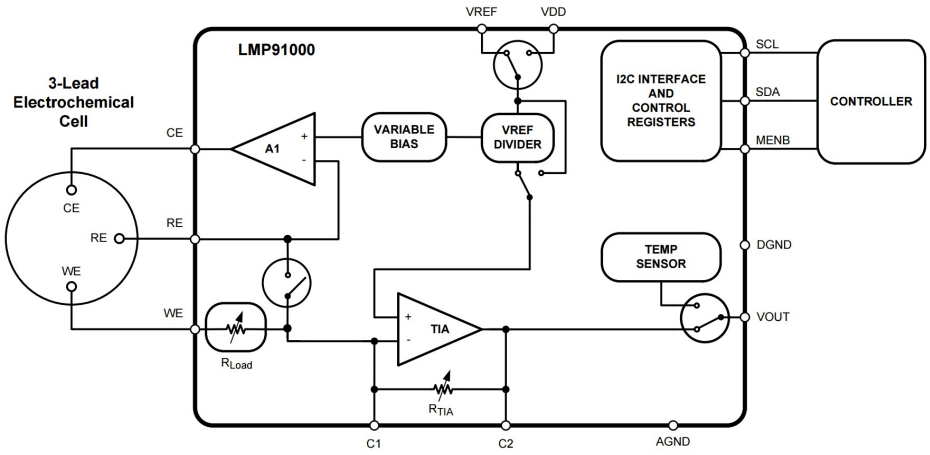

Fig. 1: Block diagram of LMP91000, the core of portable potentiostat.

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Abstracts of poster presentations

P1
Detection of chymotrypsin activity using QCM method

Ivan Piovarči[1], Alexandra Poturnayová[1,2], János Nagy[3], Réka Sarok[3], Attila Hucker[3], Katalin Szabó[3], Róbert Kocsis[3], Tibor Hianik[1]

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Chymotrypsin is a serine protease acting as a digestive enzyme. It is synthesized in pancreas and its purpose is to breakdown milk proteins. The composition of milk proteins consists of several types, but are mainly divided to casein and whey family of proteins. Casein proteins make up about 80% of bovine milk proteins and consist mostly of α-s1, α-s2, β and κ-casein. In natural milk the casein is in micellar form. The micelles have porous structure and water flows in and out of them. The surface of the β-casein micelles is terminated by κ-casein. Degradation of casein is of great importance in milk industry because it is responsible for clotting of the milk or ripening of cheese. In order to study this process, we used in vitro model of β-casein micelles bound to a gold surface of quartz crystal of fundamental frequency 8 MHz. For this purpose we modified the gold with self-assembled monolayer (SAM) of mercaptoundecanoic acid (MUA). Then we activated the surface using carbodiimide chemistry. Quartz crystal microbalance (QCM) has been used to monitor the changes in resonant frequency following changes in the mass at the surface of quartz crystal transducer. The binding of β-casein to the MUA layer resulted in decrease of the frequency. Introduction of chymotrypsin to the surface of casein layer caused an in increase of resonant frequency. This is due to the cleavage of casein and removal its fragments in a flow system. This method allowing study also other proteases (plasmin or trypsin), various types of casein (κ-casein or micellar casein), or change of medium (different salt composition or different pH). We studied cleavage of β-casein by chymotrypsin at pH 7,4 and 8,3. Chymotrypsin was detected in the range of concentrations 1 pM – 20 nM. Limit of detection was rather low: 1 pM. At this concentration, approx. 6 % of casein was cleaved (this corresponds to frequency increase by approx. 9 Hz). Topography of casein layers and its changes following chymotrypsin cleavage was studied also by atomic force microscopy.

Acknowledgments: This work was financially supported by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 690898.

P2
High sensitive electrochemical aptasensor for detection aflatoxin M1 in milk

Stefanos Karapetis[1,2], Marianna Eliášová Sohová[1], Lenka Bábelová[3], Alexandra Poturnayova[1,3], Attila Hucker[4], Katalin Szabó[4], Robert Kocsis[4], Dimitrios P. Nikolelis[5], Tibor Hianik[2]

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Mycotoxins are toxic fungal metabolites that under specific conditions are associated with diseases or mouldy crops [1]. Among mycotoxins the most relevant group are aflatoxins. There have been identified four types of aflatoxins: AFB1, AFB2, AFG1, AFG2, plus two additional metabolites: AFM1 and AFM2, being AFB1 classified as the most abundant and carcinogenic. The presence of AFB1 in feed and its exposure of lactating animals lead to the contamination of milk by its hydroxylated metabolite AFM1 [2]. Because milk is the main nutrient for children, the presence of AFM1 in milk is considered as one of the most serious problems of food safety. Therefore the development of rapid and sensitive method of detection AFM1 is among priorities in the milk analysis. Currently the AFM1 is detected only in specialized laboratories using HPLC or ELISA methods. However, for widespreading of the milk analysis also in small farms the development of less expensive and friendly to use system is among priorities. The biosensor technology based on specific receptors - DNA aptamers can be one of the option. In this work we report simple impedimetric aptasensor for detection AFM1. The DNA aptamers specific to AFM1 have been immobilized at the surface of poly (amidoamine) dendrimers (PAMAM) of four generation covalently attached to a gold surface. Impedance spectroscopy at presence of 5 mM redox indicator $K[Fe(CN)_6]^{-3/-4}$ allowed detection of charge transfer resistance which increased following addition of AFM1. This method has been successfully used for detection of AFB1 in or recent work [3]. The biosensor allowed detection of AFM1 with high sensitivity of approx. 15 ng/L, while the maximum permitted level in milk in EU is 50 ng/L. The sensitivity of aptasensor has been compared with traditional ELISA test.

Acknowledgments: This work was financially supported by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 690898.

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P3
Enzymatic degradation of lipid-embedded β-casein in two dimensions: Langmuir monolayer studies

Loránd Románszki[1], Zsófia Keresztes[1], Sofia Melikishvili[2], Natália Ružičková[2], Tibor Hianik[2]

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The *controlled* enzymatic degradation of caseins is an important factor in the dairy industry, as the reaction products contribute to the texture and flavour development of the product [1]. However, uncontrollable and unpredictable degradation is undesired. For the quality control of the milk is therefore important to gain more knowledge on these reactions. One of the interesting questions is how milk fat, associated with the proteins, influences the enzymatic degradation [2]. Here we present the concept and first results of a model experimental system for the study of lipid influence on the enzymatic degradation of milk proteins, consisting of β-casein as milk protein, trypsin as model enzyme and a Langmuir monolayer of dipalmitoylphosphatidylcholine (DPPC) as model lipid (Fig. 1). For the study of lipid–protein interaction, two different approaches have been devised, both based on monitoring the surface pressure changes induced by the penetration of protein either 1) injected into the aqueous subphase (i.e., Gibbs type, equilibrium adsorption) or 2) spread over the DPPC superphase (i.e., Langmuir type, non-equilibrium adsorption), at different DPPC packing densities. The enzymatic degradation was studied in this second configuration, by spreading the protein *over*, and injecting the trypsin *beneath* the DPPC monolayer. The potential of this approach for milk protease sensor development is also discussed.

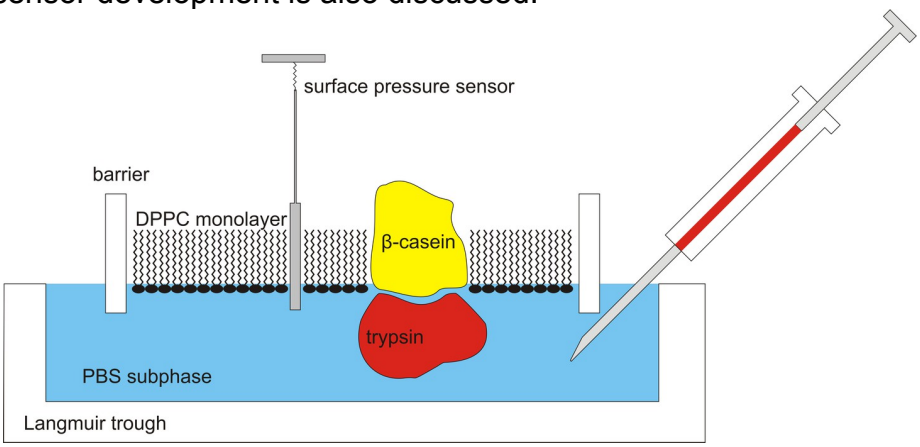

Fig. 1: Model system to study β-casein proteolysis under the influence of lipids

This work was financially supported by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No. 690898

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P4
High resolution ultrasonic spectroscopy in the use of sensor development for monitoring enzyme activity

Tamás Szabó[1], Breda O'Driscoll[2], Vitaly Buckin[2], Nikoletta Molnár[1], Zsófia Keresztes[1]

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The role of RCNS (of HAS, Hungary) group in FORMILK partnership is based on the research of electrochemical characteristics of micro and nanoelectrodes with selective 2D layers on top, that would be sensitized for enzyme-catalysed reactions with optical or electroactive mediators (e.g. ferrocen). Besides, application of electrochemical and optical mediators in the bulk sample phase is also in the focus area. High resolution ultrasound spectroscopy (HRUS) was tested on our next two concept models in order to discover its relevance.

Bulk-phase solidification and structure of further hydrogel coatings, that are intended to use as masking or sensitized layers on electrodes, was monitored by HRUS. The gel forming model solutions were composed of gelatin (1,2 and 5% w/w; 30°C), all able to solidify at room temperature (22°C). In this case, calibration was possible for the different concentrations. Further blending of gel backbone with mediator can be eased by the addition of carrier molecules (β-cyclodextrins, β-CDs) that are good hosts for hydrophobic agents. Thus gelling of gelatin solution in itself (5% w/w) and with β-CD (staurated) was also compared. The phase transitions were clearly visible, with difference in case of added CD.

Gold nanoparticle (GNP) sols have unique colour which is strongly related to the size of GNPs. Though, aggregation may occur in lack of stabilizers, implying a red shift in the absorbance. GNP sols, prepared *via* microwave irradiation (0, 20, 40, 110 and 150 W) in the presence of casein molecules, were proven to be stable, but it was shown optically that in the presence of plasmine (in phosphate buffered saline) the stability weakens and GNP aggregation starts. This is an important information about enzyme activity, but only extractable from transparent medium, therefore, in order to improve its expedience in more realistic, even food samples, HRUS method was applied to characterize GNP sols and to monitor the effect of plasmin added to GNP sol. Also, casein itself was exposed to plasmin. In both plasmin-treatments, enzyme activity was well-reported by the changing of differential relative velocity and attenuation values.

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P5
Buttermilk as carrier system for dietary supplement agents such as curcumin

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Curcuminoids are the major phenolic compounds that which are isolated from turmeric (*Curcuma longa*) and contain curcumin (the bioactive component of turmeric), demethoxycurcumin and bisdemethoxycurcumin. It can said, that the natural dye of curcumin is one of the strongest active ingredients in the curcuma root. Thanks to its impact on body health (antioxidant property; slows/kills the growth of tumour cells; lowers cholesterol level and reduces obesity; stimulates muscle regeneration after trauma; corrugate skin diseases; it is a healing herb for the liver, spleen, stomach, intestines, lungs and blood; can prevent and cure Alzheimer's disease and can reduce inflammatory reactions) it has been intensively investigated [1]. The low solubility of the curcuminoids in aqueous solutions, their susceptibility to degradation in aqueous environments and the low bioavailability compromise their usefulness as a bioactive ingredient in functional foods. Buttermilk is a by-product of dairy manufacture and contains milk proteins (caseins and whey proteins), milkfat and the milkfat globule membrane. The presence of proteins and lipids, including the phospholipids of the milkfat globule membrane in buttermilk, makes it an attractive carrier for curcuminoids: curcumin interacts with these proteins and lipids by moving into the hydrophobic groups on these molecules, so the interaction of curcumin with the components (proteins, lipids) in the buttermilk improved its solubility and stability. Centrifugation of buttermilk - curcuminoid mixtures demonstrated that curcuminoids were partitioned into the cream, milk serum and the casein-rich precipitate [2]. The ability of buttermilk to carry and stabilise curcuminoids has the potential to enable the delivery of these components into functional foods. In this study the potential of buttermilk as a "sovent" or carrier for curcuminoids (either 95% Alfa Aesar curcumin, or Thymos spice curcumin) was investigated using a HRUS 102 SS (High Resonance Ultrasonic Spectrometer) by measuring at 5°C the differential sound velocity and relative attenuation at different frequency ranges (frequency ranges between 2-10 MHz).

This work was financially supported by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 690898

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P6
Conduction mechanism of functionalized gold nanoparticle based electrochemical sensors

Nikoletta Molnár[1], Peter Juhasz[2], Tóthné-Csaba Ildikó[1], László Trif[1], Zsófia Keresztes[1], Martin Donoval[3], Martin Weis[2]

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Surface functionalized gold nanoparticles with 2-5 nm diameter were prepared. They have been stabilized by alkylthiols of various chain length (C6-C16). The hydrophobic gold nanoparticles are forming stable dispersion in organic solvents (Fig. 1). This gold dispersion was used for coating the surface of interdigitated electrodes. This electrodes can be used as electrochemical sensors.

For the determination of the electrical current conduction mechanism, current-voltage measurements were performed a Powertec in the 250 – 350 K temperature interval, by using a D8000 DLTS system (Fig. 2). Sub-ambient temperatures were achieved by liquid nitrogen cooling.

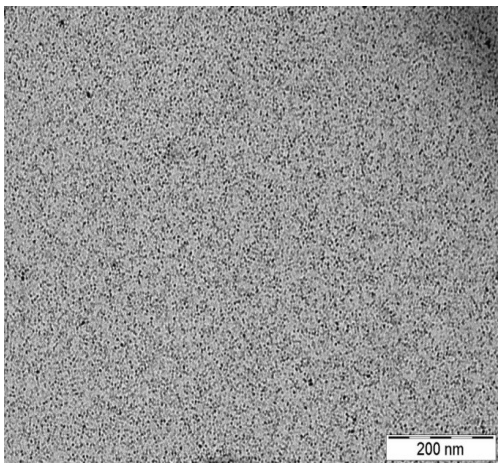

Fig. 1: TEM image of C6 functionalized gold nanoparticles

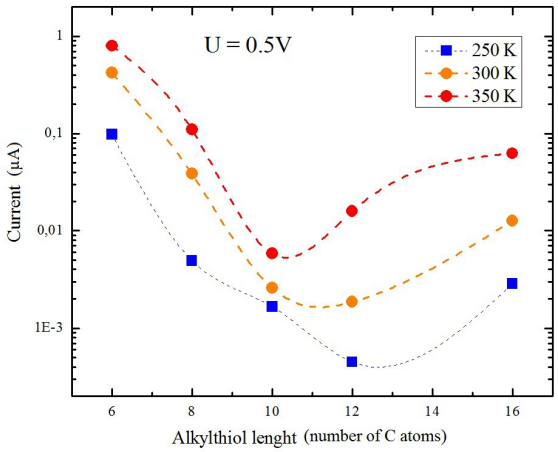

Fig. 2: Alkyl-chain length dependent conductance

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