Research Article

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Characteristics of liposomes derived from egg yolk

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Abstract: Liposomes are nanocapsules successfully applied in pharmacy and medicine. Their usage in the food industry could be increased by the development of alternative, cost-efficient lecithin materials. This work is a continuation of the previous two papers describing five different extractions of egg yolk lecithins and the pre-assessment of their usefulness for liposome formation. Physicochemical properties of extracts differed due to distinct composition. The aim of this research was to further characterise the extracts-based liposomes, especially in terms of nanomechanical properties and structural diversity. Five previously described extracts were used for liposomes preparation employing Bangham technique. Vesicles were analysed with the use of dynamic light scattering, flow cytometry, and atomic force microscopy. The results were tested for correlation with the composition of the extracts. It was proved that the chemical composition of the shell-forming material determined the size, structure, stability, and mechanical properties of the vesicles. The observed effects were found to result not only from differences in the content of major components, i.e. phospholipids, acylglycerols, and cholesterol, but also in the relative proportions. Minor constituents, i.e. tocopherols and carotenoids, were also found to be of significance. Strong correlations between size and Zeta potential of the vesicles with the content of carotenoids were determined.

Keywords: nanoparticle; nanomechanics; atomic force microscopy; flow cytometry; dynamic light scattering.

1 Introduction

Protection of bioactive compounds and drugs against environmental factors such as extreme temperature, pH, ionic strength, oxidation or enzymatic degradation is often necessary. Incorporation into lipid-based nanocarriers, such as liposomes [1], nanoemulsions [2], solid lipid nanoparticles, and nanostructured lipid carriers [3], is one of the means to improve stability of labile compounds. Moreover, nanocarriers exhibit greater solubility and surface area than microcarriers, and do not affect the sensory properties of a product significantly [4]. The importance of lipid-based carriers for both poorly soluble and permeable drugs is stressed and discussed in the literature [5].

Liposomes, bilayered phospholipid vesicles, are fully biocompatible, nontoxic, and suitable for protection of a wide range of compounds exhibiting both hydrophilic and lipophilic character. To date, many methods for encapsulation of enzymes [6], antimicrobials [7], essential oils [8], vitamins [9], antioxidants [10] into liposomes have been developed and described [11]. Nonetheless, liposome-based products are not popular on the market which is likely a result of problematic scaling up, multi-stage production, and the cost of pure phospholipids. However, promising techniques of industrial scale liposome preparation are under development, including the dense gas technique, membrane contactor, and single-stage heating method [12]. Moreover, pure phospholipids may be replaced by naturally derived lecithin extracts [13].

Complex nature of liposomes and plurality of factors influencing their properties resulted in a wide range of methods applied for vesicles assessment. Dynamic light scattering (DLS), Zeta potential measurement, and size exclusion chromatography are commonly applied for size analysis, stability determination, and encapsulation efficiency estimation respectively. Further, microscopy techniques, calorimetry, electron paramagnetic resonance spectroscopy, micro-turbidimetry, field-flow fractionation,

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^{31}P NMR, and small angle X-ray scattering are applied. Among microscopy techniques, atomic force microscopy (AFM) is recommended for imaging and characterization of fragile lipid-based particles. The resolution of AFM scanning is extremely high and samples can be scanned in physiological buffer solutions [14, 15]. Powerful tools of AFM allow the topographic and mechanical studies of biomembranes [16]. Recently, flow cytometry was proposed for liposomes evaluation as well. The technique, enable fast pre-comparison of populations of liposomes in terms of size and structure in their native environment. The key advantage of flow cytometry is analysis of vesicles in their native environment without severe manipulation of a sample [17]. The innovative application of both AFM and flow cytometry in the analysis of liposomes provides a valuable feedback on vesicles nanomechanics and whole populations diversity.

Hen egg yolk is a relatively cheap and widely available source of lecithin suitable for the production of food [18]. The mixture of phospholipids, cholesterol, carotenoids, and tocopherols present in egg yolk may also constitute a promising raw material for the preparation of liposomes [19, 20]. In our previous work, it was proved that egg yolk extracts can be successfully used for the preparation of liposomes [13]. The application of different extraction procedures give materials of distinct properties, and, consequently, of varied suitability for liposome formation [13]. The developed extracts are composed mainly of phospholipids, acylglycerols and cholesterol which comprise at least 99.5% of the compositions. Minor constituents include tocopherols and carotenoids. The extraction procedure applied affects also the ratio between fractions of main constituents i.e.: acylglycerols to phospholipids ratio; the ratio of polar to nonpolar fraction of acylglycerols; the share of phosphatidylcholine, sphingomyelin, and L- α -lysophosphatidylcholine in total phospholipids.

The composition of the vesicle membrane influences the liposomal membrane permeability, Zeta potential, and size of multilayer vesicles, prepared by Bangham method [13]. Analyses employing Langmuir trough: the π -A isotherm, dilatational and stress rheology, as well as surface potential, prove that all extracts were able to form a surface active film at the air/water interface, however, their interfacial behaviour was strongly affected by their composition. The phospholipids content is the most important factor determining the elastic response on area deformation of monolayer formed by extracts.

At the same time the balance between the polar and non-polar fraction of lipids and high content of phospholipids in the film are conducive for a solid-

like response on shear stress. However, the presence of lysophosphatidylethanolamine induces fluid-like behaviour of monolayers [21].

For the above mentioned outcomes, the relationship between the composition of the vesicles membranes and properties of the liposomes is complicated. The control of the liposome formation process from egg yolk extracts, based on existing data, is a tricky task. The aim of this research was to further characterise the liposomes produced with hen egg yolk extracts employing flow cytometry and atomic force microscopy, the methods recently refined for the needs of liposome technology.

2 Materials and Methods

2.1 Materials

Five different extracts were used for the preparation of liposomes. The extracts were obtained according to previously described procedures [13] and were denoted in the following manner: ethanol/acetone EA, methanol-chloroform/acetone MA, hot ethanol HE, hexane H, cold ethanol CE (Table 1). The buffer used for the formation of liposomes, and as a medium for measurements was 1% w/v phosphate buffered saline (PBS) of pH = 7.4. The following dyes were used for flow cytometry: DiD Lipophilic Carbocyanine DiI C18, Vybrant® Multicolor Cell-Labeling Kit, 1 mM solution in ethanol from Thermo Fischer Scientific, Germany, and Thiazole Orange (TO), 1 μM solution in dimethylsulfoxide (DMSO) from Sigma Aldrich, USA. For atomic force microscopy, mica sheets from Ted Pella Inc., USA, were used. Soft triangular DNP B (Bruker AFM Probes, USA) cantilevers made from silicon nitride were used for the measurements. Tipless CLFC-NOBO C (Bruker) was the calibration standard.

2.2 Methods

2.2.1 Preparation of liposomes

Liposomes were prepared by the thin film hydration (Bangham) method. The technique includes an organic phase for lipids dissolution followed by its removal under vacuum. Consequently, the thin, dry lipid film is formed. The film is further hydrated under agitation, which causes successive detachment of lipid lamellas and vesicles formation [12]. Each of the extracts used was dissolved in chloroform (20 mg/mL) and transferred to a round bottom

Table 1: Composition of extracts used for the preparation of liposomes [13].

Component/Extract	EA	MA	HE	H	CE
Phospholipids share in main components PL [%]	86.7	92.5	46.8	0.8	63.4
Acylglycerols share in main components AG [%]	12.5	6.5	41.0	95.8	29.0
Cholesterol share in main components Chol [%]	0.9	1.0	12.3	3.4	7.6
Total tocopherols content [mg/100g]	2.54	2.64	24.7	30.7	42.5
Total carotenoids content [μ g/100g]	143.3	96.3	1509	106.5	568.2

flask. The chloroform was removed with the use of rotary evaporator until complete dryness to form thin lipid film. The film was hydrated with an aqueous solution (4 mL of 1% PBS buffer) at 42°C for 2 h in a water bath under continuous rotation. Obtained liposomal suspensions were divided into two parts. The first, containing multilamellar vesicles (MLV), was collected directly. The second part was extruded through a 100 nm ISOPORE® membrane filter (LiposoFast by Avestin, Germany) in 15 cycles which resulted in the formation of small unilamellar vesicles (SUV).

2.2.2 Zeta potential and size distribution

Zetasizer Nano ZS90 from Malvern Instruments (England) equipped with a 5 mW helium/neon laser was applied for the determination of both size and Zeta potential of the liposomes. The measurements were taken at 21°C for 200 s with a 90° detection angle. The final results were calculated using the cumulant technique with normal Gaussian distribution.

2.2.3 Flow cytometry

Flow cytometry is a multiparametric laser-based technique applied to evaluate the distribution of both the size and structure in populations of liposomes. The technique was initially applied for cell analysis [22]. However, there are a few reports in which flow cytometry was employed to the study of liposomes. Firstly, it was used to estimate the size and average membrane/internal volume with the use of fluorescent labelling [23]. The idea was further

continued by Sato et al. who used double labelling to estimate membrane/internal volume as well as utilizing a fluorochrome with an affinity towards neutral lipids [24]. Alternatively, the size and structure distribution of positively charged liposomes in the size range of 100 to 1000 nm can be determined with the use of latex beads [25]. An idea of post-formation analysis of liposomes prepared from various lipids was presented recently [17]. The concept involved simultaneous labelling of vesicles with a hydrophilic dye that enters aqueous compartments and a lipophilic dye that binds to the phospholipid bilayer. Consequently, a method for fast and reliable analysis of liposomes without the need for problematic preparation or manipulation was developed [17]. The latter approach was applied in this research. The analysis was performed with the use of scattered laser light signals (irrespective of detection of fluorescence signals from hydrophilic and lipophilic dyes) detected as the side and forward scatter, respectively abbreviated as SSC and FSC. The SSC collects signal at 90° angle to the incident laser light and represents the complexity of the assessed particles. The FSC collects signal in the same direction as the incident laser light and, in general, is proportional to the particles' size [26]. The resolving power of a flow cytometer is determined by the performance of both the light detectors and digital signal processing. The instrument displays data in 262 144 channels per detector. Fluorescent staining and flow cytometry were used to compare the obtained populations of liposomes in terms of structure and size. Flow cytometric analysis was performed using BD FACS ARIA™III (Becton Dickinson, USA) flow cytometer (cell sorter) and was based on the protocol described previously [17]. Flow cytometric analyses were performed using logarithmic gains and specific detector settings (10 000 events were recorded per analysis). Each sample was analysed in triplicate. Data were acquired in a four-decade logarithmic scale as area signals (signal pulse sampling frequency was 10 MHz) and analysed with the FACS DIVA software (Becton Dickinson). Liposomes were characterised using two fluorescent parameters and two non-fluorescent parameters. Non-fluorescent parameters were forward and side scatter from FSC and SSC detectors, respectively, collected as area signals (FSC-A and SSC-A). FSC-A/SSC-A data provided the initial definition of particles' size and structure. The complexity of liposomes was then evaluated using green and red fluorescence signals measurement from the combination of hydrophilic dye – thiazole orange (TO), collected using the FITC detector as area signals (FITC-A) and lipophilic dye – DiD, collected using the APC detector as area signals (APC-A), respectively. Distinct and representative (exhibiting low

coefficient of variation values in the measured parameters) sub-populations of liposomes were discriminated.

2.2.4 Atomic force microscopy (AFM)

2.2.4.1 Preparation of the samples

AFM was originally designed to visualize nanoscopic and atomic surfaces of insulators [27]. Over the years, AFM began to offer much more than just topography imaging. Force spectroscopy (FS) is a powerful measurement mode which enables to determine nanomechanical parameters of investigated objects or surfaces, e.g.: the adhesion force, work of adhesion, elasticity (via Young's modulus) [28]. These parameters can be determined, calculated and mapped in situ without damaging the sample with resolution in the pN range. This makes the method suitable for the analysis of demanding and extremely soft biological systems, e.g.: living cells, viruses, bacteria [29]. One of the practical and important applications of this technique is the ability to early distinguish between normal and cancer cells. Each aspect of an FS measurement can be controlled with negligible lateral displacements during the force curve cycle, with given indentation force or retraction rate for instance. Optical-lever feedback of AFM enables to perform experiments in a native biological buffer.

Mechanical properties of SUV were determined with the use of an Agilent 5500 (N9410S; Keysight, USA) atomic force microscope equipped with an environmental chamber and a liquid cell. The measurements were performed in force spectroscopy contact mode. A 2 cm² sheets of freshly cleaved mica were mounted in a large liquid cell system (15 mm in diameter) and filled with 100 μ L of 1% PBS that contained 100 μ moles of a selected type of SUV or MLV. Following a 30-minute incubation on top of the mica sheet, another 400 μ L of 1% PBS was introduced to counter evaporation. The liquid cell was isolated from the influence of the external environment by a glass environmental chamber. The microscope was placed on an anti-vibration system and enclosed in an anti-acoustic chamber. The liquid cell was cleaned with ethanol before the introduction of subsequent samples.

2.2.4.2 Force spectroscopy (FS) measurements

Because of the progressive flattening processes of vesicles [17], each analysis of a sample was not longer than 40 min. During this time, it was possible to collect up to 7 FS force-curve grids. Each grid was obtained from a different part of the sample and consisted of 16 $\times$ 16 approach-retraction curves for a 16 μ m² surface area. 1792 approach-

retraction curves were thus collected per sample. Each force curve was recorded with the same separation rate of 1 μ m/s. The loading force did not exceed 20 nN in the grid measurements. However, to ensure full indentation of a vesicle, each sample was tested locally with a loading force up to 50 nN. The distance between each point at which a force curve was collected was 250 nm to ensure a single indentation event on a vesicle. Due to the very weak adhesion of the liposomes to the mica substrate, the measurements were done in the so-called blind mode in which the FS experiments were not preceded by a lateral topographical scan. Visualization of the vesicles suspended in the buffer by AFM prior to testing yielded unsatisfactory results. The thickness of lipid bilayer was assessed by indenting the bilayer patches that remained on the mica substrate. The exact procedure as applied here, was described elsewhere [30].

2.2.4.3 Cantilever details

The cantilever type was selected so as to enable the detection of very delicate vesicles (low force constant), avoid any perforation of the vesicle by a sharp and round tip (curvature of the end of the tip) and allow the collection of multiple force curves without degradation of the tip (material durability). To ensure the latter, two DNP B cantilevers were used in the experiment. Each cantilever tip was investigated under a Quanta 250 FEG (FEI, USA) scanning electron microscope before and after the measurement. Owing to the latter, the radius of the tip was determined (Figure 5f). To recalculate force units from the deflection signal for each force curve, it was necessary to find the deflection sensitivity and spring constant of the cantilever. The deflection sensitivity was determined with the use of a clean mica substrate in a buffer before each measurement. This was done for $n=100$ force curves at one point for each. The force constant of the cantilever was determined in air, according to an accurate reference method [31, 32]. The use of an interferometrically calibrated tipless reference cantilever, CLFC-NOBO C (with a spring constant of 0.112 N/m), enabled to perform the calibration with a total relative uncertainty lower than 5%. Detailed parameters that characterised the cantilever are presented in Table 2.

2.2.4.4 Force curve selection procedure

Erroneous force curves or results without visible indentation of the vesicles were rejected before calculations. Only the results that showed proper indentation depth (close to the averaged maximum

Table 2: Mechanical parameters of cantilevers and tips used for AFM.

Cantilever No. / Parameter	Type	Sensitivity [$\frac{nm}{V}$]*	Force Constant [$\frac{N}{m}$]**	Tip Radius [nm]***	Specimen
1	Bruker DNP B; (Silicon Nitride)	168.1 ± 3.4	0.100 ± 0.004 (4.3%)	21 ± 2	H, CE
2		80.6 ± 1.0	0.162 ± 0.004 (2.3%)	19 ± 2	EA, MA, HE

*averaged from 80 measurements on mica in buffer, examined before each part of the experiment, uncertainty is the standard deviation of the mean; **data obtained using the reference method [33, 34] with Bruker CLFC-NOBO C ($k=0.112$ N/m), complex uncertainty, the relative uncertainty in percent is given in parentheses; ***tip radius examined before and after the experiment, without visible mechanical wear, uncertainty estimated.

indentation depth) were taken into account. Only a small percentage of curves collected on the vesicles (no more than 6.7% per sample) were found valid. Because of the utilization of FS in blind mode, this step was necessary to make sure that the point of indentation was close to the geometric center of the tested vesicles.

2.2.4.5 Force curve analysis

Force curves were analysed quantitatively using AtomicJ 1.7.2 software [35]. The elasticity, in the form of Young's modulus, was calculated by the Johnson-Kendall-Roberts (JKR) model [36]. This mathematical model is a popular and well-established modification of Hertz theory [37] in contact mechanics. It is suitable across a range of size scales – from nano- to microscale. The model assumes a finite contact area between the contacting surfaces at zero load and predicts an external, non-zero force, needed to separate them. It is suitable only for small loading forces and soft materials, without asperities that would create interfacial gaps, which strongly affect the attractive adhesion forces. In case of this work, the surface of the studied liposomes was very soft and smooth [17]. Thus, the JKR model was suitable and characterised the indentation of a vesicle well. Due to the nature of the measurement taken in a biological buffer, it was necessary to discuss the influence of the additional solvent hydration and electrical double layer forces acting at the interface between the vesicle and AFM tip. According to simulations by Korayem et al. that regard soft biological cells, the output from the JKR model for air and liquid environment is practically the same, differences are negligible [38]. Thus, the JKR model was applied. Only the linear region of the approach indentation curve was taken into account. The radii of both tips and the Poisson's ratio, assumed to be 0.5 according to other papers [39-41], were included into calculations.

2.2.5 Statistical analysis

All experiments were performed in triplicates and the results were presented as means ± standard deviation (SD). The significance of the differences between mean values was determined by analysis of variance (ANOVA). Post-hoc analysis was done with the use of Tukey's test. Principal Component Analysis (PCA) was applied in order to analyse the relations between membrane composition [13] and liposomes properties derived from AFM experiment. Pearson coefficient was calculated in order to analyse the relations between membrane composition and liposomes size, polydispersity, nanomechanics, Zeta potential. Results in all the tests were considered significant at $\alpha=0.05$. The statistical analysis was performed using Statistica 10.0 software (StatSoft, Inc., Tulsa, USA).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 General characteristics of liposomes

Populations of liposomes obtained using the tested extracts as membrane building material were analysed by DLS in terms of Z-average size [nm], polydispersity index PDI [-], and Zeta potential [mV] before MLV and after SUV the extrusion process (Figure 1abc). The Z-average size of MLV formed with EA, MA, H, and CE samples exceeded 1 μ m. EA- and HE-derived vesicles were significantly distinct from the other liposomes as the biggest and the smallest, respectively. There were no significant differences in PDI values of the populations of MLV, thus they were comparable in terms of polydispersity. Differences in Z-average size and PDI values between

Figure 1: Dynamic light scattering and Zeta potential of liposomes measured directly after preparation: (a) Z-average size [nm]; (b) Zeta potential [mV]; (c) PDI. Bars bearing different superscripts are statistically, significantly different ($\alpha < 0.05$).

MLV and SUV populations were clearly noticeable, which proved a successful extrusion process. The Z-average size of all the samples, after post-preparation processing, were below 240 nm. The effect of extrusion on the size of HE-derived liposomes was relatively small. It was likely a result of their small initial size. The EA-, MA-, CE-derived liposomes were characterised by very low PDI values. The vesicles obtained with MA extract showed the lowest PDI value that may indicate increased stability (Figure 1c).

The Zeta potential is a parameter that reflects repulsion forces between colloidal particles, a measure of the stability of the system. Only slight differences of Zeta potential absolute values were determined between EA-, MA-, H-, and CE-derived MLV samples (Figure 1a). The HE-based vesicles were significantly different in terms of this parameter showing a potential almost two-fold higher than the other samples. This might have been related to the exceptionally high content of carotenoids in this extract in comparison to the other extracts (Table 1) [13]. Based on the similarity of Zeta potential obtained for MLV

and SUV (Figure 1b), it can be stated that the extrusion process did not affect the Zeta potential of the analysed liposomes.

The intensity distribution [%] versus the size of the liposomes [nm] (Figure 2a) is a 1st order result of dynamic light scattering (ISO 22412: 2008) used for reporting the size of each peak. Initially wide peaks (Figure 2a) became narrow after the processing and showed monomodal size distribution (Figure 2b). This confirmed the effect of extrusion on the mean diameter of the vesicles and corresponded with the data shown in the Figure 1 with the Z-average size of SUV in the range of 180-235 nm.

3.2 Flow cytometry

The size and complexity distribution of both MLV and SUV was analyzed by studying FSC and SSC signals [42]. Moreover, a combination of TO and DiD fluorochromes was used to evaluate the feasibility of the system to detect the

Figure 2: Size distribution of MLV (a) and SUV (b) populations. Intensity [%] stands for intensity distribution - the 1st order result derived from dynamic light scattering measurement and represents the intensity distribution of particles sizes. The parameter is naturally weighted corresponding to the scattering intensity of each particle subpopulation/fraction.

Figure 3: Diversity of MLV populations in terms of morphology and structure determined by flow cytometry. Bivariate contour plots in the upper row for each population FSC-A vs SSC-A, lower row – FITC-A vs APC-A. Diagrams indicate the gating strategy (structure of defined subpopulations). Sub-populations P1 and P2 (discriminated within the Q2 region using TO/DiD staining) indicate higher structural diversity of liposomes.

structural diversity of liposomes. It was assumed that TO would penetrate the liposome to its aqueous core and DiD would stain the membrane of the vesicle. The results are shown on FITC vs APC bivariate dot plots (Figure 3,4). FITC and APC axes of the dot plot present the intensity of green and red fluorescence signals, respectively. Background noise was discriminated by an analysis of the distribution of the bivariate FSC vs SSC dot plots.

According to FSC vs SSC dot plots (Figure 3), the majority of MLV liposomes represented a one disperse subpopulation Q2 (Q2 region that contained 92.3, 90, 93, 99.85, and 98.2% of particles of EA-, MA-, HE-, H-, and CE-derived liposomes, respectively). This was accompanied by a low structural diversity of MLV liposomes as indicated by the distribution of the population presented in FITC vs APC dot plots. The HE variant of MLV liposomes was an exception that manifested polydispersity in terms of both

Figure 4: Diversity of SUV populations in terms of morphology and structure determined by flow cytometry. Bivariate contour plots in the upper row for each population FSC-A vs SSC-A, lower row – FITC-A vs APC-A. Diagrams indicate the gating strategy (structure of defined sub-populations). Sub-populations P1 and P2 (discriminated within the Q2 region using TO/DiD staining) indicate higher structural diversity of liposomes.

size and structure. In the case of this variant, two separate sub-populations, P1 and P2, were distinguishable when the TO/DiD-stained samples were analysed. This corresponded to the results of the DLS analysis (Figure 1), as the HE variant of MLV liposomes showed the smallest size and highest absolute value of Zeta potential. This could stem from the exceptionally high carotenoids content [13] (Table 1). Moreover, a sub-population Q3 (Q3 region), supposedly composed of small liposomes of a relatively simple complexity, was detected in the populations of EA-, MA-, and HE-based vesicles. This sub-population amounted to 6.43, 8.27, and 6.13% of the total population of the respective samples.

FSC vs SSC bivariate dot plots revealed a transformation of the vesicles that resulted from extrusion (Figure 4). The analysis with flow cytometry demonstrated striking differences between MLV and SUV liposomes in terms of both size and structure. The observed differences were dependent on the extract variant used for the preparation of samples. EA-, MA-, and HE-derived vesicles showed striking distinction with the dominance of Q3 sub-population (Q3 region that contained 73.17, 85.47, and 68.3% of particles formed with EA, MA, and HE extracts, respectively). The liposomes prepared with H and CE extracts showed a dominance of sub-population Q2 (Q2 region). The analysis also revealed: (i) a more

complex structure of Q2 sub-population, as presented for H extract, for which two separate sub-populations P1 and P2 were distinguishable after TO/DiD staining, and (ii) higher density of sub-population Q2, as indicated for the CE extract-based sample on FSC vs SSC and FITC vs APC dot plots. Thus, the majority of the produced vesicles underwent a reduction in size and lamellarity. In the case of H- and CE-derived samples, the dominant subpopulation did not change following the extrusion process. However, after the treatment, FSC vs SSC bivariate dot plots obtained for SUV showed S-like shapes that were found to be common in a previous report [17]. FITC vs APC bivariate dot plots demonstrated the presence of P1 and P2 sub-populations within the Q2 region in the samples based on EA, HE, and H extracts. An uneven distribution of SUV liposomes was thus determined. P1 and P2 sub-populations were oriented vertically (along the axis of ordinates of the dot plot) so the differences between these sub-populations were related to the red fluorescence signal generated by the DiD lipophilic dye. Nonetheless, the majority of EA-, MA-, and HE-derived liposomes were situated within sub-population Q3 (Q3 region) characterised by low lamellarity and complexity. The liposomes obtained with H and CE extracts showed different distribution of structural features with only 7.1 and 1.15% particles of the respective samples contained

Figure 5: Quantitative analysis of nanomechanical properties of SUV by FS: (a,b) illustrative scheme of the force spectroscopy experiment – force curve and indentation (I. – physical contact between AFM tip and surface of a vesicle, II. – indentation of a vesicle, III. – indentation of the mica substrate; symbols: z – scanner position, d – cantilever deflection, V_D – cantilever deflection signal, h – height of vesicle, S – sensitivity, F – force); (c,d) typical approach (blue) – retraction (green) force curve recorded on a CE vesicle with calculated force [nN]; (d,e) the Johnson-Kendall-Roberts (JKR, red curve) model fitted to typical CE data region II. – force curve (d) and corresponding force-indentation profile (e); (f) scanning electron micrograph of Bruker DNP B tip after the experiment (cantilever no. 1, inset: radius R of the tip); (g,h) calculated Young's modulus and adhesion force histograms for CE specimen (green striated curve – fitted Gauss distribution profile); (i) typical approach (blue) – retraction (green) force curve recorded on a H sample depicts indented lipid bilayer patch (region II.).

within sub-population Q3 (Q3 region). Moreover, the CE-derived population of vesicles became more uniform because of extrusion, especially in terms of structure. Compared to the other samples, this population was also found to show increased lamellarity and complexity of vesicles.

3.3 AFM and nanomechanical study

To analyse a scope of nanomechanical properties of the prepared SUV, FS, a powerful tool of the AFM technique,

was employed. The FS measurements of the engineered egg yolk vesicles were done in the buffer, to ensure a quantitative output as close to native as possible. The vesicles were attached to the atomically flat muscovite mica in a soft manner via electrostatic interactions. To avoid any impurities, no functionalization of the substrate was employed. Separated vesicles were characterised in terms of force and work of adhesion, elasticity, bending modulus, and thickness of the lipid membrane. An attempt at correlating the mechanical properties with chemical composition was made. Only SUV were analysed (Figure 5) because they provide more approximate feedback on

single lipid bilayer properties than MLV. The percentage of the SUV detected in relation to the introduced number of vesicles (the ratio of the number of curves on a vesicle to total curves obtained) was as follows: EA (8%), MA (93%), HE (1.4%), H (22%), and CE (66%). This suggests different adhesion properties of the outer lipid membrane (Table 1). As the mica is negatively charged, the deposition of vesicles is strongly related to both charge of liposomes and pH [33]. Differences in the deposition percentage could thus be expected.

The vesicles were indented by the AFM tip (regions I. and II.) until the significantly stiffer substrate was reached (region III.) (Figure 5a-c). The higher stiffness of the substrate is represented by the steeper slope of the approach indentation curve (blue). Moreover, the presence of a single jump-in event, marked as V_D and Z_2 in Figure 5b and visible also in CE force curve on Figure 5c, is a clear information on the elasticity limit of a vesicle which facilitates interpretation of the results. This particular event is related to the detection of a force gradient near the substrate. In this part of the curve (beginning of III.), the force gradient, due to the vertical proximity of the AFM tip and the substrate, exceeded the force constant of the AFM cantilever. The attractive forces brought the tip to the substrate through the vesicle. Such jump-in events were also described in other works related to liposomes that involved FS [39, 40]. During the indentation, the AFM tip was compressing the vesicle which underwent a change in shape – from almost round (region I., vesicle's height h_0 , flattened by attractive electrostatic forces between the substrate and the vesicle) to planar (region II., vesicle's height h_1) and concave (beginning of region III., vesicle's height h_2) (Figure 5a) [30]. The retraction curve (green, Figure 5b-d) brings information about the adhesion force and its work. Adhesion peak height and its area with respect to the baseline are the measures of the respective parameters (Figure 5b-d). Elasticity was calculated from region II. of the approaching curve (Figure 5d). The red curve represents a typical JKR model fitted to the experimental data. The indentation fingerprint in form of force-indentation curve is also fitted with JKR (red curve, Figure 5e). The resulted histograms of Young's modulus and adhesion force show Gaussian distribution (green dotted curve, Figure 5g,h). Such distribution is common for measurements of many isolated objects of the same type.

Table 3 presents parameters derived from the experimental data. The deformation of vesicles is an intermediate product obtained from the indentation procedure. Calculated elasticity was the highest for EA-based vesicles (28.53 MPa) which implied one order

of magnitude higher stiffness compared to the other samples. In the case of these vesicles, the loading force (5.4 nN) caused a much smaller deformation (8.8 nm) in comparison to the other, softer vesicles. HE- and CE-derived vesicles had similar elasticity (within the margin of error) – 2.16 MPa and 2.21 MPa, respectively. The most delicate liposomes were obtained with MA (0.53 MPa) and H (1.42 MPa) extracts. The elasticity data were consistent with the values reported for different liposomes in the literature [39, 40]. Locally measured elasticity was related to the bending of the lipid bilayer. The bilayer, a building block of each vesicle, is more or less susceptible to bending [34]. This complex behaviour is described by the so-called bending modulus [43]. This parameter can be calculated if the elasticity, Poisson's ratio and the thickness of the lipid bilayer of a vesicle are known [22, 41]. Bending modulus is temperature-dependent [39]. For the investigated samples, the bending modulus was closely correlated with elasticity represented by Young's modulus (Table 3). The highest value was found for EA liposomes and equaled $1.1 \cdot 10^{-19}$ J. The values of bending modulus determined for the other vesicles were one order of magnitude smaller which indicated less rigid structure. The lowest found value was $0.09 \cdot 10^{-19}$ J for H vesicles. Noteworthy, the observed values of bending modulus data were consistent with the literature [39, 40, 43, 44].

Adhesion force recorded against the AFM tip was about 1 nN or less. The nonspecific adhesion force depends on the contact area between the sample and the AFM tip. Both utilized tips had comparable radii (Table 2). Thus, the data concerning adhesion force and work of adhesion are comparable for all the studied vesicle variants (Table 3). Notably low adhesion force of 0.17 nN was found for the H sample. These vesicles also showed one of the most delicate mechanical structures. The most rigid sample, EA, had relatively high adhesion force that equaled 0.93 nN and was comparable to HE and CE. However, the EA sample was characterised by an exceptionally low value of work of adhesion (273.3 μ J). Work of adhesion was comparable among the other tested samples. This parameter is related to the stiffness of the bilayer. Stiffer EA vesicles with their compact and rigid structure could have been easily separated during the retraction of the AFM tip. The structure of the vesicle was not susceptible to stretching.

The last assessed parameter was the thickness of the lipid bilayer (Figure 5i). The stiff EA vesicles had the thinnest lipid bilayer (3.26 nm). The bilayer of the most delicate MA vesicles was nearly twice as thick (5.98 nm). The bilayer of the delicate H vesicles, however, was similar to that of EA (3.78 nm). This suggests that the mechanical

Table 3: Parameters obtained from the quantitative force curve analysis of SUV.

Data Type / Sample	EA	MA	HE	H	CE
Loading Force [nN]*	5.4 ± 0.3^a	2.4 ± 0.1^b	5.6 ± 0.3^a	6.8 ± 0.2^c	5.8 ± 0.1^a
Deformation [nm]*	8.8 ± 0.6^a	70.6 ± 1.8^b	67.1 ± 9.9^b	71.4 ± 1.5^b	49.7 ± 1.2^c
Young's Modulus [MPa]*	28.53 ± 1.88^a	0.53 ± 0.01^b	2.16 ± 0.36^c	1.42 ± 0.03^d	2.21 ± 0.05^c
Bending Modulus (10^{-19}) [J]**	1.10 ± 0.30	0.13 ± 0.04	0.18 ± 0.03	0.09 ± 0.02	0.17 ± 0.03
Adhesion Force [nN]*	$0.93 \pm 0.11^{a,d}$	0.79 ± 0.02^b	1.14 ± 0.05^a	0.17 ± 0.01^c	0.99 ± 0.03^d
Work of Adhesion [μ J]*	273.3 ± 35.5^a	$351.9 \pm 22.7^{a,b}$	398.3 ± 56.2^b	398.4 ± 20.1^b	359.5 ± 17.9^b
Data Count***	21	96	16	97	117
Thickness of the Lipid Double Layer [nm]*	3.26 ± 0.24^a	5.98 ± 0.63^b	4.18 ± 0.23^c	$3.78 \pm 0.21^{a,c,d}$	$4.10 \pm 0.23^{c,d}$
Data Count	16	9	16	20	20

Values (means $\pm$ SD) with different superscripts are significantly different ($\alpha < 0.05$), *uncertainty is standard deviation of the mean;

complex uncertainty; *data from separated vesicles (the thickness of the lipid double layer is given from a separate statistics).

properties of the studied liposomes are not solely dependent on the vertical dimensions of the lipid bilayer.

3.4 Correlation analysis

As indicated in our previous work [13], the extracts used for obtaining the tested liposomes varied greatly in terms of chemical composition. Generally, phospholipids, acylglycerols and cholesterol were their main constituents. The phospholipid fraction of the extracts consisted of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and sphingomyelin (SM). The extracts also contained tocopherols and carotenoids [13]. PCA analysis of the mechanical characteristics of the tested vesicles and the data on their chemical composition was performed to analyse the main factors determining the properties of the analysed liposomes. The two principal components (factor 1 and 2) explained 82.19% of the total variance, Figure 6.

Projection of the cases on the factor plane (Figure 6b) pointed to a similarity of MA and EA extracts as well as CE and HE extracts. At the same time the H extract differed from the others. Similar results were found in our previous works describing in details extraction procedures and composition of extracts [13] as well as surface behaviour of monolayers they formed [21]. The H extract was obtained in simple one step procedure

and was rich in acylglycerols but scant in carotenoids due to the use of nonpolar hexane as the extraction solvent. The CE and HE extracts were also obtained in a simple, one-step extraction procedure differing only in the temperature used. The liposomes obtained from the CE and HE extracts did not differ significantly in loading force, Young's modulus, bending modulus, work of adhesion, thickness of lipid double layer values (Table 3). Nonetheless, these extracts differed in surface behaviour as the compression process of the HE monolayer is irreversible, whereas the CE monolayer revealed only small hysteresis [21]. This corresponds to the higher α -tocopherol content in CE extract [13]. We believe special attention should be paid to the HE extract, as this material enabled, even by simple Bangham procedure, to obtain relatively small vesicles of the highest absolute value of Zeta potential (Figure 1). This phenomenon is related to a distinctly high content of carotenoids [13]. Liposomes from EA and MA extracts of similar composition, obtained in the course of a few-step procedure and rich in phospholipids were characterised by significantly different mechanical properties excluding work of adhesion. Projection of the variables on the factor plane (Figure 6a) proved, however, that the phospholipid content had a positive but slight effect on Young and bending moduli, while the share of lysophosphatidylethanolamine in total phospholipids was of predominant influence. Cholesterol and minor constituents (tocopherols and carotenoids) were also

Figure 6: PCA loading plot (a) and score plot (b) of characteristics and nanomechanical properties of liposomes formed from different extracts (Table 3). The data on the composition of extracts were derived from our earlier work [13]. Abbreviations: PL – phospholipids; AG – acylglycerols; Chol – cholesterol; PC/PL – phosphatidylcholine share in total phospholipids; LPC/PL – lysophosphatidylcholine share in total phospholipids; PE/PL – phosphatidylethanolamine share in total phospholipids; LPE/PL – lysophosphatidylethanolamine share in total phospholipids; SM/PL – sphingomyelin share in total phospholipids; PF/AG – polar fraction share in total acylglycerols; NPF/AG – non-polar fraction share in total acylglycerols; α -T – alfa-tocopherol; γ -T – gamma-tocopherol; L – lutein; Z – zeaxanthin; Young – Young modulus; Bend – bending modulus; Af – adhesion force; Aw – work of adhesion; h – thickness of the lipid double layer.

relevant to the liposomes' mechanical properties. Their effect on Young and bending moduli, in contrast to the impact of lysophosphatidylethanolamine, was negative. Cholesterol is a widely used additive in liposomal formulations as it improves membrane rigidity [40, 45, 46]. It interacts with the phospholipid molecules thus resulting in the modulation of membrane properties [47]. This explains the change in the mechanical strength of liposomes of varying cholesterol content. With regards to the shares of other phospholipids in total, the fraction with the biggest effect were phosphatidylcholine and lysophosphatidylcholine. However, their effects on the mechanical strength of liposomes were opposite, negative and positive respectively. Sphingomyelin also revealed small effect on vesicles mechanics while there was no effect of phosphatidylethanolamine share (Figure 6a). In general, the presence of the hydrolyzed forms of phospholipids, which are more polar than their non-hydrolyzed counterpart, stiffened the structure of the vesicle bilayer making it stronger mechanically.

Liposomes and cells membrane surface interactions are, in living organisms, responsible for fusion and absorption [48]. However, in liposomal systems they may influence stability of vesicles. Adhesion force is determined by the outer part of a vesicle, and

was negatively correlated with the contents of the acylglycerol fraction and positively, but to a lesser extent, phospholipids. At the same time, the work of adhesion was mostly influenced by total tocopherols content. Both acylglycerols and phospholipids strongly determine the properties of the surface of liposomes as their polar heads form the superficial layer of the shell of the vesicles.

Comparison of the biomechanical analyses results (Table 3) with rheology data described in our previous work [21] indicated a correlation between the properties of mono- and bilayer vesicles. As evidenced by the Pearson correlation coefficients (Table 4), mechanical properties of liposomes described by Young and bending moduli did not show any significant correlation except for the one between bending and dilatational elastic moduli. In contrast, parameters relating to the stability of liposomal dispersion correlated with the values describing adhesion phenomena. The strongest and positive correlation was observed between the adhesion force and collapse surface pressure. It is worth to mention that H extract, rich in acylglycerols, formed monolayer of the lowest CSP value [21]. Moreover, the adhesion force correlated also with the dilatational elastic modulus and the Zeta potential, whereas the work of adhesion – with the dilatational elastic and the shear loss moduli as well as with the

Table 4: Pearson correlation coefficients for the interfacial rheological properties of monolayer [21] and nanomechanical properties of liposomes.

	Dilatational Elastic Modulus	Dilatational Loss Modulus	Shear Elastic Modulus	Shear Loss Modulus	Collapse Surface Pressure	Zeta Potential	Z-Average Size
Young's Modulus	0.605*	-0.037*	-0.030*	0.625*	0.102	0.133	-0.437
Bending Modulus	0.653	-0.006	-0.025*	0.630*	0.164	0.105	-0.433
Adhesion Force	0.648	0.071	0.306	0.385	0.969	-0.562	0.108
Work of Adhesion	-0.761	-0.394	-0.133	-0.703	-0.256	-0.348	0.705
Thickness of the Lipid Double Layer	0.212	0.731	-0.219	-0.512	0.300	0.003	0.046

*Significance at $\alpha=0.05$.

Z-average size. Lipid double layer thickness correlated with both loss moduli.

4 Conclusions

As a result of this and previous work, it was proved that the chemical composition of a bilayer determined the mechanical properties, structure, and stability of liposomes. However, the effect of minor constituents was just as important as of main components. Moreover, the share of individual phospholipid fractions in total phospholipids or polar to non-polar fractions ratio of acylglycerols exerted a significant effect. In terms of its positive effect on the nanomechanical properties, described by Young and bending moduli, lysophosphatidylethanolamine share proved to be the most important of the phospholipids. Significantly weaker, but also a positive effect was observed for total phospholipids content as well as the share of lysophosphatidylcholine and sphingomyelin in the total phospholipids. Cholesterol, tocopherols, and carotenoids exerted the strongest negative effect on Young and bending moduli. The other negative, but significantly weaker, the effect on Young and bending moduli was a result of acylglycerol content as well as the share of phosphatidylcholine in total phospholipids.

The share of individual phospholipid fractions in total phospholipids or polar to non-polar fractions ratio of acylglycerol affected not only nanomechanical properties of liposomes but also surface behaviour of analysed extracts. The components improving liposomes nanomechanical properties also increased dilatational elastic moduli.

Similarly, minor constituents affected nanomechanical properties of liposomes and surface

behaviour of extracts. Tocopherols negatively influenced both interfacial shear moduli and dilatational elastic moduli. Contrarily, the presence of carotenoids positively affected interfacial shear elastic moduli. The presence of carotenoids was crucial for the stability of liposomes, represented by Zeta potential. The liposomes structure as well as the susceptibility to changes under the influence of the extrusion process also reflected carotenoid content. Lipid double layer thickness depended mainly on the α -tocopherol content, which positively affected loss moduli of monolayer films too.

According to the presented results, MA and EA extracts as well as CE and HE extracts proved similar to each other, whereas H extract differed significantly. The H extract was rich in acylglycerols but deficient in carotenoids due to the use of nonpolar hexane as the extraction solvent. It formed liposomes of the lowest mechanical strength, stability, permeability, and heterogeneous in terms of structure.

The CE and HE extracts showed different surface properties but formed liposomes of approximate nanomechanical properties. The above corresponded to the higher α -tocopherol content in CE extract. HE extract, containing an exceptionally high amount of carotenoids, enabled, to obtain relatively small vesicles of the highest absolute value of Zeta potential, even by simple Bangham method. Mechanical strength of liposomes, made from MA and EA extracts, the richest in phospholipids, differed from each other due to differences in the share of lysophosphatidylethanolamine in the total phospholipid content. However, they did not show structural differences. Their structure was found to be of the lowest lamellarity and complexity.

Considering the potential application of developed extracts in technological practice it can be stated that,

four of them i.e. MA, EA, CE, and HE could be used for the production of functional foods. The HE extract deserves special attention, as it was obtained by simple, one-step extraction and enabled the formation of small, stable liposomes by a simple procedure. At the same time, we would not recommend the H extract, as it resulted in liposomes of low stability and deficient permeability.

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Abbreviations

Af	adhesion force
ANOVA	analysis of variance
Aw	work of adhesion
AFM	atomic force microscopy
AG	acylglycerols
APC	allophycocyanine red fluorescence signal
Chol	cholesterol
CSP	collapse surface pressure
DiD	1,1'-diocetadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate
DLS	dynamic light scattering
DMSO	dimethylsulfoxide
FITC	fluorescein 5(6)-isothiocyanate green fluorescence signal
FS	force spectroscopy
FSC	forward scatter
h	thickness of the lipid double layer
JKR	Johnson-Kendall-Roberts model
L	lutein
LPC/PL	lysophosphatidylcholine share in total phospholipids
LPE/PL	lysophosphatidylethanolamine share in total phospholipids
MLV	multilamellar vesicle

NPF/AG	non-polar fraction share in total acylglycerols
PBS	phosphate buffered saline
PCA	principal component analysis
PC/PL	phosphatidylcholine share in total phospholipids
PDI	polydispersity index
PE/PL	phosphatidylethanolamine share in total phospholipids
PF/AG	polar fraction share in total acylglycerols
PL	phospholipids
SD	standard deviation
SM/PL	sphingomyelin share in total phospholipids
SSC	side scatter
SUV	small unilamellar vesicle
TO	thiazole orange (1-Methyl-4-[(3-methyl-2(3H)-benzothiazolylidene)methyl]quinolinium p-tosylate)
Z	zeaxanthin
γ-T	gamma-tocopherol
α-T	alfa-tocopherol

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