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THE USE OF IR SPECTROSCOPY AFTER REHYDRATION TO FOLLOW TERNARY LIPOPLEX FORMATION AND DESIGN AS A METAL-BASED DNA NANOPHARMACEUTICALS

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A b s t r a c t: IR spectroscopy was used to follow the dynamic structural transitions of the transfection competent 100 nm DNA-divalent metal cation-phospholipid ternary complex upon recognition of individual molecules engaged. The selection of small charged metal ions as complexing agents and zwitterionic L-α-phosphatidylcholine as an alternative to the currently used problematic cationic lipids in gene transfection is emphasized. Spectra of unbound components were compared with those of nucleic acid-lipid and lipid-metal ion binary mixtures, as well as with the ternary complex. Data obtained for carbonyl, phosphate, choline and CH groups was used for deductions of DNA-phospholipid recognition profiles, induced by Mn^{2+}. Ion effects were considered as dehydrations of phosphates and H-bonding of carbonyls. The possible structure of the ternary complex is discussed with its further potential to be utilized as a nonviral gene delivery formulation.

Key words: DNA-lipid recognition, nucleic acid-divalent metal cation-phospholipid ternary complex, IR spectroscopy, Mn^{2+}, nonviral gene delivery, lipoplexes.

Introduction

Self-assemblies of various lipids with nucleic acids are currently being tested in the search for reliable nonviral gene delivery vectors [1–4]. Lipofection assays performed with commonly employed cationic lipids often lead to undesirable cytotoxicity, difficult to control macromolecular properties, and low transfection rates, as well as other discouraging results. Information concerning

physicochemical characteristics of lipid-poly(ribo)nucleotide complexes, which is a prerequisite for designing more efficient cell transfection protocols, is insufficient. Despite frequent reports on lipid-nucleic acid interations [4, 5], the electrochemical, colloidal and intermolecular forces responsible for their complex formation are not well established. Fluorescent assays, X-ray scattering, various electron microscopic, thermodynamic and spectroscopic techniques employed until now in their characterization studies have resulted in abstract and frequently contradictory data, which is difficult to relate to cellular mechanisms of transfection.

Among the spectroscopic approaches, infrared spectroscopy (IR) and Raman scattering are regarded as those offering the potential of providing useful information on structural changes occurring upon the association of biomacromolecules [6]. FTIR spectroscopy is the commonly used method for gaining valuable structural information on macromolecules [7–9]. IR spectroscopy and IR microspectroscopy of human cells and tissues have attracted scientific interest, since these can detect the chemical composition and structural changes of cells and tissues in health and in pathology [10–12]. FTIR spectroscopy has been used for studying genome damage [13, 14] and for diagnostic applications such as cancer detection and monitoring [15,16].

Such approaches employ drying cells and tissues in a vacuum or treatment with dehydrating agents to prevent bacterial degradation of cells and to improve sample preservation for future uses [17]. However, such a water removal from biomacromolecules often leads to artifacts concerning interpretations of their IR spectra, since this procedure affects their conformations. On the other hand, interactions of nucleic acids with ions and water molecules are important for understanding nucleic acid recognition events including drug-nucleic acid and protein-nucleic acid associations [18, 19]. In general, these cellular events are accompanied by the release or uptake of ions or water molecules. This holds true also in the case of complexation of lipids with nucleic acids [20]. Determination of the effects of hydration on the physicochemical features of the nucleic acids studied depends on the evaluation of the amount of bound water. Therefore, we studied the secondary structural transitions of DNA and phospholipids following their recognition and complex formation, trying to correlate hydration and structural data. The obtained information can be used for deducing their spatial structural arrangements and for comparison with our previous thermodynamic and microscopic studies of their complexes [21–23]. More specifically, these methods permit working without the use of bulky extrinsic probes, and provide a possibility of avoiding peak fluctuations, as well as scattering and absorption flattering artifacts. IR spectroscopy is frequently applied in following nucleic acid structure and kinetics [8, 18, 19, 24, 25]. Although it does not provide the atomic details of

X-ray crystallography and NMR spectroscopy, it is widely used as it can distinguish between different nucleic acid secondary structures and motifs. In addition, little sample material is needed, spectra are collected rapidly, and the samples are utilized in liquid, membraneous and solid form or on surfaces [26]. Therefore, we have employed IR spectroscopy to study the secondary structural motifs of lipid-DNA assemblies. The objective is to relate and further use the obtained data for physicochemical profiling and design of lipid based therapeutic nucleic acid delivery vehicles.

Materials and Methods

Materials

Calf thymus DNA (D-4764; 5 units) of approximate size > 13 kb (A_{260}/A_{280} = 2.0) was obtained from Sigma-Aldrich Chemie GmbH, 89552 Steinheim, Germany and stored at 4°C. 1 unit yields an A_{260} of 1.0 in 1.0 ml of 1mM NaCl, 1mM Tris-HCl; pH = 7.5, and 1mM EDTA in 1 cm light path of spectrophotometric cuvette. 1 mg of calf thymus DNA is equivalent to approx 20 units. L-α-phosphatidylcholine (P3556), FT-IR-grade KBr (22.186) and $MgCl_2.2H_2O$ (M8266) was obtained from the same supplier. All reagents were of the highest analytical grade and were used without further purification.

Liposome preparation

Liposomes were prepared according to the procedure described by Popova and Hincha [27]. Briefly, the lipids (10 mg) were dried from chloroform under a stream of N_2 gas and stored overnight under a vacuum to avoid traces of remaining solvent. They were hydrated afterwards in 250 µl of HEPES (*N*-(2-Hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid) buffer to form multilamellar vesicles (MLV). Unilammelar vesicles (ULV) were obtained by extruding (10 times) of multilamellar vesicle suspension though two stacked 100-nm pore-size Nucleopore® polycarbonate filters using a miniextruder (Avanti Polar Lipids, Inc., Alabaster, AL 35007, USA). 50 µl of the ULV suspension were spread on an FTIR sample holder window and dried at 28 °C for 48 hours in the dark, as described [27, 28]. The absence of water absorption peak at 1650 cm^{-1} was used as an indication that samples were anhydrous. The dried lipids were rehydrated by storing them in a closed container upon treatment with water vapour phase overnight at 28°C in the dark.

FTIR spectroscopy

Rehydrated lipids were deposited between two sample holder windows and mounted towards the infrared beam. These were cooled on ice and then

equilibrated at 28°C for 20 minutes. Infrared spectra were recorded from 500–4000 cm^{-1} with a Vectra 22 FTIR spectrometer (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany), equipped with He-Ne laser. After normalization of absorbance and baseline correction, the peak frequencies of the symmetric CH_2 stretching, the asymmetric P = O stretching and the asymmetric $N-(CH_3)_3$ stretching peaks were determined by using the original software OPUS 3.0, provided by the manufacturer. FTIR spectra were taken for each single component engaged in the DNA-lipid complex formation for three combinations of binary mixtures (Mn^{2+}-DNA, DNA-phosphatidylcholine, phosphatidylcholine-Mn^{2+}) and for the ternary mixture (DNA-Mn^{2+}-phosphatidylcholine) in equimolar ratios for comparison with our recent calorimetric studies [21]. The evaluations of the parallel effects of Mn^{2+} and lipids on DNA absorption spectra, or alternatively of the metal cation and DNA on the lipid structure are emphasized. The spectral comparisons and interpretations were performed in those IR regions where the lipids and DNA do not interfere (phosphate, carbonyl and CH vibrations). Thus, the ternary complex is compared with controls of binary mixtures trying to depict the driving forces engaged in ternary complexations.

Results and Discussion

We employed FTIR spectroscopy to study the structure of model zwitterionic lipids in unbound form and upon their complexation with DNA in the absence and in the presence of Mn^{2+}. After fresh preparation of the unilamellar vesicle suspensions, their further treatment under vacuum was performed to ensure that the remaining water content was eliminated, as suggested [27].

Fig. 1. shows an overall IR absorption spectrum of L-*α*-phosphatidylcholine. This zwitterionic lipid was selected for this study, since its physicochemical characteristics are well-known (http://www.lipidat.chemistry.ohio-state.edu) both in the gel and in the liquid-crystalline state. Approximately 15 well-seen bands can be employed in studies of structual changes of this phospholipid following association with various ligands. In this respect, those observed between 2750–3100 cm^{-1}, representing CH stretching modes, those between 1150–1400 cm^{-1} and 1465–1415 cm^{-1} depicting CH_2 wagging progression and CH_2 scissoring vibrations are considered particularly useful [32–34]. The former is used as a measure of the degree of the lipid acyl chain order, whereas the latter is regarded as an indication of chain packing effects. The signal at 1258 cm^{-1} is ascribed to antisymmetric PO_2^- stretching and that at 1736.6 cm^{-1}- to *ν*[C = O] stretching. The well-seen scissoring vibration at 1465–1475 cm^{-1} shows the existence of hexagonal packing of acyl chains [29, 30–34]. The peak

at 1242 cm^{-1} corresponds to antisymmetric PO_2^- stretching and provides information about the interfacial region of the phospholipid. *v*[C = O] stretching vibration (1736.6 cm^{-1}) is split into two separate bands, ascribed to hydrogen unbound and to H-bonded C = O groups, respectively [24]. The band at 970. 4 cm^{-1} depicts stretching of the asymmetric terminal choline group. The other minor bands of L-*α*-phosphatidylcholine are located in the region of 700–1100 cm^{-1}.

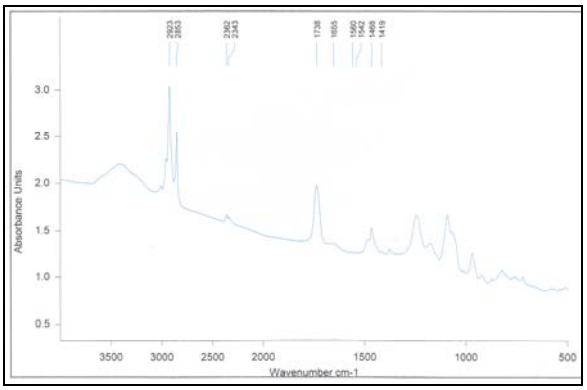

Figure 1 – Overall IR absorption spectrum of L-α-phosphatidylcholine
Слика 1 – ИЦ апсорпциски спектар на L-α-фосфатидилхолин

The vibrational spectra of DNA (Fig. 2) contains a substantial amount of overlapping bands. The IR absorption spectra of DNA mostly depicts vibrational bands of PO_2^- groups. Arround 10 absorption frequencies are seen in the 530-3425 cm^{-1} region. Of these, the OPO group stretching motions, sugar vibrations, CO stretches and existence of particular helices, bond conformations, as well as C = N stretchings are regarded as the most informative prior to and after any complexation. Thus, the vibration band seen at 835 cm^{-1} indicates S-type sugar, that of 1068.7 cm^{-1}-C form of DNA and the bands occurring in the region of 530–800 cm^{-1}-out of plane base vibrations, respectively. Artificial Z-helices seem to dominate the spectrum of DNA, the evidence being based on the band seen at 1407.4 cm^{-1}. Residual water possesses the band at 3425 cm^{-1}, as well as below 850 cm^{-1}, which bands are ascribed to O-H bending vibrations [20]. The region between 1450–1750 cm^{-1} refers to sensitive to H-bonding C = O, C = C and C = N stretchings [25]. As opposed to our recent studies performed with DNA samples prepared as KBr pellets [23], these in the form of thin films yield peaks located at lower wavenumbers, in agreement also with Pevsner and Diem [24].

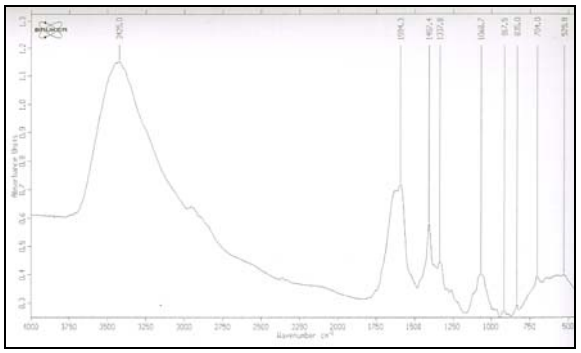

Figure 2 – Overall IR absorption spectrum of DNA. The vibrational frequencies are authomatically assigned by the original softhware OPUS 3.0, as described in Materials and Methods.

Слика 2 – ИЦ апсорпциски спектар на ДНК. Вибрациските фреквенции автоматски се означени со оригиналниот софтвер OPUS 3.0 (опишано во делот Материјали и методи)

Overall IR absorption patterns of the binary mixtures

Major structural transitions of DNA-Mn^{2+} binary mixture (Fig. 3) are seen in the 500–1800 cm^{-1} region. These are ascribed mainly to wagging of planar C-NH_2 bond (616 cm^{-1}) and ribose backbone vibration (1051.8 cm^{-1}), as well as C = N and C = C ring vibrations (1634.8 cm^{-1}). Those at 3240.4 cm^{-1} and 3405.7 cm^{-1} are linked to residual water or to antisymmetric stretching of the C-NH_2 bond. Mn^{2+} affects the two water bands in the DNA spectrum and represents the effect of hydration shell of ions leading to phase separation due to specific distributions of H-bonds of various stregth. These result in increased binding sites and inter- and intra-molecular vibrational coupling within the water structure. Fig. 4 depicts DNA-phosphatidylcholine recognition. The lipid dominated absorbance spectrum with a very little contribution from DNA is obtained. CC stretch frequency of the B-form of the DNA backbone is clearly seen as a single peak. Vibrations observed at 1175–1188 cm^{-1}, 1244.6 cm^{-1} and 1453–1457 cm^{-1} are indications of an A-form helix. Thus, upon recognition with phosphatidylcholine moiety, DNA undegoes B→A helical transition. Fig. 5 shows the IR spectrum of lipid-Mn^{2+} binary mixture representing the biologically important case of membrane phospholipid-metal cation binding. The observed effects are concerned with phosphate, carbonyl (850–1850 cm^{-1}), CH_2 stretchings (1000–2500 cm^{-1}) and water bands (2800–3600 cm^{-1}). The phosphate moiety (1000–1175 cm^{-1}) is slightly affected, whereas H-bonded and unbonded carbonyls are shifted to a greater extent (1635.3 cm^{-1} and 1734.5 cm^{-1}).

The effects could be due to a reduction of H-bonds as a result of lowering the interaction of carbonyl groups with water molecules after rehydration of the samples. Lipid CH_2 signals are only slightly shifted and are less informative regarding the lipid chain order. The IR spectra of lipid carbonyls are more useful. The peak observed at 1736.6 cm^{-1} indicates H-bonded carboyls and its water surrounding. Metal ion shifts the C = O vibrational peaks towards smaller wave numbers due to the increase of the accessability of free carbonyl groups to water molecules or due to interfacial conformational changes of carbonyls. Apparently, a non-random distributions of ions arround the lipids leading to Mn^{2+} insertion into the polar region of phosphatidylcholine takes place.

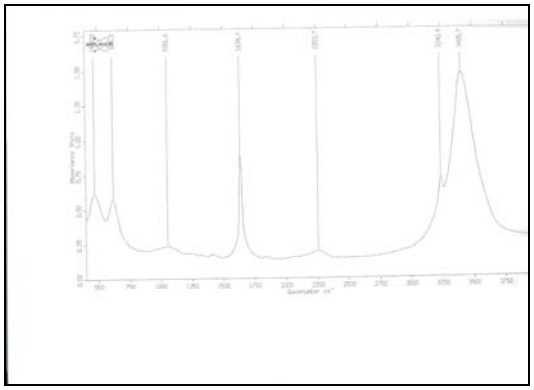

Figure 3 – IR absorption spectrum of DNA-Mn^{2+} binary complex
Слика 3 – ИЦ апсорпциски спектар на двојниот комплекс на ДНК и Mn^{2+}

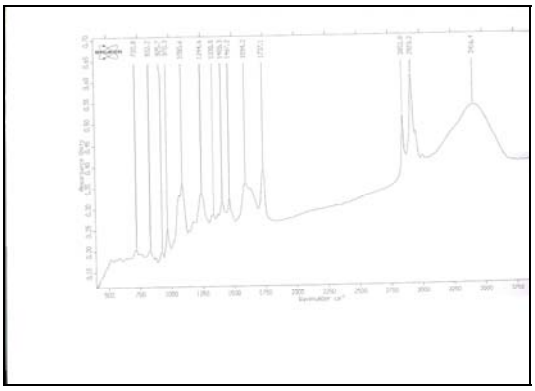

Figure 4 – IR absorption spectrum of DNA- L-α-phosphatidylcholine binary mixture
Слика 4 – ИЦ апсорпциски спектар на двојниот комплекс на ДНК и L-α-фосфатидилхолин

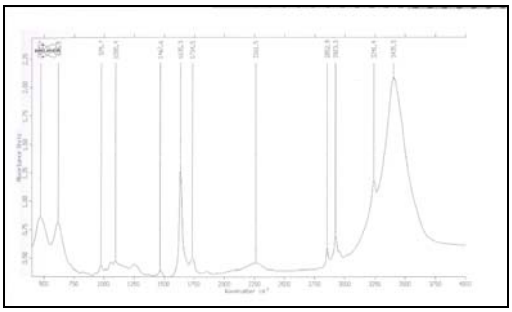

Figure 5 – IR absorption spectrum of L-α-phosphatidylcholine-Mn^{2+} binary mixtute
Слика 5 – ИЦ апсорпциски спектар на двојниот комплекс на L-α-фосфатидилхолин и Mn^{2+}

Spectral Features of Ternary Complex Formations

1. Effects of nucleic acid-lipid recognition on C = O moiety

IR spectra in the carbonyl stretching region of L-α-phosphatidylcholine unilamellar liposomes is shown in Fig. 6. Following our preliminary thermodynamic studies [21] on divalent metal cation driven DNA-phospholipid self-assembly, it is interesting to study the recognition between the engaged molecules in this association by FTIR spectroscopy, first at the level of carbonyl groups, located at the interphase between the hydrophobic hydrocarbon chains and the more hydrophilic headgroup region. The C = O band in the IR absorption spectrum of the lipid is split into several bands, two of which are useful for spectra evaluation. The highest peak appearing at 1732 cm^{-1} is due to unbonded C = O groups, while the downfield peak results from H-bonded C = O groups [27]. In the liquid-crystalline state (60ºC), the narrow peak indicates a more homogeneous distribution of C = O groups after lipid chain melting. The lower peak at 1700 cm^{-1} belonging to carbonyl groups is more diminished, which is interpreted as decreased H-bonding between lipids and residual water molecules in more condensed bilayers under these liquid-crystalline conditions due to increased lipid spacings. Having considered this fact based on the well-establishedestablished phase transition of L-α-phosphatidylcholine at ~39ºC [27], it is expected, on the other hand, that upon hydration of lipid film, the C = O group frequencies change in consistence with an increased possibility of H-bonding due to the presence of water, which could be used for evaluating H-bonding interactions between C = O and OH groups in the dry and rehydrated state. In the spectra of the lipid prepared in KBr pellet form, the main peak appearing at 1736.6 cm^{-1} shifts to 1732 cm^{-1}. Hydration causes νC = O to decrease, as seen by this shift of 4 cm^{-1} towards lower wavenumbers (Fig. 1 vs. Fig. 6). Interestingly, the lower peak at 1652 cm^{-1} did not change. This can be interpreted as

a depiction of the effect of directly bound water molecules. However, the long-range influences of water on lipid backbone accompanying phase transitions cannot be excluded [32]. This demonstrates that the interfacial region of the lipid is tightly linked to the acyl chain part, allowing it to be affected by the phase transition. Such direct water-carbonyl interactions in liquid-crystalline lipids deserve to be exploited further. Apparently, the observed lipid phase transition effects on νC = O stretching modes is composed of a lyotropic phase transition, e. g. A chain melting, followed by water binding to the lipid polar moiety existing in a more hydrophilic L_α phase, in agreement with Selle and Pohle [32]. This sort of detection of such lyotropically driven chain-melting structural transitions by IR spectroscopy demonstrates the excellent suitability of FTIR method for studying such macromolecular systems.

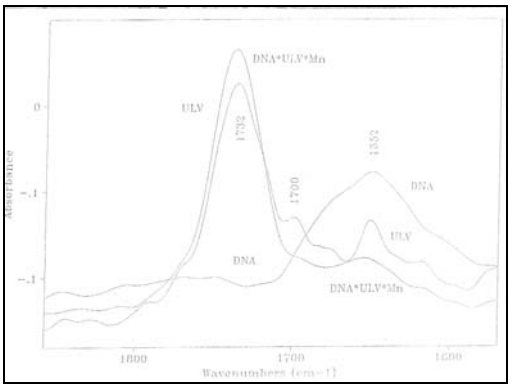

Figure 6 – IR absorbance spectra of DNA, L-α-phosphatidylcholine and their ternary complex with Mn^{2+} depicting effect of hydration on C = O moiety

Слика 6 – ИЦ апсорпциски спектри на ДНК, L-α-фосфатидилхолин и нивен троен комплекс со Mn^{2+} кои го покажуваат ефектот на хидратација врз C = O

Within the employed spectral region DNA possesses one major band (Fig. 6). The wavenumber range employed contains mostly purinic and pyrimidinic vibrations, situated in the 1500–1800 cm^{-1} spectral region, the bands observed being sensitive markers for base pairing and stacking effects. The major nucleic acid band seen at 1652 cm^{-1} is a hydration-induced shift of 3 cm^{-1} of DNA C = O stretching and N-H bending, which usually arises within the 1655–1660 cm^{-1} wavenumber region [9]. The relatively medium height of the peak indicates the existence of thymine base pairing into a double stranded conformation. This is further supported by the diminished intensity of C4 = O4 vibration upon complexation with lipids (Fig. 6), in agreement with Banyay *et al.* [25].

Mn^{2+} ions induce ternary complex formation between DNA and phosphatidylcholine. Comparing the spectra of their complex formed with that of the unbound unilamellar vesicles and DNA alone shows that the IR absorption

spectrum of their ternary complex is a lipid signal dominated with little contribution from DNA. This result is in agreement with more detailed studies on a similar ternary complex with cationic liposomes [33]. The IR spectra of DNA in the complex could be regarded in terms of water activity upon hydration of this isoelectric 1 : 1 : 1 complex. However, besides the marker band indicating thymine base pairing and double stranded structure, it is not clear from the remaining spectrum in which particular conformation DNA exists. Interestingly, our recent IR analysis of anhydrous phosphatidylcholine-divalent metal cation-DNA mixture showed the B→A helical transition of the nucleic acid upon ternary complex formation [23]. Obviously, such a less hydrated A-form of DNA is expected to form under these water-removing conditions. Following hydration, however, the polymorphic nature of DNA becomes apparent, resulting in the adoption of various forms -A, B, C or D, depending on its intrinsic primary structure, as well as degree of hydration and ionic state, as outlined [33]. These structures can be classified into different families, e.g. A and B families, populating different regions of conformational space.

2. Nucleic acid-lipid recognition effects on the phosphatidylcholine headgroup region: interactions of P = O and choline groups

Fig. 7. shows a structural comparison of FTIR spectra of the ternary lipid-Mn^{2+}-DNA complex and its components. The wavenumber range under consideration is that of P=O and choline groups in the phosphatidylcholine headgroup region. The substantial absorption in the range of 1200–1250 cm^{-1} in phospholipids is mainly due to the antisymmetric PO_2^- stretching mode [33]. The spectra of the asymmetric P = O stretching region can be utilized as a vibrational mode providing structural information about the interfacial region of the bilayer [27]. Compared with the anhydrous phosphatidylcholine spectrum (Fig. 1), Fig. 7 shows the appearance of two new bands at 1059 cm^{-1} and 1198 cm^{-1} specific for hydrated unilamellar vesicle suspension. The shift from 1091 cm^{-1} and 1242.6 cm^{-1} towards lower wavenumbers indicates the sensitivity of these vibrations to the formation of H-bonds. Thus, such shifts are observed usually with increased H-bonding [34, 35]. Rehydration inserts a profound effect especially on the latter P = O frequency band. The peak is shifted nearly 5 cm^{-1} to a unilamellar vesicle specific peak of 1237 cm^{-1}. New DNA bands at 1198 cm^{-1} and 1051 cm^{-1} clearly show the different nucleic acid spectra obtained in comparison with KBr pellet samples (Fig. 2). The former depicts the A-form of DNA which moves to this position from 1188 cm^{-1} upon water treatment and the latter band indicates furanose CO stretching of the backbone. However, the substantial height of this peak shows that probably an intermediary short-lived Z-form of DNA predominates, which is surprising since calf thymus DNA normally does not adopt such a conformation. This is an interesting transition,

since until now in similar conditions the lipid induced A-helix was usually the most frequently found form [33]. The data confirms the conclusion that the presence of water molecules in polar surroundings may significantly increase their IR absorption intensity [17].

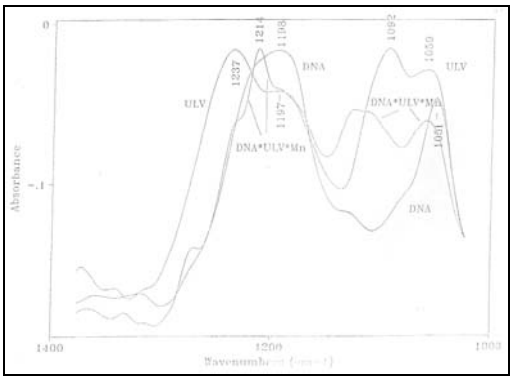

Figure 7 – IR absorbance spectra of DNA-Mn^{2+}-L-α-phosphatidylcholine ternary complex and its components, with particular reference to effect of hydration on P = O moiety
Слика 7 – ИЦ апсорпциски спектри на тројниот комплекс на ДНК-Mn^{2+}-L-α-фосфатидилхолин и неговите компоненти, со посебен осврт на ефектот на хидратација врз P = O

Examination of the IR absorption spectrum of the unilamellar vesicle-Mn^{2+}-DNA ternary complex also indicates the lipid driven nucleic acid helical transition into such an unexpected Z-form conformation. The two major DNA specific bands seen are those at 1214 $cm^{-1,}$ the main marker for the Z-form of helix, and at 1197, which is a DNA hydration-induced shift of 1 cm^{-1}. Water molecules also shift the unilamellar vesicle signal from 1237 cm^{-1} to 1214 cm^{-1}, creating an overlap band. The band at 1170 cm^{-1} is due to CO-O-C asymmetric stretching of phospholipids. The frequencies at 1059 cm^{-1} and 1060 cm^{-1} are phospholipid signals, the latter of which would otherwise be thought of as a DNA ribose CO stretching mode. Thus, apparently, phosphatidylcholine induces at least some part of the nucleic acid to adopt a structure towards the artificial Z-form.

3. IR absorption of the DNA-Mn^{2+}-L-α-phosphatidylcholine ternary complex νOH region occupied by the O-H stretching vibration band of water

To study the role of hydration in following the spectral differences in structures of lipid-bound DNA in contrast to free DNA, the spectra in the *ν*OH

region occupied by the O-H stretching vibration band of water were examined (Fig. 8). Such an approach permits evaluating the effect of the simultaneous presence of Mn^{2+} ions and phosphatidylcholine on the DNA-specific bands. Moreover, the effects of metal ions and DNA on phospholipids is possible in CO and CH vibration regions, where the nucleic acid and phospholipid bands do not overlap. Spectral interpretation in this wavenumber range should be performed with caution, since rather complicated and not well understood relationships exist between aggregations in aqueous systems and their spectroscopic responses [33].

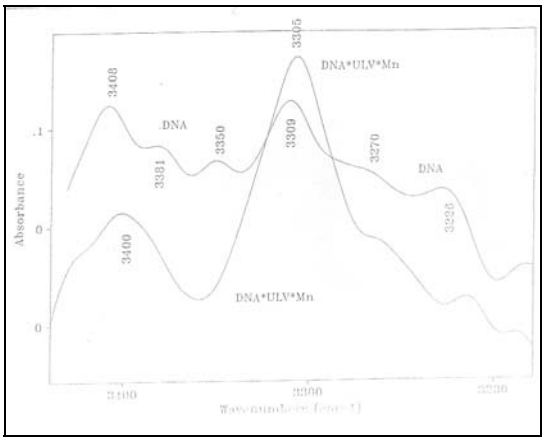

Figure 8 – IR spectra of Mn^{2+}-induced lipid-associated DNA in the vOH region occupied by the O-H stretching vibration band of water

Слика 8 – ИЦ спектар на комплексот липид-ДНК формиран со посредство на Mn^{2+} во vOH регионот заземен од издолжената О-Н вибрациска лента на водата

The DNA band at 3408 cm^{-1} is a vibration of the planar C-NH_2 bond [19]. The rest of the peaks are relevant subtransitions resulting from hydration-dependent downshifts of asymmetric phosphate group stretchings due to the H-bonding of water. The signal is also regularly detected in the substance or in KBr pellets (Figs. 1, 2 vs. Fig. 8) [9]. The broad *v*OH appears to be due to the overall superposition of several single bands representing a number of coexisting subpopulations of water aggregates of various sizes and binding affinities. The next band specific for lipid-bound DNA situated at around 3226 cm^{-1} depicts the existence of strong H-bonds, since in general the lower the wavenumber of the *v*OH water band the stronger the hydrogen bonding [33]. The formation of different types of hydration water is highly probable in lipid-complexed DNA in comparison with unbound DNA, as seen by its higher peak

at 3226 cm^{-1}. The 3220 cm^{-1} frequency peak can be ascribed to more tightly bound water molecules engaged in long-lived and stronger H-bonds, probably linking DNA double strands and lipid headgroups. The greater peak observed at 3305 cm^{-1} represents some fraction with weaker H-bonds, obtained under these conditions of raised temperature, in contrast to the diminished peak of 3309 cm^{-1} of unbound DNA (Fig. 8). The role of particular bound and unbound water molecules in maintaining the thermodynamically more favourable conformations of lipid-nucleic acid assemblies remains to be determined.

Interestingly, in contrast to the rest of the spectrum of the analysed DNA-lipid complex, where the domination of lipid vibrational frequencies is obvious, in this case certain bands appear to originate from DNA. Thus, the broad peak at around 3400 cm^{-1} could have arisen due to DNA. This possibility is further strengthened by the overlapping bands of ULV- Mn^{2+}-DNA (3305 cm^{-1}) and free nucleic acid (3309 cm^{-1}). However, applying a more accurate optimization of spectral subtraction [33], as well as optimization of drying, rehydrating samples and humidity control is necessary to avoid erroneous deductions [25].

Structure of ULV- Mn^{2+}-DNA ternary complex

The appearance of two distinct IR absorption bands of water indicates the existence of two different water moieties within the lipid-DNA complex. The first is that at the interface between DNA double stranded helices sandwiched between lipid arrays. Besides this tightly bound water, another type is represented by interstitial water linking lipid layers facilitating DNA-DNA interactions. The self-assembly form consists of highly ordered multilamellar arrays in which the hydrated DNA is located between lipid bilayers bridged together by the employed metal cations linking the phosphate groups of the nucleic acid with lipid polar headgroups. In this respect, the model is similar to that deduced for DNA-Mg^{2+}-phospholipids [21–23]. Mg^{2+} and Mn^{2+} seem to act in a similar way in DNA-lipid recognition events, the difference being in the more restricted features of the Mn^{2+} of preferential mode of binding to nucleic acid groups [18].

Implications in designing non-viral gene delivery formulations

The present work describes FTIR measurements of interfacial DNA-neutral phospholipid recognition in the presence of divalent metal cations. The aim is to use such preliminary studies for the deduction and design of alternative zwitterionic lipid-based nucleic acid delivery vectors. In addition, these binding data could be utilized for the development of immobilized liposome chromatography for sequence-specific DNA purification and analyses [23]. This issue is also of biopharmaceutical relevance [29]. Thus, the instability of lipid-based pharmaceutical formulations is a major problem to bypass before their

commercial application in gene therapy. Therefore, the manufacturing of stable and easy to handle lipoplex preparations becomes an important item. For this reason, it is useful to examine them in different physical states. The expected huge potential of non-viral gene therapy vectors can be realized provided that vehicles with controllable physicochemical properties are manufactured on an industrial scale. A proper understanding of these features governing nucleic acid-lipid assembly formation becomes a prerequisite for designing improved lipoplexes. Hence, the stability testing of DNA-lipid arrays under both hydrating and anhydrous conditions, as perfomed in this study is important.

The choice of a suitable, easy to use and user-friendly measuring system for following lipid-DNA complex formations is necessary in most of the biophysical studies of this sort. By further clarifying the precise mechanisms of these interactions, a better understanding of their cellular pharmacological targets can be obtained, as well as making possible the designing of novel lipid based gene delivery formulations with improved bioavailability properties. The analytical approaches currently employed in studying nucleic acid-liposome associations are limited either by the requirement of experienced personnel, sophisticated instruments or by methodological difficulties. Therefore, developing novel experimental procedures utilizing complementary, easier to apply techniques is to be emphasized. IR spectroscopy is frequently applied for following biomolecule structure and kinetics [6–9,17–20]. Besides its potential for being employed as a tool for identifying types of chemical bonds, the method permits determination of the orientation of a functional group and its location in the particular structure [16, 17, 20, 21].

Trying to simulate the mode of action of more efficient viruses in cell transfections, our emphasis is on designing vehicles acting through target-cell membrane destabilization and lipid fusion, instead of following the problematic receptor-mediated pathways, where endosomal degradation is a barrier difficult to overcome. Such fusion-based intracellular delivery of therapeutic nucleic acids would also facilitate avoiding the low membrane permeability, instability in serum, sequence non-specific recognitions, as well as the short half-life in circulation following injection of lipoplexes [36]. It is hoped that chemical conjugations and relevant manipulations could help in overcoming these cellular barriers. Since an excessive positive charge often leads to cytotoxicity [1–4], optimizing electrostatic control of the lipoplex formation by designing cationic compounds usually facilitates nucleic acid transfer through the negatively charged surfaces of target cells. The utilization of more naturally encountered biointerfaces is emphasized instead of applying bulky polymers. In this respect, it is worth studying the design of polyelectrolyte complex formations between zwitterionic lipid vesicles and nucleic acids, mediated by various small inorganic cations, acting as DNA compacting and condensing agents. Moreo-

ver, our previous thermodynamic studies on ternary complexes formed between nucleic acid, divalent metal cations and phospholipid vesicles has already shown the stabilization of nucleic acid helices upon recognition with lipids in the presence of inorganic ions [21–23].

In such designs, before switching to biologically more relevant *in vitro* transfection experiments utilizing numerous gene reporter systems, it is important first to achieve a stable and well characterized DNA-lipid formulation with controllable physicochemical properties. The objective is to deduce alternative mechanisms of therapeutic gene delivery to the proper sites and facilitating binding to their target sequences in the nucleus while avoiding the receptor-mediated intake. In this respect, our model focuses on electrostatic and hydrophobic control of membrane destabilization, using lipidic carriers, in terms of their well documented potential as drug and gene delivery systems [1–4]. In addition, we have used small metal cations' charge for achieving electrostatic nucleic acid-lipid recognition, which with its slightly positively charged surface would facilitate cellular penetration through the negatively charged cell membrane of the target cells. The precise mechanisms of this membrane fusion related internalization of polyribonucleotides has not yet been studied in detail and deserve to be exploited further.

Designs of this sort rely on controlling the interfacial behaviour of the engaged biomacromolecules and hence, knowledge of their surface structure is crucial for constructing efficient lipid-based gene delivery systems. Studies on these carriers would also promote research on proposing new clues to their interactions with various cellular surfaces, as well as internalization mechanisms of the entrapped therapeutic DNA through membranes. The appearance of gel-like microdomains in such a liquid-crystalline environment could serve as sites for larger lipid phase separation undertaken by cations and could further contribute to fusion of lipid carriers with a plasma membranes of cells [21]. The suggested model considers interfacial recognition and phase separation and is based on the complexation of several lipid surface curvatures resulting in their fusion, induced by polynucleotide chain unwinding. In this highly transfection competent structure, divalent metal cations bridge the DNA polyanionic helix to charge reversed liposomes, further facilitating their membrane destabilizing features.

Our model relies on the several distinct ways through which inorganic metal cations can enhance gene transfection efficiency. Thus, their effect on nuclear stability and the topology of genomic DNA seems to be one cellular mechanism for controlling gene transcription. Metal cations target complexed DNA via nuclear localization signals (NLS) to transcriptionally active chromatin regions. Thus, gene carrier-mediated nuclear targeting could represent a way of facilitating the gene expression profiles. Moreover, metal cations can

govern the conformation of the transfected DNA, indicating the role of nucleic acid topology control in gene transfection. The model hypothesizes that, based on these properties of Mn^{2+} as an interfacial complexing agent, similarly to cell-penetrating cationic peptides, an inorganic cation-mediated cell membrane destabilization could take place due to the electrostatic and hydrophobic property of the forces engaged. The model envisages the desired properties of Mn^{2+} of bridging the neutral lipid vesicles with the entrapped nucleic acid in close proximity to a negatively charged target cell membrane. Upon reaching the optimal combination of surface forces and amphiphilicity needed for bilayer destabilization, the DNA-inserted fusion between zwitterionic lipids and cell membrane occurs. The proposal needs to be explored further with relevant gene-reporter systems for *in vitro* transfection efficacy, as well as with fluorescent assays for the intracellular fate of the lipoplexes. On the other hand, the described ability of divalent metal cations in both DNA and lipid binding, partitioning, phase separation and the role in nuclear delivery of exogenous DNA undertaken by helix-binding molecules supports the hypothesis that divalent metal cations can confer membrane-permeant properties on complexed DNA. The exact mechanism of nucleic acid exerted phospholipid fusion in the presence of metal cations remains to be elucidated. Apparently, similarly to cationic peptides, the electrostatic screening of the hydration shell of inorganic cations compensates for the intervesicle repulsive forces, leading to lipid mixing. The model receives further support from the recent interesting results of Sato *et al.* [37] on divalent cation-induced DNA attachment to phospholipids. Our fluorescent microscopy work on these DNA-lipid nanoassemblies is in progress and will be reported separately.

Conclusions

Despite the significant progress in gene therapy trials, the cytotoxicity issues, serum degradation problems and cellular resistance to a broad spectrum of structurally diverse therapeutic nucleic acid designs is of major concern. The precise mechanisms of the involvement of the target cell membrane in the control and regulation of the intracellular fate of lipoplexes remain poorly understood. In this respect, studying the interactions of various nonviral lipid based formulations with model and biomembranes attracts research efforts. The choice of a suitable technique to monitor dynamic structural transitions of the lipoplexes under study is crucial for formulating genosomes with improved *in vitro* transfection properties. Vibrational spetroscopy represents a powerful but still unappreciated approach to the study of different lipid-DNA complexes, as it provides detailed information about the dynamic changes of both lipids and

nucleic acids simultaneously without significant perturbations. The current work presents our recent IR measurements on the surface recognition between rehydrated individul species of the newly suggested therapeutic nanoscale DNA-divalent metal cation-neutral phospholipid ternary complex. It is shown that by employing such a simple spectroscopic approach, valuable data on the degree of conformational order, on the hydration state of interfacial groups and on the headgroup conformation of lipids can be obtained. On the other hand, the effects of lipid recognition on DNA structure can also be envisaged. The basic applications of IR spectroscopy in following nucleic acid-lipid interactions show promising results in terms of high signal-to-noise ratio, as well as for the improvement of spectral resolution of the lipoplex complex formations.

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REFERENCES

1. Templeton NS. (2001): Developments in liposomal gene delivery. *Expert Opin Biol Ther;* 1: 1–4.

2. Templeton NS. (2002): Liposomal delivery of nucleic acids in vivo. *DNA Cell Biol;* 12: 857–867.

3. Templeton NS. (2003): *Gene and Cell Therapy-Therapeutic Mechanism and Strategies*. 2nd ed. Marcel Dekker.

4. Ulrich AS. (2002): Biophysical apsects of using liposomes as delivery vehicles. *Bioscience Rep;* 22, 2: 129–149.

5. Safinya CR. (2001): Structures of lipid-DNA complexes: supramolecular assembly and gene delivery. *Curr Opin Struct Biol;* 11: 440–448.

6. Zundel G. (1983): *Polar Interactions, Hydration, Proton Conduction and Conformation of Biological Systems – Infrared Results*, pp. 243, in: W. Hoppe, W. Lohmann, H. Markl, H. Ziegler (Eds.), *Biophysics*, (Springer-Verlag, Berlin, Hedelberg, New York, Tokyo).

7. Johnston SF. (1991): *Fourier Transform Infrared – A Constantly Evolving Technology*. Ellis Horwood Limited, UK.

8. Taillandier IE., Liquier J. (2002): In: J.M. Chalmers and Griffith (eds.), *Handbook of Vibrational Spectroscopy* (John Wiley & Sons, Chichester), Vol. 5, Chapter 2, pp. 3465.

9. Günzler H. and Gremlich H.-U. (2002): *IR Spectroscopy – An Introduction*. Wiley-VCH Verlag GmbH, 69469, Weinheim (Germany).

10. Diem M., Romeo M., Boydston-White S., Miljkovic M. *et al.* (2004): A decade of vibrational micro-spectroscopy of human cells and tissue (1994–2004). *Analyst;* 129, 10: 880–885.

11. Lasch P., Naumann D. (2006): Spatial resolution in infrared microspectroscopic imaging of tissues. *Biochim Biophys Acta;* 1758, 7: 814–829.

12. Walsh MJ., German MJ., Singh M. *et al.* (2006): IR microspectroscopy: potential applications in cervical cancer screening. *Cancer Lett;* May 18.

13. Dovbeshko GI., Chegel VI., Gridina NY. *et al*. (2002): Surface enhanced IR absorption of nucleic acids from tumor cells: FTIR reflectance study. *Biopolymers;* 67, 6: 470–486.

14. Repnytska OP., Dovbeshko GI., Tryndiak VP. *et al*. (2004): Structural organisation of nucleic acids from tumour cells. *Faraday Discuss;* 126: 61–76.

15. Sahu R., Mordechai S. (2005): Fourier transform infrared spectroscopy in cancer detection. *Future Oncol;* 1, 5: 635–647.

16. Andrus PG. (2006): Cancer monitoring by FTIR spectroscopy. *Technol Cancer Res Treat;* 5, 2: 157–167.

17. Pevsner A., Diem M. (2001): Infrared spectroscopic studies of major cellular components. Part II: The effect of hydration on the spectra of nucleic acids. *Appl Spectrosc;* 55: 1502–1505.

18. Wolfgang Saenger (1987): *Principles of Nucleic Acid Structure*, Chapter 17: Water and Nucleic Acids. (Mir Publishers, Moscow), pp. 393 (Russian translation).

19. Bloomfield VA., Crothers DM. and Tinoco I. Jr. (2000): *Nucleic Acids-Structures, Properties, and Functions*. (University Science Books, Sausalito, CA 94965) pp. 210.

20. Choosakoonkriang S., Wiethoff CM., Anchordoquy TJ. *et al*. (2001): Infrared spectroscopic characterization of the interaction of cationic lipids with plasmid DNA. *J Biol Chem;* 276, 11: 8037–8043.

21. Süleymanoğlu E. (2004a): A nanoscale polynucleotide-neutral liposome self-assemblies formulated for therapeutic gene delivery. *Electron J Biomed;* 2: 13–35.

22. Süleymanoğlu E. (2004b): Divalent cation induced DNA-zwitterionic vesicle formulation compacted for gene delivery: thermodynamic aspects. *Pak J Pharm Sci;* 17, 2: 129–133.

23. Manavbaşı Y., Süleymanoğlu E. (2006): *Assessment of structures of poly(ribo)nucleotide-phospholipid self-assemblies and their implications in gene transfection and DNA chromatography*, Proc. of the 8th International Symposium on Pharmaceutical Sciences, Ankara, Turkey, pp. 213.

24. Pevsner A., Diem M. (2003): IR spectroscopic studies of major cellular components. III. Hydration of protein, nucleic acid, and phospholpid films. *Biopolymers (Biospectroscopy);* 72: 282–289.

25. Banyay M., Sarkar M., Gräslund A. (2003): A library of IR bands of nucleic acids in solution. *Biophys Chem;* 104: 477–488.

26. Krummel AT., Zanni MT. (2006): DNA vibrational coupling revealed with two-dimensional infrared spectroscopy: insight into why vibrational spectroscopy is sensitive to DNA structure. *J Phys Chem B;* 110: 13991–14000.

27. Popova AV., Hincha DK. (2003): Intermolecular interactions in dry and rehydrated pure and mixed bilayers of phosphatidylcholine and digalactosyldiacylglycerol: a Fourier transform infrared spectroscopy study. *Biophys J;* 85: 1682–1690.

28. Hincha DK., Zuther E., Hellwege EM. *et al*. (2002): Specific affects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. *Glycobiology;* 12: 103–110.

29. Sorgi F., Huang L. (1995): A dry powder formulation for gene therapy. *Pharm Res;* 12: S266.

30. Lai E., van Zanten JH. (2002): Evidence of lipoplex dissociation in liquid formulations. *J Pharm Sci;* 91: 1225–1232.

31. Clement J., Keifer K., Kimpfler A. *et al*. (2005): Large-scale production of lipoplexes with long shelf-life. *Eur J Pharmaceut Biopharm;* 59: 35–43.

32. Selle C., Pohle W. (1998): Fourier transform infrared spectroscopy as a probe for the study of the hydration of lipid self-assemblies. II. Water binding versus phase transitions. *Biospectroscopy;* 4: 281–294.

33. Pohle W., Selle C., Gauger DR. *et al*. (2000): FTIR spectroscopic characterization of a cationic lipid-DNA complex and its components. *Phys Chem Chem Phys;* 2: 4642–4650.

34. Lewis RNAH., McElhaney RN. (1998): The structure and organization of phospholipid bilayers as revealed by infrared spectroscopy. *Chem Phys Lipids;* 96: 9–21.

35. Lewis RNAH., McElhaney RN. (1996): Fourier transform infrared spectroscopy in the study of hydrated lipids and lipid bilayer membranes. In: H.H. Mantsch and D. Chapman (Eds.), *Infrared Spectroscopy of Biomolecules.* Wiley-Liss, New York, pp. 159.

36. Dass R. (2002): Cytotoxicity issues pertinent to lipoplex-mediated gene therapy *in vivo*. *J Pharm Pharmacol;* 54: 593–601.

37. Sato Y., Nomura S-I., Yoshikawa K. (2003): Enhanced uptake of giant DNA in cell-sized liposomes. *Chem Phys Lett;* 380: 279–285.

Р е з и м е

ПРИМЕНА НА ИНФРАЦРВЕНА СПЕКТРОСКОПИЈА ЗА АНАЛИЗА НА ЛИПИДЕН КОМПЛЕКС СО ДНК ПО РЕХИДРАТАЦИЈА И НЕГОВА ПОДГОТОВКА ВО НАНОФАРМАЦЕВТСКА ФОРМА СО КОМПЛЕКСИРАЊЕ СО МЕТАЛ

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Во испитувањето беше користена ИЦ спектроскопија со цел да се следи динамичкиот структурен премин на тројниот комплекс, со потенцијал за трансфекција, составен од ДНК, двовалентен метал и катјонски фосфолипид со големина од 100 nm, преку препознавање на одделните молекули вклучени во комплексот. Особено внимание е посветено на изборот на малите наелектризирани метални јони како комплексирачки средства и цвитерјонот L-α-фосфатидилхолин како замена за актуелно користените проблематични катјонски липиди во генската трансфекција. Во актуелното испитување, споредувани се спектрите на одделните компоненти со спектрите на бинарните смеси составени од нуклеинска киселина-липид, односно липид-метал, како и со тројниот комплекс. Податоците добиени за карбонилната, холинската и CH групата се користени за определување на профилот на комплексот ДНК-фосфолипид создаден со посредство на Mn^{2+}. Јонските ефекти се должат на дехидратација на фосфатите и водородно врзување на карбонилите. Анализирана е можната структура на тројниот комплекс, вклучително и потенцијалот истиот да биде користен како невирусна формулација за генска испорака.

Клучни зборови: идентификација на ДНК-липид, троен комплекс на нуклеинска киселина-двовалентен метал-катјонски фосфолипид, ИЦ спектроскопија, невирусна генска испорака, липоплекс.

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