HKD 1926 CROATICA CHEMICA ACTA

CROATICA CHEMICA ACTA
CCACAA, ISSN 0011-1643, e-ISSN 1334-417X
Croat. Chem. Acta **83** (4) (2010) 387–393.
CCA-3431

Original Scientific Article

Interaction of High Density Lipoprotein with Nicotine – an IR and Raman Study

Dubravka Krilov,* Marin Kosović, Maja Balarin, Ozren Gamulin, and Jasminka Brnjas-Kraljević

Department of Physics and Biophysics, School of Medicine, University of Zagreb, Šalata 3b, Zagreb, HR-10000 Croatia

RECEIVED JULY 22, 2009; REVISED FEBRUARY 24, 2010; ACCEPTED MAY 18, 2010

Abstract. The direct interaction of high density lipoprotein (HDL) with nicotine, one of the major components of cigarette particulate matter, has been studied here at molecular level. Nicotine affects the vibration modes of HDL due to its embedment within the lipid monolayer. The changes in the positions and intensities of vibration bands in protein and lipid domain of the particle were studied by IR and Raman spectroscopy. Three types of samples were prepared: native HDL sample and two HDL samples with added nicotine. The molecular ratio of nicotine/phospholipids in two samples was 1 : 12 and 1 : 6. The same types of samples were prepared from liposomes containing phosphatidylcholine, and sphingomyelin with addition of cholesterol. Spectra of liposome samples were used for distinguishing and attribution of lipid bands in spectra of HDL samples, where the majority of changes were observed. The incorporation of nicotine into lipid monolayer induces changes in the lipid bands from the vibrations in acyl chains and head groups of phospholipids. The changes in vibration bands from particular amino acids' residues confirm that nicotine molecule is located within lipid monolayer but close to lipid-protein interface.

Keywords: HDL, nicotine, liposome, IR spectroscopy, Raman spectroscopy

INTRODUCTION

Many clinical studies present the evidence that cigarette smoking is one of important risk factors for the development of cardiovascular disease (CVD).[1,2] Atherosclerosis as a cause of CVD is enhanced by abnormal lipid and lipoprotein profiles. Today it is well known that one of major negative influences of cigarette smoking is its impact on lipoprotein metabolism. It was found that smokers have higher concentrations of total cholesterol, triglycerides and very low density lipoprotein (VLDL) and lower concentrations of high density lipoprotein (HDL) and apolipoprotein AI (apo A-I).[3] The decrease of HDL, a major cardioprotective lipoprotein can alter the reverse cholesterol transport, promoting the development of atherosclerosis. Nicotine is one of the major components of particulate matter (tar) and is responsible for smoking addiction, but there are different opinions about its role in atherosclerosis.[4] However, the studies of lipoprotein profile after smoking cessation while the person was on nicotine replacement therapy showed that HDL level was not normalized until the patch was removed.[3] In order to better understand the influence of nicotine on HDL, the possible direct interaction of nicotine with HDL particles should be investigated. In this study we wanted to find out whether nicotine added in HDL solution *in vitro* will interact with the particle. We expected that the incorporation of non polar nicotine molecules within particle's lipid monolayer will induce structural changes which could affect the vibration modes in protein and lipid domain of HDL. These changes were analyzed in IR and Raman spectra of HDL samples with and without added nicotine.

EXPERIMENTAL

HDL was isolated by the sequential ultracentrifugation of pooled plasma from normolipidemic donors.[5] The cut-off density (ρ = 1.125 g cm^{-3}) was adjusted by adding KBr. Each centrifugation procedure was performed at 50 000 rpm for 24 hours at 10 °C in a Beckman 70 Ti rotor. EDTA (1 g L^{-1}) was added in the solution through all preparation steps to protect lipoprotein against peroxidation. Before spectroscopic measurement, the solution was dialyzed against degassed 0.01 M phosphate buffer, pH = 7.4 and stored at 4 °C. The purity of isolated HDL was checked by gel-electrophoresis as described elsewhere.[6] The concentration of HDL solution was determined by the standard Lowry method as 54.19 μg of protein per μL, *i.e.* 0.55 mmol L^{-1}.

* Author to whom correspondence should be addressed. (E-mail: krilov@mef.hr)

Liposome samples were prepared by mixing solutions of phosphatidylcholine (PC), sphingomyelin (SM) and cholesterol (C) dissolved in chloroform-methanol (2 : 1, *v/v*). The aliquots were combined to obtain the molar ratio of PC : SM : C as 2 : 1 : 0.3 and calculated to achieve final concentration of lipid solution as 6.7 mmol L^{-1}. The solvent was slowly removed from the lipid solution under a stream of nitrogen and additionally under vacuum for 1 hour. The lipid film was further dispersed in phosphate buffer by shaking for 5 minutes in vortex. Sonication of the resultant liposomal solution led to formation of unilamellar vesicles.

Three types of HDL and liposome samples were prepared: sample of native solution, and two samples with added nicotine. Nicotine was added to HDL to obtain the molecular ratio of nicotine/phospholipids 1 : 12 (about 5.5 nicotine molecules to one lipoprotein particle, concentration of nicotine 3.025 mmol L^{-1}) and 1 : 6 (about 11 nicotine molecules to one lipoprotein particle, concentration of nicotine 6.05 mmol L^{-1}). The concentration of nicotine in liposome solution for the molecular ratio of nicotine / phospholipid 1:12 was 0.55 mmol L^{-1} and for the ratio 1:6 was 1.10 mmol L^{-1}. The concentration of pure nicotine solution was 6.05 mmol L^{-1}. Spectra of three types of samples in HDL or liposomes are labelled A, B and C respectively in all figures. For IR measurements 50 μL lipoprotein, liposome or nicotine solution was placed on ZnSe window and dried under nitrogen to obtain thin film. For Raman measurements, the samples as solutions were used. Before the analysis, the spectrum of buffer was subtracted from the experimental spectrum. IR and Raman spectra of all prepared samples were recorded at room temperature in the region 2000–600 cm^{-1} with Perkin Elmer Spectrum GX FT-IR with 4 cm^{-1} resolution. IR spectra were recorded in transmission mode equipped with DTGS detector. For liposome and nicotine samples 50 scans were co added and for HDL samples 100 scans were co added. Raman spectra were recorded in backscattering mode, equipped with Ultra-InGaAs detector and Nd:YAG laser at 1064 nm. For liposome and nicotine samples 2500 scans were co added using laser power of 1000 mW, while for HDL samples 500 scans were co added using laser power 500 mW. The spectrometer was continually purged with nitrogen to remove the water vapour from the detector and sample compartment.

RESULTS

IR Spectra

The dominant bands in IR spectra of HDL arise from the vibrations in lipid domain, mostly from phospholipids and cholesterol in lipid monolayer. The protein bands, amide I and amide II, are well expressed while the vibrations from the amino acids' residues are usually rather weak. The liposome spectrum was used as the additional source of information for the assignment of lipid bands[7] in HDL. IR spectra of HDL, liposome (LS) and nicotine thin films in the region 2000–600 cm^{-1} are presented in Figure 1.

The phospholipid bands from LS spectrum are labelled by asterix in HDL spectrum. The spectrum of nicotine was not observable.

In the region 1800–1500 cm^{-1} (Figure 2), there is

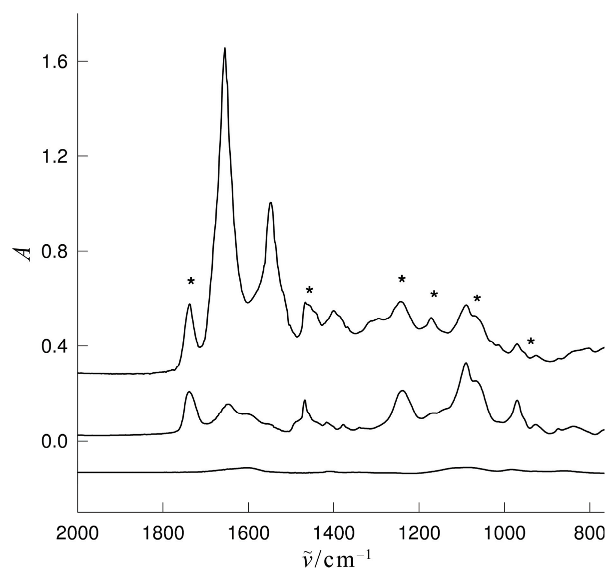

Figure 1. IR spectra of native samples, HDL, LS and nicotine in the region 2000–600 cm^{-1}. The phospholipid bands, labelled by asterix in HDL spectrum are assigned by comparison with LS spectrum.

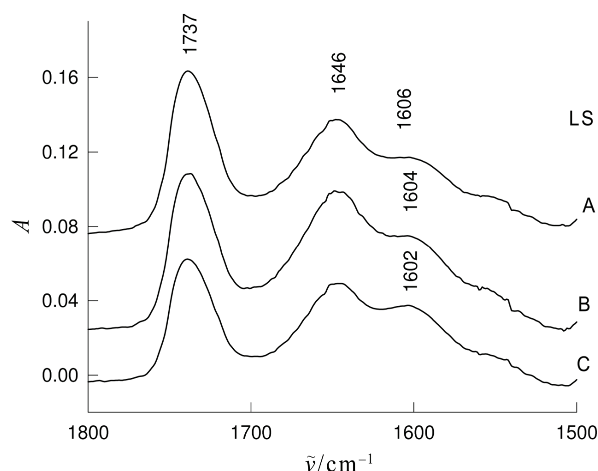

Figure 2. IR spectra of liposome samples in the region 1800–1500 cm^{-1}: native (A), with added nicotine (1 molecule to 12 molecules of phospholipids) (B), with added nicotine (1 molecule to 6 molecules of phospholipids) (C). The positions of three lipid bands are indicated in the figure and their assignation is listed in Table 1.

Table 1. Phospholipid bands from IR spectra of liposomes in the interval 1800 − 1500 cm^{-1}

$\tilde{\nu}$ / cm^{-1}	vibration
1737	C=O stretching
1646	C=C stretching
1606	C=C stretching, sample A
1604	C=C stretching, sample B
1602	C=C stretching, sample C

Table 2. Bands from IR spectra of HDL below 1000 cm^{-1}

	$\tilde{\nu}$ / cm^{-1}	vibration
lipid bands	874	C–C stretching, acyl chains
	833	C–C stretching, choline
	808	O–P–O asymmetric stretching
	768	O–P–O symmetric stretching
	737	CH_2 bending, core lipids
	722	CH_2 rocking, acyl chains, sample A
	717	CH_2 rocking, acyl chains, sample C
	702	CH_2 rocking, acyl chains
protein bands	808	CH_2 bending, Trp
	737	ring, Tyr
	665	skeletal
	624	skeletal

no difference among the spectra of native HDL sample and those with added nicotine (not shown).

In liposome spectra of three samples, C=O stretching band from ester group in phospholipids, and doublet C=C stretching band from phospholipids[8] were observed. Their positions are listed in Table 1.

These bands are overlapped by much stronger amide I band in HDL spectrum. One component of doublet is shifted from 1606 cm^{-1} in spectrum of native liposome (A), to 1604 cm^{-1} (B) and further to 1602 cm^{-1} (C) in the samples with nicotine. The intensity of this component increases relatively to the intensity of the other component, at 1646 cm^{-1}.

The region 1400 − 1300 cm^{-1} contains a spectral pattern which is the source of information about the conformational states of acyl chains in lipids.[9] It is composed of methyl deformation band and several methylene wagging bands. In HDL samples, CH_3 band is overlapped by strong COO^- stretching vibration from Glu and Asp.[10] On the contrary, in liposome spectra the lipid deformation bands in this region are clearly visible, Figure 3.

The decomposition and curve fitting procedure were performed in order to check the influence of nicotine on the conformation of acyl chains. The spectrum of native liposome, was fitted with three component bands: methyl deformation band (methyl umbrella) at 1378 cm^{-1} and methylene bands from two chain conformers, kink (*gtg′*) + *gtg* at 1368 cm^{-1} and end *gauche* at 1340 cm^{-1}.[9] The incorporation of nicotine causes the change in the spectrum which is reflected as the additional component in fitting procedure. This component is attributed to *gg* conformer at 1353 cm^{-1} (B) or at 1355 cm^{-1}(C).[9]

In the region below 1000 cm^{-1} several bands from lipid and protein domain were observed in HDL samples, Figure 4. The positions of lipid and protein bands are listed in Table 2.

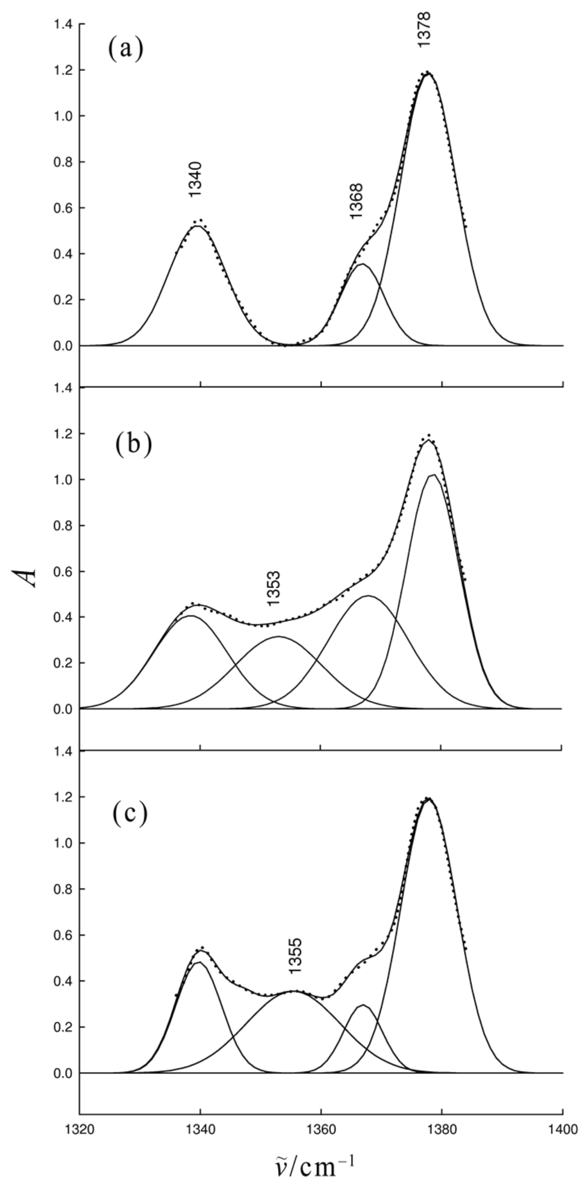

Figure 3. IR spectra of liposomes from three samples marked as in Figure 2, in the region 1400 − 1300 cm^{-1}. The component bands of different conformers were obtained by decomposition and curve fitting procedure. Experimental spectrum is presented by dots and calculated curve by solid line.

Table 3. Bands from Raman spectra of HDL in the interval 1200 – 1000 cm^{-1}

	Raman shift / cm^{-1}	vibration
lipid bands	1130	*trans* C–C stretching
	1107	*gauche* C–C stretching
	1087	*trans* C–C stretching
	1068	*trans* C–C stretching
	1055	P–O–C stretching, sample C
protein bands	1180	Tyr, ring bending
	1158	Phe, ring bending
	1030	Phe, ring breathing
	1006	Phe, ring breathing

Table 4. Bands from Raman spectra of HDL below 1000 cm^{-1}

	Raman shift / cm^{-1}	vibration
lipid bands	900	C–C stretching skeletal
	882	C–C stretching skeletal
protein bands	958	C–C stretching, skeletal
	941	C–C stretching, skeletal, sample A
	935	C–C stretching, skeletal, sample B
	933	C–C stretching, skeletal, sample C
	878	Tyr, ring
	853/837	Tyr, Fermi resonance, sample A
	853/836	Tyr, Fermi resonance, sample B
	853/829	Tyr, Fermi resonance, sample C

Skeletal C–C stretching band from acyl chains[8] at 874 cm^{-1} and band from stretching vibrations in choline head[11], at 833 cm^{-1}, decrease with the addition of nicotine in HDL. Two bands from stretching O–P–O vibrations, asymmetric at 808 cm^{-1} and symmetric at 768 cm^{-1}, are present in the spectra.[8] The incorporation of nicotine increases the asymmetric stretching band but decreases the symmetric stretching one. However, the first one is probably mixed with Trp CH_2 bending vibration.[12] That could explain the overall increase and change in shape of this band. The two bands between 730 cm^{-1} and 700 cm^{-1}, observed also in liposome spectrum, are attributed to rocking vibrations from CH_2 groups in phospholipids' chains.[8] The vibration at 722 cm^{-1} in sample A is shifted to 717 cm^{-1} in sample C and increased, while the component at 702 cm^{-1} remained the same. The vibration at 737 cm^{-1}, which decreased in the HDL samples with nicotine was not present in liposome spectrum. This component could be attributed to the ring vibration from Tyr[12] or to the rocking band from other lipids in HDL core, triglycerides and cholesterol esters, which are not present in liposome. The remaining two bands observed only in HDL samples, at 665 cm^{-1} and at 624 cm^{-1}, are attributed to the protein skeletal vibrations.

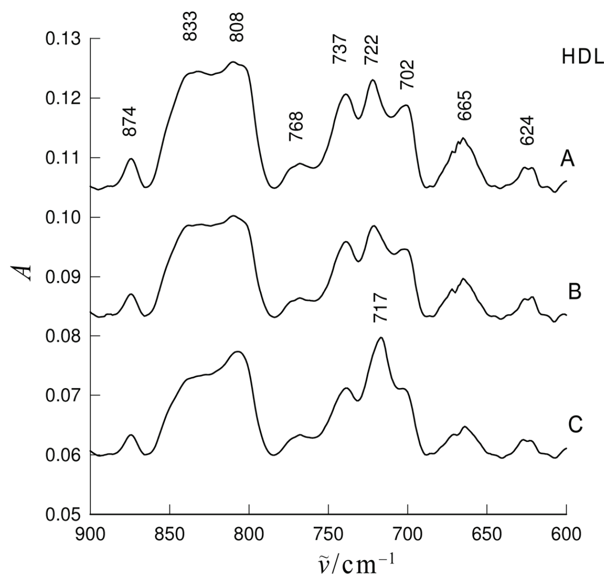

Figure 4. IR spectra of HDL samples: native (A), with added nicotine (1 molecule to 12 molecules of phospholipids) (B), with added nicotine (1 molecule to 6 molecules of phospholipids) (C), in the region below 1000 cm^{-1}. The positions of individual vibrations are labelled in the figure and their assignation is listed in Table 2.

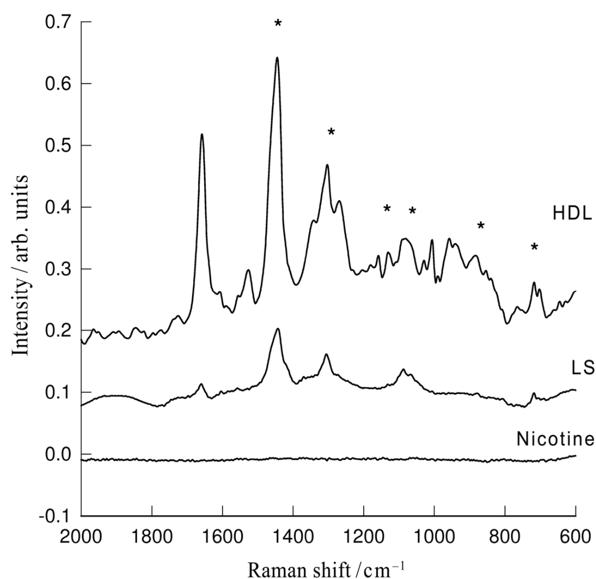

Figure 5. Raman spectra of native samples, HDL, LS and nicotine in the region 2000 – 600 cm^{-1}. The phospholipid bands, labelled by asterix in HDL spectrum are assigned by comparison with LS spectrum.

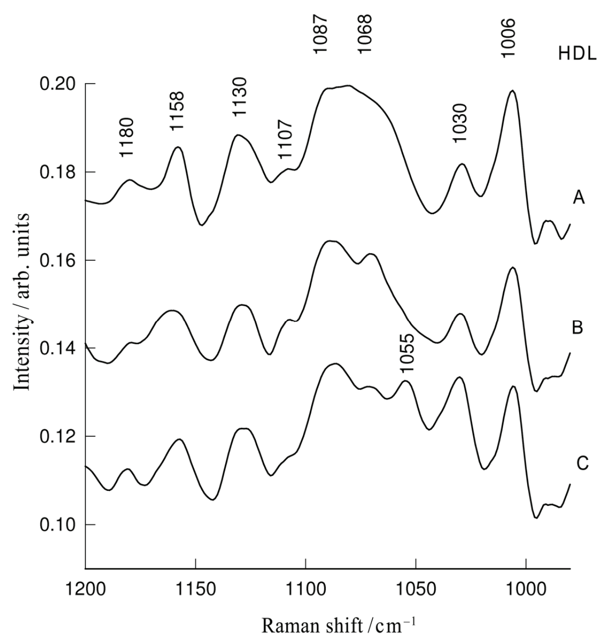

Figure 6. Raman spectra of three HDL samples marked as in Figure 4, in the region 1200−1000 cm^{-1}. The positions of vibrations are labelled in the figure and their assignation is listed in Table 3.

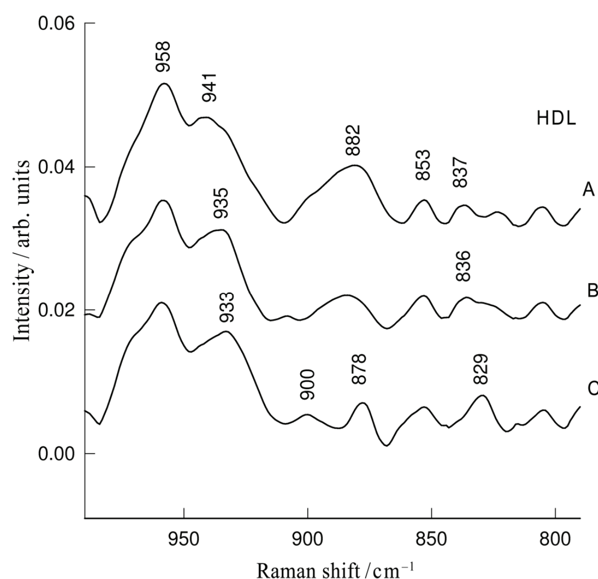

Figure 7. Raman spectra of HDL samples marked as in Figure 3, in the region below 1000 cm^{-1}. The positions of vibrations are labelled in the figure and their assignation is listed in Table 4.

Raman Spectra

HDL, LS and nicotine spectra in the region 2000−600 cm^{-1} are presented in Figure 5.

As in Figure 1, the phospholipids' bands from LS spectrum are labelled by asterix in HDL spectrum. In the spectrum of nicotine no bands were observed.

Raman spectra of HDL in the region 1200−1000 cm^{-1} contain more information about the vibrations in protein domain of the particle, Figure 6. The positions of lipid and protein bands are listed in Table 3.

Tyr band[13] at 1180 cm^{-1} is not sensitive to the presence of nicotine, but it has influence on three bands attributed to Phe[13]: at 1158 cm^{-1}, 1030 cm^{-1} and 1006 cm^{-1}. The band at 1158 cm^{-1} changes its intensity and shape and the other two bands change their relative intensities in the samples with added nicotine. The changes are more pronounced in the spectrum C. The absence of these bands in liposome spectrum confirms their attribution to the vibrations from protein moiety. Three bands from skeletal *trans* C–C stretching vibrations in acyl chains, Table 3, are usually observed in the Raman spectra of lipid compounds and are also present in liposome spectrum. The middle band and its weak shoulder at 1107 cm^{-1} have additional contribution from vibrations of *gauche* conformers. These three bands are often used for the study of conformational changes in acyl chains.[14] The incorporation of nicotine significantly changes the middle band. In addition, the band at 1055 cm^{-1}, present as a non resolved weak shoulder in spectra A and B, appears distinguished in the spectrum C. This band is attributed to P–O–C stretching vibration.[15]

Several bands in low frequency region, below 1000 cm^{-1} are sensitive to the presence of nicotine, Figure 7. Their positions are listed in Table 4.

One of two bands from C–C stretching vibrations of protein backbone[8], that at 941 cm^{-1} is sensitive to the presence of nicotine. It is shifted to 935 cm^{-1} (sample B) and further to 933 cm^{-1} (sample C). The stretching C–C vibrations in this band are usually attributed to α-helix structure which is predominant in apo A-I, present in more than 75 % of HDL particles. Two weak bands at about 900 cm^{-1} and at 882 cm^{-1} were observed at similar frequencies in Raman spectra of PC and SM from brain lipids.[16] They are attributed to C–C skeletal vibrations in phospholipids. The structural changes of these two bands in spectra B and C could be the consequence of decreasing intensity of these vibrations and better expression of Trp ring vibration[13] which is mixed with phospholipids' band. A well known doublet from Tyr Fermi resonance[8] is sensitive to the environment. The bands become more intense in spectra of the samples with nicotine, and the right one is also shifted from 837 cm^{-1} (sample A) to 836 cm^{-1} (sample B) and further to 829 cm^{-1} (sample C).

DISCUSSION

HDL is a particle built of hydrophobic core and outer monolayer. The core contains triglycerides and cholesterol esters. The monolayer is composed of phospholipid

molecules with incorporated molecules of free cholesterol. The significant part of the surface of monolayer is occupied with apo A-I which is wrapped around monolayer as a double belt and partly embedded into it. Nicotine is a non polar molecule and it is expected that, in contact with lipoprotein surface, it will tend to enter into lipid monolayer.

The changes observed in IR and Raman spectra of HDL and liposome samples with nicotine confirm this hypothesis. Nicotine affects the conformation and dynamics of acyl chains which is reflected in its influence on skeletal C–C stretching bands observed in Raman spectra (Table 3 and Figure 6, Table 4 and Figure 7) and methylene rocking vibrations (Table 2 and Figure 4). The increase of vibrations from *gauche* conformers reflects the conformational change in acyl chains (Figure 2, Table 3 and Figure 6). In addition, the changes in vibrations close to the polar part of phospholipids, from choline and phosphate group (Table 2 and Figure 4) and C–O stretching from head (Table 3 and Figure 6) indicate that nicotine is not deeply buried within monolayer. On the other hand, it was found earlier that nicotine has affinity towards some amino acids' residues, presumably Lys, His, Tyr and Cys.[17] We observed its influence as the changes in bands from Tyr vibrations (Table 4 and Figure 7), from Phe (Table 3 and Figure 6) and Trp vibrations (Table 4 and Figure 7). There are other bands from these amino acids of lower intensity which are not mentioned here. We could not observe the bands from Lys, because they are overlapped by much stronger bands from lipids in the same region. The influence of nicotine on Lys was observed indirectly because Phe and Trp are located as immediate neighbours to Lys in primary structure of apo A-I. The number of His residues is very low in apo A-I, while Cys is present in low percentage only in apo A-II and we did not observe any vibrations from these two amino acids.

CONCLUSION

On the basis of above analysis, we suggest that nicotine molecule is residing within lipid monolayer, but close to polar head of phospholipids and lipid-protein interface. There is a lot of evidence[18] that carboxyl domain of apo A-I, consisting of 10 α helices is in close contact with lipid monolayer. Helices 1 and 10 are partly buried within the lipid chains, anchoring the protein at the surface of the particle. Localization of nicotine in these regions could explain its influence on both, lipid and protein vibration bands. Nicotine molecule alone does not affect the secondary structure of apo A-I, because the protein amide I bands in the spectra were the same for the samples without and with added nicotine. However, if majority of nicotine molecules entered the lipid monolayer, surface enlargement could be expected and the conformation of apolipoprotein should adjust to that change. It is well documented[19] that apolipoproteins of HDL directly mediate binding to the scavenger receptor SR-BI which enables selective lipid uptake from the particle and a recognition pattern for the binding to the receptor is the helical structure. Therefore, nicotine induced surface changes should be considered for the possible binding dysfunction of HDL.

Acknowledgements. This work was supported by Ministry of Science, Education and Sports of the Republic of Croatia (project No. 108-1080134-3105). We acknowledge the Laboratory for Neurobiochemistry, Clinical Hospital Zagreb for the donation of plasma and for the performing of electrophoresis, the Laboratory for Magnetic Resonance, Institute Ruđer Bošković for the use of facilities for the lipoprotein isolation as well as Chemistry and Biochemistry Department of University of Zagreb Medical School for the performing of concentration measurements of lipoproteins.

REFERENCES

1. J. B. Lakier, *Am. J. Med.* **93** (1992) 8S–12S.
2. T. Neunteufl, S. Heher, K. G. Mitulović, S. Lehr, G. Khoschsorur, R. W. Schmid, G. Maurer, and T. Stefenelli, *J. Am. Coll. Cardiol.* **39** (2002) 251–256.
3. S. Cheland Campbell, R. J. Moffatt, and B.A. Stamford, *Atherosclerosis* **201** (2008) 225–235.
4. J. A. Ambrose and R. S. Barua, *J. Am. Coll. Cardiol.* **43** (2004) 1731–1737.
5. J. Brnjas-Kraljević, G. Pifat, and J. N. Herak, *Croat. Chem. Acta* **66** (1993) 547–554.
6. G. Jürgens, G. J. Knipping, P. Zipper, R. Kayushina, G. Degovics, and P. Laggner, *Biochemistry* **20** (1981) 3231–3237.
7. D. Krilov, M. Balarin, M. Kosović, O. Gamulin, and J. Brnjas-Kraljević, *Spectrochim. Acta Part A* **73** (2009) 701–706.
8. F. S. Parker, *Applications of Infrared, Raman, and Resonance Raman spectroscopy in Biochemistry*, Plenum Press, New York, 1983.
9. L. Senak, M. A. Davies, and R. Mendelsohn, *J. Phys. Chem.* **95** (1991) 2565–2571.
10. A. Barth, *Progr. Biophys. Molecul. Biol.* **74** (2000) 141–173.
11. S. Krishnamurty, M. Stefanov, T. Mineva, S. Begu, J. M. Devoisselle, A. Goursot, R. Zhu, and D. R. Salahub, Available via Cornell University Library at: *http://arxiv.org/ftp/arxiv/papers/0806/0806.0774.pdf*
12. M. Wolpert and P. Hellwig, *Spectrochim. Acta Part A* **64** (2006) 987–1001.
13. J. De Gelder, K. De Gussem, P. Vandenabeele, and L. Moens, *J. Raman Spectrosc.* **38** (2007) 1133–1147.
14. I. W. Lewin, *Vibrational spectroscopy of membrane assemblies*, in: R. J. H. Clark and R. E. Hester (Ed.), *Advanced Infrared and Raman Spectroscopy*, Wiley Heyden, New York, 1984, pp 1–48.
15. R. C. Spiker Jr and I. W. Lewin, *Biochim. Biophys. Acta* **388** (1975) 361–373.
16. C. Krafft, L. Neudert, T. Simat, and R. Salzer, *Spectrochim. Acta Part A* **61** (2005) 1529–1535.
17. T. L. Lentz, V. Chaturvedi, and B. M. Conti-Fine, *Biochem. Pharmacol.* **55** (1998) 341–347.
18. C. G. Brouillette, G. M. Anantharamaiah, J. A. Engler, and D. W. Borhani, *Biochim. Biophys. Acta* **1531** (2001) 4–46
19. S. Xu, M. Laccotripe, X. Huang, A. Rigotti, V. I. Zannis, and M. Krieger, *J. Lipid Res.* **38** (1997) 1289–1298.

SAŽETAK

Međudjelovanje lipoproteina visoke gustoće i nikotina – IR i Raman studija

Dubravka Krilov, Marin Kosović, Maja Balarin, Ozren Gamulin i Jasminka Brnjas-Kraljević

Medicinski fakultet, Katedra za fiziku i biofiziku, Sveučilište u Zagrebu
Šalata 3b, Zagreb, HR-10000, Croatia

U ovom radu proučavala se na molekularnoj razini direktna interakcija lipoproteina velike gustoće (HDL) s nikotinom, jednom od glavnih čestičnih komponenti u cigareti. Nikotin utječe na vibracijske modove HDL zbog svoje ugradnje unutar monosloja lipida. Promjene u položajima i intenzitetima vibracijskih vrpci u proteinskom i lipidnom dijelu čestice proučavane su IR i Raman spektroskopijom. Pripremljene su tri vrste uzoraka: čisti uzorak HDL i dva uzorka s dodanim nikotinom. Molekularni omjer nikotina i fosfolipida u dva uzorka je bio 1:12 i 1:6. Iste vrste uzoraka pripremljene su s lipozomima koji sadrže fosfatidilkolin i sfingomijelin s dodatkom kolesterola. Spektri uzoraka lipozoma poslužili su za uočavanje i određivanje lipidnih vrpci u spektrima HDL uzoraka, u kojima je opaženo najviše promjena. Ugradnja nikotina unutar lipidnog monosloja unosi promjene u lipidne vrpce koje potječu od vibracija u acilnim lancima i glavama fosfolipida. Promjene u vibracijskim vrpcama pojedinih aminokiselinskih ostataka potvrđuju da je molekula nikotina smještena u lipidnom monosloju, ali blizu dodirnog područja lipida i proteina.