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Detekcija porifirina karijesne lezije pomoću fluorescencije inducirane ljubičastim laserom

The Detection of Carious Lesion Porphyrins Using Violet Laser Induced Fluorescence

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Sažetak
U istraživanju se željelo odrediti spektralna područja za lasersku ekscitaciju otopina protoporfirina IX (PP-a), koproporfirina (CP-a) i uroporfirina (UP-a) s različitim pH-vrijednostima. Drugi je cilj bio usporediti spektre laserski inducirane fluorescencije (LIF-a) otopina PP-a, CP-a i UP-a različitih pH-vrijednosti s fluorescencijom prirodne karijesne lezije i rezultatima ostalih istraživanja. **Materijali i postupci:** Apsorpcijski spektri otopina PP-a, CP-a i UP-a s pH-vrijednostima u rasponu od 0,6 do 13 izmjereni su spektrofotometrom (model Varian). Laser od 405 nm (10mW) odabran je prema najvišoj apsorpciji porfirina za mjerenja spektra laserski inducirane fluorescencije (LIF-a) PP-a, CP-a i UP-a te prirodne karijesne lezije. **Rezultati:** Ovisno o pH-vrijednosti, LIF-ove otopine PP-a, CP-a i UP-a pokazuju pomak prema višim valnim duljinama. U kiseloj otopini PP-a uočeni su fluorescencijski vrški na 601 nm i 655 nm nakon ekscitacije lasera na valnoj duljini od 405 nm. **Zaključak:** *In vitro* mjerenja LIF-a porfirinskih otopina ljubičastim laserom pokazuju sličnosti s valnim duljinama prave karijesne lezije i vrhovima fluorescencijskih vrpci prirodnih zuba poznatih iz literature. Ipak, fluorescencija testiranih porfirina ne objašnjava potpuno fluorescencijske spektre zubnog karijesa te su potrebna daljnja istraživanja. Čini se da pH ima važan utjecaj na svjetlosnu apsorpciju i emisiju porfirina.

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Ključne riječi
protoporfirin; koproporfirin; uroporfirin; zubni karijes; spektrometrija, fluorescentna

Uvod

Moderna stomatologija zahtijeva brzu i pouzdanu dijagnostiku ranog karijesa te odgovarajuće liječenje. Za to je potreban dijagnostički sustav za detekciju najranijih stadija razvoja karijesa kada je remineralizacija najučinkovitija i još je moguć povratak u prvotno stanje, odnosno kada su još mogući minimalno invazivni postupci. Vizualno-taktilni postupak jako je nepouzdan jer se temelji na subjektivnoj interpretaciji. Današnja, a i buduće tehnologije, stavljaju naglasak na objektivna mjerenja svojstava svjetlosnih valova u interakciji sa zubom (1).

Potraga za preciznijim postupcima detekcije karijesa provodi se u mnogobrojnim laboratorijima. Svjetlo je posebice prikladno sredstvo za proučavanje zuba zbog izrazito pravilnog, kristalastog ustrojstva cakline i tubularne strukture dentina, zbog čega je omogućeno dobro provođenje svjetla (1). Fluorescencija je dobro poznat fenomen i njome se stomatolozi često služe u dijagnostici karijesa. Osnovni princip temelji se na tome da se svjetlo jedne valne dužine (ekscitacijska

Introduction

The modern dentistry requires quick and reliable diagnostics of early caries and appropriate subsequent treatment. Adequate diagnostic system is required for the detection of the early stages in caries development, when the remineralization treatment is the most efficient and the reversal of the process or minimally invasive procedures are still possible. The visual-tactile method is highly unreliable since it is based on the subjective interpretation. Current and future technology places the emphasis on the objective measurements of the properties of the light waves in the interaction with tooth (1).

The search for more accurate methods of caries detection is carried out in many laboratories. Light is particularly suitable tool for the study of teeth, because of highly regular, crystalline structure of enamel and tubular structure of dentine, both of which ensure good light propagation (1). Fluorescence is a well known light phenomenon which has been often exploited for the caries diagnostics. Basic principle of it

valna dužina) apsorbira u tkivu koje emitira drugi pojas više valne dužine (emisijska valna dužina) (2). Činjenice da zubi prirodno fluoresciraju pri iradijaciji ultraljubičastim i ljubičastim svjetlom (3) te izostanak fluorescencije u karijesnim područjima intrigirale su znanstvenike od početka 20. stoljeća – željeli su iskoristiti te informacije kako bi otkrili karijes (4, 5). No, u karijesu je fluorescencija također pronađena, što su potvrdila kasnija istraživanja (6, 7).

Nedavno provedena dijagnostička ispitivanja pomoću laserski inducirane fluorescencije (LIF-a) pokazala su zanimljive rezultate. Nekoliko novih pristupa pridonijelo je detekciji karijesnih lezija. Kvantitativna laserska fluorescencija (eng. "Quantitative laser fluorescence" - QLF) sustav je za detekciju karijesa kod kojega se zubi izlažu plavom svjetlu (od 488 do 514 nm), a detekcija karijesa temelji se na izostanku fluorescencije u karijesnim lezijama, vjerojatno zbog raspršivanja svjetlosti (1, 8). Za detekciju fluorescencije temeljenu na diodnom laseru (DD-u) (DIAGNODent), KaVo, Biberach, Njemačka) (9) rabi se crveni laser (655 nm) kako bi se izazvala emisija svjetlosti iz samog karijesa i da bismo je detektirali u valnim duljinama višima od 680 nm (1).

Točan identitet sastojaka odgovornih za fluorescenciju karijesa još intrigira znanstvenike. U literaturi se može naći nekoliko teorija. König i njegovi suradnici (10) predložili su da fluorescencija u karijesnim lezijama odgovara produktima bakterijskog metabolizma, porfirinskim monomerima bez metalnih iona. Prema njihovu istraživanju, profirini karijesne lezije fluoresciraju samo u crvenom spektralnom području. Nakon toga su Hibst i Paulus (11) identificirali fluorescirajuće sastojke kao protoporfirin IX (PP), uroporfirin (UP) i koproporfirin (CP) (Slika 1.). Zanimljivo je istaknuti da je ustanovljeno kako mutans-streptokoki nisu odgovorni za crvenu fluorescenciju plaka. Oni pokazuju zelenu fluorescenciju, slično kao i zdravo zubno tkivo. Umjesto njih, obvezne anaerobne bakterije, poput Prevotella melaninogenica i Actinomyces israelii te Candida albicans u zrelom plaku, jesu mikroorganizmi koji stvaraju porfirine (12, 13).

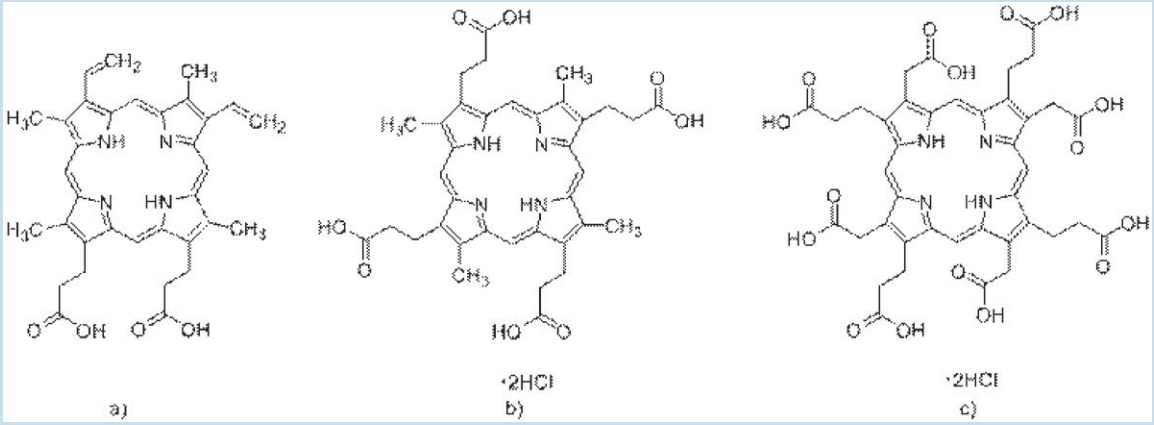
Prema spoznajama autora, još nije obavljeno istraživanje koje bi uzimalo u obzir kiselost karijesa i utjecaj na porfirine karijesne lezije. To bi moglo djelovati na njihove apsorpcijske i emisijske fluorescencijske spektre te interferirati s rezultatima trenutačno dostupnih dijagnostičkih uređaja.

is that the light of one wavelength (excitation wavelength) is absorbed by the tissue which emits in second band of longer wavelength (emission wavelength) (2). The facts that teeth naturally fluoresce upon irradiation with ultraviolet and visible light (3) and the absence of the fluorescence in carious regions have intrigued the scientists since the beginning of the 20th century to use these information to detect caries (4, 5). However, the fluorescence is found also in caries, as the subsequent investigations have shown (6, 7).

The recent initial diagnostic trials by means of laser induced fluorescence (LIF) presented interesting results. A few new approaches have contributed to caries lesions detection. Quantitative laser fluorescence (QLF) is a caries detection system where the teeth are exposed to blue light (488-514 nm) and the detection of caries is based on the absence of fluorescence in carious lesions, probably due to light scattering (1, 8). The diode laser-based fluorescence detection (DD) (DIAGNODent), KaVo, Biberach, Germany) (9) operates using red laser (655 nm) to provoke the light emission from caries itself and detects it in wavelengths higher than 680 nm (1).

The exact identity of compounds responsible for caries fluorescence still puzzles the scientists. Several theories can be found searching the literature. König et al. (10) proposed that the fluorescence in carious lesions conforms to the products of bacterial metabolism, metal-free porphyrin monomers. According to their research, porphyrins in carious lesions fluoresce only in red spectral area. Furthermore, Hibst and Paulus (11) have identified fluorescing compounds of caries as being protoporphyrin IX (PP), uroporphyrin (UP) and coproporphyrin (CP) (Figure 1). Interestingly, it has been established that mutans streptococci are not responsible for the red fluorescence in plaque. They exhibit green fluorescence, similar to healthy tooth tissue. Instead, obligate anaerobic bacteria, like Prevotella melaninogenica and Actinomyces israelii, as well as Candida albicans in mature plaque are porphyrin-producing species (12, 13).

To the authors' knowledge, there is still no investigation which has taken into concern the acidity of the carious environment and its influence on the fluorescence of the porphyrins in carious lesion. This might present differences in their absorption and emission fluorescence spectra and interfere with the results of currently available diagnostic devices.



Slika 1. Molekularne strukture: a.) protoporfirina IX (PP-a); b.) koproporfirina (CP-a) i c.) uroporfirina (UP-a) (www.sigmaaldrich.com)
Figure 1 Molecular structures of a) protoporphyrin IX (PP), b) coproporphyrin (CP) and c) uroporphyrin (UP) (www.sigmaaldrich.com).

U našem prethodnom radu predstavljeno je moguće objašnjenje podrijetla fluorescencije karijesnih lezija pomoću laserski inducirane fluorescencije CP-a i UP-a ekscitiranih laserima od 420 nm, 473 nm i 532 nm (14). Prikazano je da su fluorescencijski spektri uvelike slični spektralnim profilima prirodnih karijesnih lezija proučavanih na izvađenim zubima (7). Ipak, određeni vršci nisu se mogli objasniti fluorescencijom CP-a i UP-a te je istaknuto da su potrebna dalja istraživanja kako bismo upotpunili spektralnu sliku karijesa. Neki autori (11) tvrde da PP igra presudnu ulogu u fluorescenciji karijesnih lezija. Osim toga, analiza apsorpcijskih spektara CP-a i UP-a upućuje na to da bi najbolji ekscitacijski izvori bili u području od 370 do 405 nm (14).

Prvi zadatak našeg istraživanja bio je ustanoviti najprikladniju valnu dužinu za učinkovitu ekscitaciju PP-a, UP-a i CP-a u različitim pH-uvjetima. Nakon toga, željeli smo ustanoviti do koje su mjere PP, CP i UP odgovorni za apsorpciju i emisiju svjetla u karijesu te kako na njih utječe pH. Dakle, drugi je zadatak bio provesti mjerenja spektra laserski inducirane fluorescencije otopina PP-a, CP-a i UP-a različitih pH-vrijednosti i usporediti ih s fluorescencijom prirodne karijesne lezije te s rezultatima ostalih istraživanja.

Materijali i postupci

Kemikalije

Protoporfirin IX (PP), uroporfirin I dihidroklorid (UP) i koproporfirin I dihidroklorid (CP) nabavljeni su od tvrtke Sigma-Aldrich Co. (St. Louis, Missouri, SAD). Osnovne otopine porfirina bile su pripremljene prema postupku Komagoe i suradnika (15). PP (0,6 mg), CP (3,6 mg) i UP (1,0 mg) otopljeni su bili u 10 mmol/L NaOH, otopine neutralizirane sa 100 mmol/L HCl i razrijeđene destiliranom vodom. Koncentracije ishodnih otopina bile su $1{,}0\times10^{-4}$ mol/L, $5{,}0\times10^{-4}$ mol/L i $1{,}1\times10^{-4}$ mol/L za PP, CP i UP.

Otopine različitih pH-vrijednosti pripremljene su razrjeđivanjem ishodnih otopina odgovarajućom kiselinom, bazom ili puferskom otopinom. Jako lužnate otopine pripremljene su razrjeđivanjem ishodnih otopina porfirina s 10 mmol/L NaOH-a. Razrjeđivanje otopinama pufera 10 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 7,0), 10 mmol/L NaH_2PO_4/H_3PO_4 (pH 5,0) i 10 mmol/L NaH_2PO_4/H_3PO_4 (pH 4,0) rezultiralo je neutralnim i blago kiselim mjernim otopinama. Za pripremu jako kiselih otopina porfirina rabljeni su 1 mol/L H_3PO_4 i 1 mol/L HCl-a. Koncentracija porfirina u mjernim otopinama bila je 1×10^{-5} mol/L.

Instrumentacija

Apsorpcijski spektri mjereni su spektrofotometrom Varian (model CARY 3, Varian Inc., Palo Alto, SAD). Za mjerenja su bile odabrane konvencionalne kvarcne ćelije (10 mm×10 mm). Spektri su uvijek bili snimljeni odmah nakon pripreme mjernih otopina. Molarni apsorpcijski koeficijenti ε, izračunati su za apsorpcijske maksimume prema Beer-Lambertovu zakonu $A = \varepsilon bc$, u kojem je A apsorbancija, b je duljina puta (1 cm) i c je molarna koncentracija u mjerenim otopinama (1×10^{-5} mol/L).

In our previous paper, the possible explanation of the origin of the fluorescence in carious lesions was given by studying LIF of CP and UP, which were excited by using 420 nm, 473 nm and 532 nm lasers (14). It was shown that the fluorescence spectra greatly coincide to the spectral profiles of natural caries lesions studied on extracted teeth (7). However, certain peaks could not be explained by the fluorescence of CP and UP and it was established that, in order to complete the spectral imaging of caries, further investigation is needed. Other authors (11) claim that PP plays the crucial role in the fluorescence of caries lesions. Also, the analysis of absorption spectra of CP and UP indicates that the best excitation sources should be in range from 370-405 nm (14).

The first aim of our investigation was to establish the most appropriate laser wavelength for the efficient excitation of PP, CP and UP solutions under different pH conditions. After that, we wanted to see to what extent are PP, UP and CP responsible for the light absorption and emission in caries and the influence of pH on them. Hence, the second aim was to perform LIF of PP, CP and UP solutions with various pH and compare it to the fluorescence of natural carious lesion and the results of other studies.

Materials and methods

Chemicals

Protoporphyrin IX (PP), uroporphyrin I dihydrochloride (UP) and coproporphyrin I dihydrochloride (CP) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Stock solutions of the porphyrins were prepared according to Komagoe et al. (15). PP (0.6 mg), CP (3.6 mg) and UP (1.0 mg) were dissolved in 10 mmol/L NaOH, neutralized by 100 mmol/L HCl and diluted with distilled water. The concentration of the resulting stock solutions were 1.0×10^{-4} mol/L, 5.0×10^{-4} mol/L and 1.1×10^{-4} mol/L for PP, CP and UP, respectively.

Solutions of different pH values were prepared by dilution of the stock solutions with an appropriate acid, base or buffer solution. To obtain highly alkaline solutions, the porphyrin stock solutions were diluted with 10 mmol/L NaOH. Dilution with the buffer solutions of 10 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 7.0), 10 mmol/L NaH_2PO_4/H_3PO_4 (pH 5.0) and 10 mmol/L NaH_2PO_4/H_3PO_4 (pH 4.0) resulted in neutral and mild acidic working solutions. For preparation of highly acidic porphyrin solutions 1 mol/L H_3PO_4 and 1 mol/L HCl were used. The concentration of the working solutions was 1×10^{-5} mol/L.

Instrumentation

Absorption spectra were taken on a Varian spectrophotometer (model CARY 3, Varian Inc., Palo Alto, USA). Conventional quartz cells (10 mm×10 mm) were used throughout the measurements. Spectra were always recorded immediately after the preparation of the working solution. Molar absorption coefficients, ε, were calculated at the absorption maxima according to the Beer-Lambert's law $A = \varepsilon bc$, where A is absorbance, b is the path length (1 cm) and c is the molar concentration of the measured solutions (1×10^{-5} mol/L).

Za mjerenja pH upotrijebljen je pH-metar Mettler Toledo (model MP 220, Mettler Toledo Inc., Greifensee, Švicarska) kombiniran sa staklo-kalomel elektrodom Mettler Toledo InLab®413. pH-metar je bio kalibriran standardnim vodenim puferskim otopinama koje su imale pH-vrijednosti 7,00 i 4,01. Vrijednosti pH-radnih otopina bile su izmjerene nakon snimanja apsorpcijskih spektara.

Mjerenja laserski inducirane fluorescencije (LIF)

Nakon što su dobiveni rezultati apsorpcije testiranih porfirina, potvrđeno je da bi laser od 405 nm bio prikladan za mjerenja LIF-a. Diodni laser bio je zapravo laserski uređaj u obliku olovke s fiksnom valnom dužinom od 405 nm i snagom od 10 mW (CNI laser, Changchun New Industries Optoelectronics Tech. Co., Ltd., Changchun, Kina). Laserski uređaj proizvodio je kontinuirane laserske valove.

Mete mjerenja LIF-a bile su karijesni zub i otopine porfirina. Za mjerenje spektra laserski inducirane fluorescencije rabljen je jedan ljudski ekstrahirani zub koji je prije mjerenja i tijekom njega bio pohranjen na suhom. Spektri LIF-a učinjeni su u tamnoj sobi. Inducirana fluorescencija nakon iluminacije laserom od 405 nm prikupljena je s različitih dijelova zuba: cementa korijena, zdrave cakline i karijesnog dentina. Također su obavljena mjerenja laserske fluorescencije otopina PP-a s pH 0,6, 1,7 i 11,7, otopina CP-a s pH 0,8, 1,7 i 12,0 i otopina UP-a s pH 0,8, 1,6 i 12,0.

Laserska zraka bila je usmjerena u ćeliju s porfirinskom otopinom. Bila je fiksirana u crnom anodiziranom aluminijskom držaču koji je omogućavao mjerenje fluorescencije pod kutom od 90°, koristeći se vrhom optičkog vlakna povezanog sa spektrometrom. To je olakšalo mjerenja LIF-a mnogobrojnih ćelija s različitim porfirinima i pH-vrijednostima.

Sva mjerenja obavljena su digitalnim spektrometrom visoke rezolucije HR4000CG-UV-NIR (OceanOptics, Dunedin, FL, SAD) koji pokriva spektralni raspon od 200 do 1100 nm i ima rezoluciju od oko jednog nm. Spektralna osjetljivost u području promatrane fluorescencije bila je konstantna.

Rezultati

Apsorpcijski spektri

Apsorpcijski spektri vodenih otopina protoporfirina IX svjedočili su o prisutnosti različitih vrsta koje su posljedica pH-ovisne agregacije i protoniranja molekula (Slika 2.) (16, 17). Apsorpcijski maksimum pri 407 nm, ε = 17,47×10^4 L mol^{-1} cm^{-1}, u spektru PP-a pri pH 0,6 uputio nas je na to da postoje neagregirane vrste s protoniranim krajnjim propionatnim skupinama te unutarnjim dušikovim atomima. Smanjenje apsorbancije pri pH 1,7 (λ = 405, ε = 4,74×10^4 L mol^{-1} cm^{-1}) navijestilo je progresivni gubitak protona iz porfirinskog prstena. Vrlo široki apsorpcijski pojas s maksimumom oko 355 nm (ε ~ 3,6×10^4 L mol^{-1} cm^{-1}) i ramenom oko 450 nm, pri blago kiselim i neutralnim uvjetima (3,8, 4,7 i 7,0), pripisana je interakciji kromofora u višim agregatima. U jako bazičnom mediju (pH 11,7), karboksilne skupine su disocirane, što je rezultiralo formiranjem „glava-rep“ dime-

For pH measurements, a Mettler Toledo pH meter (model MP 220, Mettler Toledo Inc., Greifensee, Switzerland) with a Mettler Toledo InLab®413 combined glass-calomel electrode was used. The pH meter was calibrated with standard aqueous buffer solutions of pH 7.00 and 4.01. pH values of the working solutions were measured after the absorption spectra were recorded.

Laser induced fluorescence measurements (LIF)

After obtaining the absorption results for the tested porphyrins, laser at 405 nm was confirmed to be the appropriate for the LIF measurements. The laser diode was a pen like laser device having fixed wavelength of 405 nm and the power of 10 mW (CNI laser, Changchun New Industries Optoelectronics Tech. Co., Ltd., Changchun, China). The laser device performed continuous wave lasing.

The targets of LIF measurements were carious tooth and porphyrin solutions. One human extracted tooth was used in LIF measurements, which was kept dry previous and during measurements. The LIF spectra were taken in dark room. The fluorescence induced after illumination with 405 nm laser was collected from different parts of the tooth: root cement, healthy enamel and carious dentine tissue. Also, laser fluorescence measurements of PP solutions with pH 0.6, 1.7 and 11.7, CP solutions with pH 0.8, 1.7 and 12.0 and UP 0.8, 1.6 and 12.0 were taken.

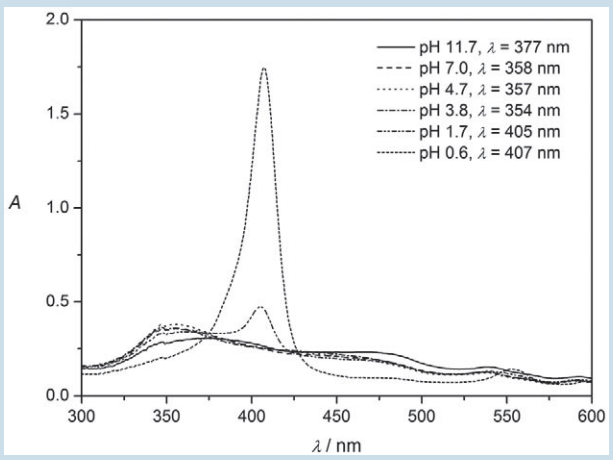
The laser beam was focused into the cell with porphyrin solution. The cell was fixed by the black anodized aluminum holder, which allowed the fluorescence measurement at an angle of 90° using the tip of the fiber connected to the spectrometer. This enabled easy LIF measurement of a number of cells with different porphyrins and pH values.

For all spectral measurements HR4000CG-UV-NIR high resolution digital spectrometer (OceanOptics, Dunedin, FL, USA) was used, which covers the spectral range 200 - 1100 nm, with a resolution of about 1 nm. The spectral sensitivity in the region of observed fluorescence bands was constant.

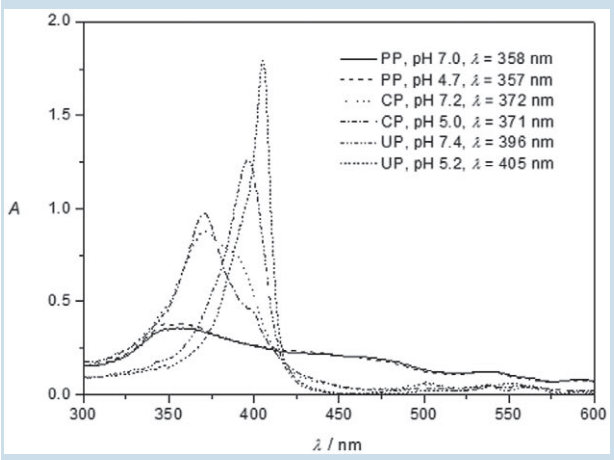
Results

Absorption spectra

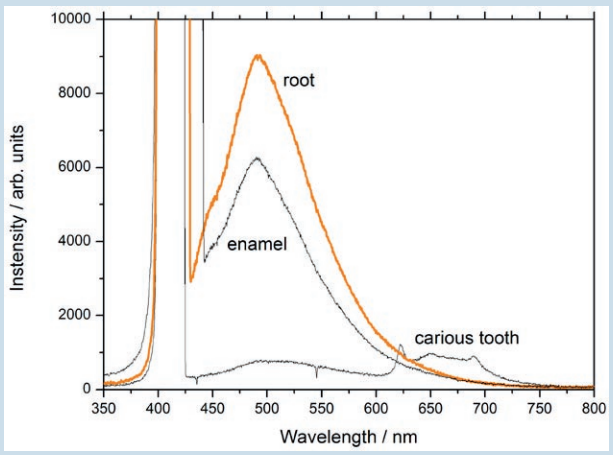
Absorption spectra of aqueous solutions of protoporphyrin IX evidenced the presence of different species as a result of the pH dependent aggregation and protonation of the molecules (Figure 2) (16, 17). An absorption maximum at 407 nm, ε = 17.47×10^4 L mol^{-1} cm^{-1}, in the spectrum of PP at pH 0.6 indicated the existence of nonaggregated species having protonated both the propionate end groups as well as the inner nitrogen atoms. Decrease in absorbance at pH 1.7 (λ = 405, ε = 4.74×10^4 L mol^{-1} cm^{-1}) implied progressive removal of protons from the porphyrin ring. Under mild acidic and neutral conditions (3.8, 4.7 and 7.0) very broad absorption band with a maximum at around 355 nm (ε ~ 3.6×10^4 L mol^{-1} cm^{-1}) and a shoulder around 450 nm were associated with interacting chromophores in higher aggregates. In highly alkaline medium (pH 11.7), dissociation of the carboxyl-



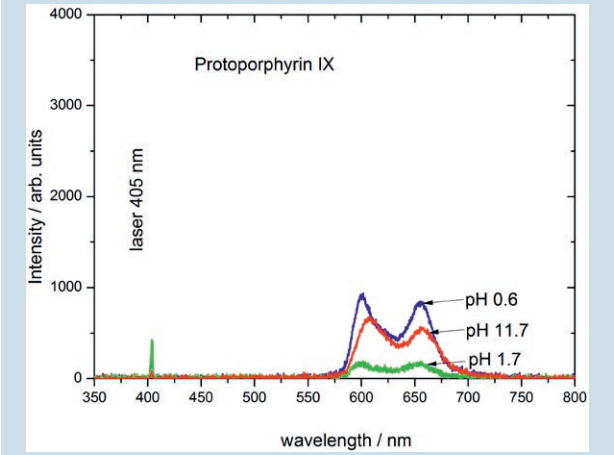
Slika 2. Apsorpcijski spektri PP-a, $c = 1\times10^{-5}$ mol/L, pri različitim pH-vrijednostima
Figure 2 Absorption spectra of PP, $c = 1\times10^{-5}$ mol/L, at various pH.



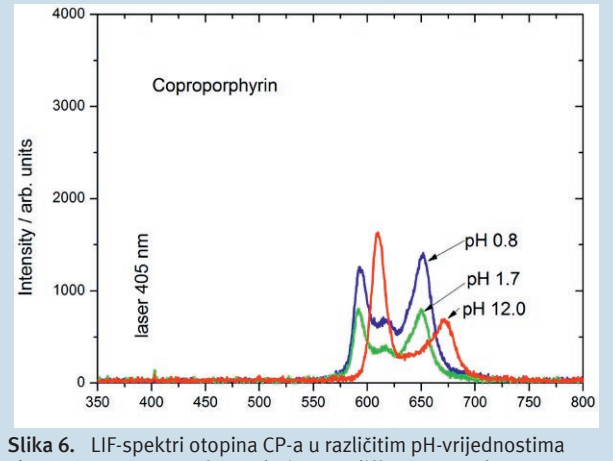
Slika 3. Apsorpcijski spektri PP-a, CP-a i UP-a, $c = 1\times10^{-5}$ mol/L, u neutralnim i lagano kiselim pH-uvjetima
Figure 3 Absorption spectra of PP, CP and UP, $c = 1\times10^{-5}$ mol/L, at neutral and slightly acid pH.



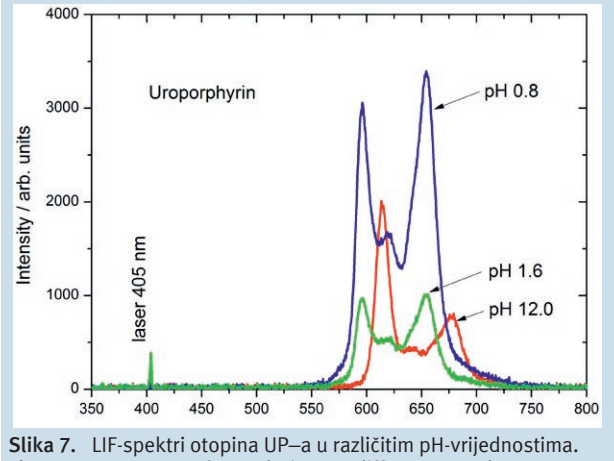
Slika 4. Laserski inducirana fluorescencija različitih dijelova ekstrahiranog karijesnog zuba osvijetljenog 405 nm laserom
Figure 4 Laser induced fluorescence of different parts of the extracted carious tooth illuminated by the 405 nm laser.



Slika 5. LIF-spektri otopina PP-a u različitim pH-vrijednostima
Figure 5 LIF spectra of PP solutions at different pH values.



Slika 6. LIF-spektri otopina CP-a u različitim pH-vrijednostima
Figure 6 LIF spectra of CP solutions at different pH values.



Slika 7. LIF-spektri otopina UP–a u različitim pH-vrijednostima.
Figure 7 LIF spectra of UP solutions at different pH values.

ra karakteriziranog apsorpcijskim maksimumom pri 377 nm (ε = 3,08×10^4 L mol^{-1} cm^{-1}).

Za razliku od dominantnih agregiranih molekula PP-a pri neutralnom i blago kiselom pH, u otopinama CP-a apsorpcijski maksimum pri 372 nm (pH=7,2, ε = 8,77×10^4 L mol^{-1} cm^{-1}) s ramenima pri višim valnim dužinama upućuje na ravnotežu između monomera i dimera sa slaganjem „lice-u-lice“ (Slika 3.) (15-20). No, kod UP-a se intenzivna apsorpcija pri 396 nm u neutralnoj otopini (ε = 12,58×10^4 L mol^{-1} cm^{-1}) povezala samo s monomernim vrstama koje se protoniraju u već blago kiselim uvjetima i jako apsorbiraju zračenje pri 405 nm (ε = 17,96×10^4 L mol^{-1} cm^{-1}) (19-22).

Spektri laserski inducirane fluorescencije

Na Slici 4. predstavljamo spektre LIF-a karijesom nezahvaćenih područja na korijenu i caklini karijesnog zuba, od kojih oba imaju maksimum na oko 500 nm. No, kada se laser od 405 nm usmjerio na karijesnu leziju, pojavio se drugačiji spektar laserski inducirane fluorescencije. Istina, ispod karijesa vidi se fluorescencija dentina s maksimumom na 500 nm, no sam karijes ima vrlo izražen oblik s vrhuncima na 622, 650 i 689 nm.

LIF vrlo kiselih i vrlo alkalnih otopina PP-a (Slika 5.) upućuje na vrhunce na 600 nm i 655 nm. Različite pH-vrijednosti otopina PP-a pokazuju pomak u zamijećenim vršcima. Tako se za pH jednak 11,7 dva vrška pomiču prema 607 nm i 657. Slično se događa i u otopinama CP-a te UP-a. No, i CP i UP posjeduju tri vrhunca. Oni se kod CP–a nalaze na 592 nm, 615 nm i 651 nm (Slika 6.) te na 596 nm, 620 nm i 655 nm kod UP-a (Slika 7.). Sve te vrijednosti dobivene su za najniže pH-vrijednosti oko 0,8. Za najviše pH-vrijednosti maksimumi se pomiču prema dužim valnim dužinama te se u slučaju CP-a pojavljuju u samo dvama vrhuncima na 609 nm i 670 nm, dok se kod UP-a tri vrhunca pomiču na 613 nm, 641 nm i 679 nm.

Na Slici 8. fotografije su istoga karijesnog zuba pod običnim prostornim osvjetljenjem (lijevo). Desno je zub osvijetljen laserom (od 405 nm) usmjerenim na karijesnu leziju, a fotografija je snimljena korištenjem laserskih naočala koje eliminiraju lasersku radijaciju od 405 nm. Emitirano fluorescentno svjetlo ima žućkasto-crvenu boju.

ic groups took place leading to the formation of head-to-tail dimers characterized by an absorption maximum at 377 nm (ε = 3.08×10^4 L mol^{-1} cm^{-1}).

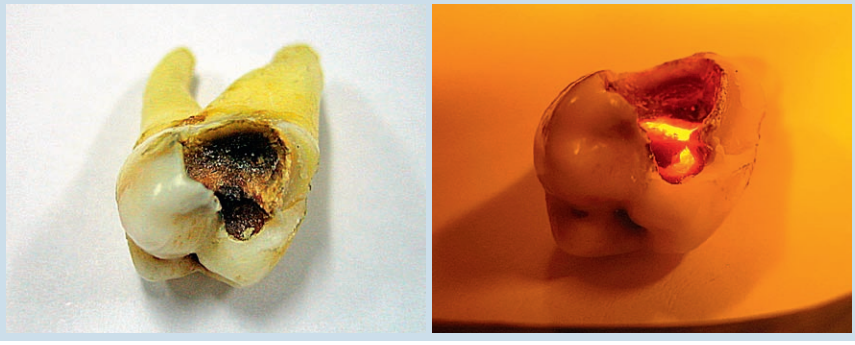
Unlike dominant presence of PP aggregated molecules at the neutral and slightly acidic pH, an absorption maximum at 372 nm (pH=7.2, ε = 8.77×10^4 L mol^{-1} cm^{-1}) with shoulders at higher wavelengths indicated equilibrium between dimers with face-to-face stacking and monomers in CP solutions (Figure 3) (15-20). For UP, however, an intense absorption at 396 nm in neutral solution (ε = 12.58×10^4 L mol^{-1} cm^{-1}) was associated only with monomeric species, which were protonated already under the mild acidic conditions highly absorbing at 405 nm (ε = 17.96×10^4 L mol^{-1} cm^{-1}) (19-22).

Laser induced fluorescence spectra

In Figure 4 we present the laser induced spectra of the caries unaffected areas on the root and enamel of the carious tooth, which both have a maximum at about 500 nm. However, when the 405 nm laser was pointing to the carious lesion different LIF spectra appeared. It is true that beneath the caries there is dentin fluorescence with the maximum at 500 nm, but caries alone has very prominent shape with peaks at 622, 650 and 689 nm.

The LIF of very acidic and very basic solutions of PP (Figure 5) indicate the peaks at 600 nm and 655 nm. Different pH values of the PP solutions exhibit shift in observed peaks. So for pH equal to 11.68 the two peaks move toward 607 nm and 657 nm, respectively. The similar behavior exists in solutions of CP and UP. However, both CP and UP posses three peaks. They are at 592 nm, 615 nm and 651 nm for CP (Figure 6) and at 596 nm, 620 nm and 655 nm for UP (Figure 7). All these values have been obtained for the lowest pH values of about 0.8. For the highest pH values the maxima move toward the longer wavelengths and in the case of CP only two peaks appear at 609 nm and 670 nm, whereas for UP three peaks move to 613 nm, 641 nm and 679 nm.

In Figure 8 we show the photos of the same carious tooth under normal room light (left). On the right, the tooth was illuminated by the laser at 405 nm aiming at the carious lesion and the photo was taken using laser goggles that eliminate 405 nm laser radiation. The emitted fluorescence light has yellowish-red color.



Slika 8. LIF velike karijesne lezije na ekstrahiranom zubu: a.) karijesni zub; b.) osvijetljen laserom od 405 nm i fotografiran kroz laserske naočale koje zaustavljaju lasersko ljubičasto svjetlo, a dopuštaju da se vidi žuti i crveni dio spektra

Figure 8 LIF of large carious lesion of the extracted tooth: a) carious tooth, b) tooth illuminated by 405 nm laser and observed through the laser goggles, which stops the violet laser light and allows the yellow and red spectrum to be seen.

Rasprava

Stručnjaci su se u mnogobrojnim studijama bavili podrijetlom fluorescencije u karijesu i porfirinima, većina njih koristeći se retrogradnom metodom rada – proučavajući direktno karijesnu leziju i pripisujući fluorescencijske maksimume određenim porfirinima (6, 7, 10, 11). Dosad nitko nije proučio svaki porfirin posebno kako bi ustanovio njihov stvarni doprinos fluorescenciji karijesa, niti su se proučavale moguće promjene emisijskog spektra ovisno o pH. Ovo istraživanje provedeno je kao nastavak našega prijašnjeg rada u kojemu su detaljno opisani apsorpcijski i emisijski spektri CP-a i UP-a, dvaju važnih porfirina karijesne lezije (14). Kako su mnogi autori istaknuli da je PP najvažniji i najrasprostranjeniji porfirin (10, 11, 23), u ovom je istraživanju analiziran posebice PP te njegova interakcija s CP-om i UP-om, a zatim je uspoređen s prirodnom karijesnom lezijom zuba.

Važnost definiranja apsorpcijskih spektara fluorescirajućih molekula jest u određivanju najučinkovitije ekscitacijske valne duljine. Kako je istaknuto u našem prijašnjem članku (14), različite eksitacijske valne duljine daju zapravo iste izlazne fluorescencijske vrške. Prema stajalištu Buchalle (7), koji je proučavao fluorescenciju prirodnih karijesnih lezija s ekscitacijskim valnim duljinama u rasponu od 360 do 580 nm u koracima od 20 nm, određene valne duljine daju viši emisijski intenzitet od drugih. Korištenje tog određenog dijela spektra za ekscitaciju olakšava detekciju fluorescencije. Rezultati ovog istraživanja upućuju na to da su apsorpcijski maksimumi u rasponu od 357 do 407 za PP, od 370 do 401 za CP i od 390 do 405 nm za UP. Zbog toga je laser s diskretnom valnom duljinom od 405 nm odabran kao prikladan za sva tri porfirina. To je u skladu s vrijednostima određenima za prirodne karijesne lezije od 400 do 420 nm i oko 520 nm (7).

Iz rezultata apsorpcijskih spektara PP-a, CP-a i UP-a može se zaključiti da pH-vrijednost ima velik utjecaj, što se objašnjava pH-ovisnim protoniranjem i agregacijom molekula. Monomeri učinkovitije apsorbiraju svjetlo od visoko agregiranih formi (15). U jako kiselim i bazičnim uvjetima apsorpcijski vrhovi PP-a mogu se jasno razlikovati, za razliku od klinički relevantnijih neutralnih i blago kiselih uvjeta u kojima se PP nalazi u obliku visoko agregiranih vrsta. Taj podatak, uz nizak apsorpcijski koeficijent, upućuje na slabiju podložnost ekscitaciji. Također navješćuje da doprinos PP-a fluorescenciji karijesnih lezija, uobičajeno blagih pH-uvjeta od oko 5,5, možda nije tako velik kao što se prije spominjalo (11, 23). To je potvrđeno i u ovom istraživanju u kojem su neutralne i blago kisele otopine PP-a bile neprikladne za ekscitaciju laserom od 405 nm, što bi moglo stvarati velike probleme za detekciju PP-a u normalnim uvjetima kakve nalazimo u usnoj šupljini.

Slično apsorpcijskim spektrima, emisijske valne duljine PP-a, CP-a i UP-a također su pod utjecajem pH. Za sve porfirine u ovom istraživanju bio je karakterističan „crveni pomak“ – pomak vrhova LIF-a prema višim valnim duljinama. Bio je posebice uočljiv kod CP-a i UP-a. Možemo teoretizirati o tome kako bi razlozi mogli biti slični onima spomenutima za apsorpcijske spektre. Fenomen „crvenog pomaka“ također je poznat kao batokromni pomak i može se pripisati utjecaju supstitucije ili promjeni u okolišu (24). Agregacija

Discussion

A number of studies have dealt with the origin of fluorescence in caries and porphyrins, most of them using the “backward method” – studying the carious lesion directly and assigning the fluorescence peaks to certain porphyrins (6, 7, 10, 11). So far, neither addressed each of the porphyrins from caries lesions individually to observe their actual contribution to the carious fluorescence, nor did they consider the possible alterations in the emitted spectra influenced by the pH. This study was conducted as the proceeding of our previous work, where detailed absorption and emission spectra of CP and UP, two important porphyrins of carious lesions, were given (14). Since many authors have stressed out that PP is the most important and abundant porphyrin (10, 11, 23), the present study was concentrated to this particular ingredient, as well as to the interaction with CP and UP and the comparison to the natural carious lesion on tooth.

The importance of defining the absorption spectra of fluorescing molecules is to determine of the most efficient excitation wavelength. As shown in our previous article (14), different excitation wavelengths give essentially the same output fluorescence peaks. According to Buchalla (7), who studied the fluorescence of natural carious lesions with excitation wavelengths in range from 360-580 nm in 20 nm steps, certain wavelengths give higher emission intensity than others. The use of that particular excitation wavelength range facilitates the detection of fluorescence. The results of the present study indicate that the absorption maxima are in the range 357-407, 370-401 and 390-405 nm for PP, CP and UP, respectively. Therefore, the laser with discrete wavelength at 405 nm is selected as appropriate for all three porphyrins. This corresponds well to the values given for natural caries, 400-420 nm and around 520 nm (7).

It can be extrapolated from the results of the PP, CP and UP absorption spectra that the pH value has great influence, which can be explained by the pH dependent protonation and aggregation of the molecules. The monomers have the higher efficiency than the highly aggregated forms to absorb light (15). In highly acidic and basic conditions, absorption peaks of PP are clearly distinguishable, unlike the clinically more relevant neutral and mild acidic pH values, where highly aggregated forms of PP are present. This information, along with low absorption coefficient, indicates lower susceptibility to excitation. This suggests that the contribution of PP to the fluorescence of carious lesion, with the usually mild pH values around pH 5.5, might not be as high as previously mentioned (11, 23). This is also confirmed by this study, where those neutral and slightly acidic PP solutions were inappropriate for 405 nm laser excitation, which could present problems for PP detection in conditions normally found in oral cavity.

Similar to absorption spectra, the emission wavelengths of PP, CP and UP were also under the influence of pH. For all porphyrins used in the current study, the characteristic “red shift” – the shift of LIF peaks toward higher wavelengths was obvious. The shift was especially notable in CP and UP and only minor for PP, We might speculate that the reasons could be similar as those mentioned for the apsorption spectra. The phenomenon

porfirinskih monomera i promjena u molekularnoj strukturi zbog promjena u pH, sigurno pridonose pomaku fluorescencije, no potrebna su dalnja istraživanja tog područja kako bi se razjasnili detalji.

Uspoređujući spektre laserski inducirane fluorescencije PP-a, CP-a i UP-a s LIF-om karijesnog zuba, evidentno je da se vrhovi na 622 i 650 nm mogu jednostavno objasniti. Naime, svi testirani porfirini imaju emisiju na 650 nm ili slične vrijednosti, dok je vrh na 620 nm tipičan za CP i UP. Široka vrpca na 500 nm u zelenom spektralnom području potječe od dentina ispod karijesne lezije. Vrh na 689 nm u LIF-karijesu posebice je zanimljiv, osobito zato što se, suprotno očekivanju, nije mogao objasniti prisutnošću PP-a. Osim u ovom istraživanju, samo je još Buchalla (7) pronašao taj vrh, također na prirodnim zubima. To bi se pitanje trebalo razjasniti u budućim istraživanjima. U Tablici 1. uspoređujemo spektre LIF-a u ovom istraživanju s drugim sličnim studijama provedenim na zubima.

Usporedba podataka spektara laserski inducirane fluorescencije PP-a, CP-a i UP-a u otopinama s različitim pH vrijednostima daje vrijedne informacije, ali bilo bi jako teško zaključivati o sastavu porfirina u pravoj karijesnoj leziji. Zbog toga bi buduće opsežne kliničke studije bile od velikog značenja.

of "red shift" is also known as bathochromic shift and can be attributed to the influence of substitution or a change in environment (24). The aggregation of porphyrin monomers and the change of molecular structure due to changes of pH certainly contribute to the shift of the fluorescence, but further investigation of the subject is needed to clarify the details.

Comparing the LIF spectra of PP, CP and UP to the LIF of the carious tooth, it is evident that 622 and 650 nm peaks can be easily explained. Namely, all tested porphyrins have the emission at or around 650 nm, whereas peak at 620 nm is typical for UP and CP. The 500 nm broadband peak in green spectral region stems from the dentin lying underneath caries lesion. The 689 nm peak in caries LIF is especially interesting since, opposite to expected, PP did not provide the explanation for it. Except here, only Buchalla (7) found this peak, also in natural teeth. This issue remains to be resolved in future investigations. LIF spectra of this research are compared to the results of other similar studies conducted on natural teeth in Table 1.

The comparison between LIF spectra of PP, CP and UP in solutions with different pH values provides valuable information, but it would be very difficult to judge about the porphyrins composition in a real caries lesion. Therefore, in the near future extensive clinical study of different carious teeth would be of considerable importance.

Tablica 1. Usporedba podataka o spektrima LIF-a u trenutačnom i ostalim istraživanjima. Svi su bili ekscitirani ljubičastim svjetlom od 377 do 410 nm; *samo u alkalnom pH, ** samo u početnim karijesnim lezijama.
Table 1 Comparison of the LIF spectra data of the present and the other studies, all excited by violet light from 377-410 nm; *only in alkaline pH, ** only in white spot lesions

		emission wavelength / valna duljina emisije (nm)							
Present study/ Trenutno istraživanje	PP (pH 1.7)				600			655	
	CP (pH 1.7)				591	617		650	671*
	UP (pH 1.6)				595	620		654	677*
	Caries / karijes		500			622		650	689
Other studies/ Druga istraživanja	Buchalla (7)		500			624	635**	650	690
	Zezell et al. (6)	455	500	582		622			
	Borisova et al. (25)	440	490		590		635	650	

Zaključci

Najprikladnije valne dužine za eksitaciju protoporfirina IX, koproporfirina i uroporfirina u klinički bitnim neutralnim i blago kiselim uvjetima jesu od 358 do 405 nm. Fluorescencija karijesa može se pripisati protoporfirinu IX, koproporfirinu i uroporfirinu. Rezultati ovog istraživanja potvrđeni su u mnogobrojnim studijama provedenima na prirodnim karijesnim lezijama. Izvori određenih valnih dužina ipak ostaju neotkriveni te je potrebno detaljno istraživanje prirodnih karijesnih zuba.

Ovo istraživanje otkrilo je da bi doprinos protoporfirina IX laserski induciranoj fluorescenciji karijesne lezije mogao biti manje važan, što je suprotno očekivanom. Razlog je vjerojatno niska apsorpcija u blago kiselom i neutralnom pH. Ovo temeljno istraživanje fluorescencije porfirina u karijesnoj leziji važno je za mnoge nove uređaje za rano otkrivanje karijesa.

Conclusions

The most appropriate wavelengths for the excitation of protoporphyrin IX, coproporphyrin and uroporphyrin in clinically relevant neutral and mild acidic conditions is from 358-405 nm. The fluorescence of carious tissue might be contributed to protoporphyrin IX, coproporphyrin and uroporphyrin. The results of our study are confirmed by numerous studies performed on the natural carious lesions. However, sources of certain fluorescence wavelengths still remain to be elucidated and a detailed study of natural carious teeth is needed. This study revealed that the contribution of the protoporphyrin IX to the laser induced fluorescence of carious lesion might be of minor importance, contrary to expected. This is probably due to its low absorption in mild acidic and neutral pH. This basic research of porphyrin fluorescence in carious lesion is important for many novel devices for early caries detection.

Zahvale

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Abstract

Objectives: The aim of the study was to establish spectral regions for laser excitation of protoporphyrin IX (PP), coproporphyrin (CP) and uroporphyrin (UP) solutions under different pH conditions. The second aim was to compare laser induced fluorescence (LIF) spectra of PP, CP and UP solutions with various pH to the fluorescence of natural carious lesion and the results of other studies. **Materials and methods:** Absorption spectra of PP, CP and UP solutions with pH values in range from 0.6-13.0 were taken on a Varian model spectrophotometer. According to the peak absorption of the porphyrins used, laser at 405 nm (10 mW) was selected for LIF measurements of PP, CP and UP solutions, as well as the natural carious lesion. **Results:** Depending on the pH value, the LIF of the PP, CP and UP solutions exhibited the red shift toward higher wavelengths. Using excitation at 405 nm laser wavelength, fluorescence bands peaking at 601 nm and 655 nm of the acidic PP solution were observed. **Conclusions:** Violet LIF peaks of porphyrin solutions measured in vitro are similar to the wavelengths of a real carious lesion and fluorescence band peaks of natural teeth known from literature. However, the fluorescence of tested porphyrins does not completely explain the fluorescence spectra of dental caries and further studies are needed. pH seems to have an important influence on the light absorption and emission of porphyrins.

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Protoporphyrin; Coproporphyrin; Uroporphyrin; Dental Caries; Spectrometry, Fluorescence

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