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Isotope method for the determination of stoichiometry between compounds forming polypyrrole and glucose oxidase composite

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The stable isotope ratio mass spectrometry is a conventional method used in archaeology, medical, environmental and paleoenvironmental reconstruction studies. However new insights and applicability of the equipment often open new research areas and improve our understanding of the ongoing processes. Therefore the stable isotope ratio mass spectrometry method was applied for the stoichiometry determination of the complex polypyrrole and glucose oxidase composite (PPy/GOx composite). Enzyme glucose oxidase and conducting polymer polypyrrole are reported to form a composite, which was evaluated in time using dynamic light scattering method. The consistent enlargement of the PPy/GOx composite and the relative decrease of the spare enzyme molecules were observed in the polymerization solution. UV-VIS spectrometry was employed to follow the polymerization process. The isotope mixing model was applied for the evaluation of the constitution of the PPy/GOx composite. According to the obtained results the determination of the PPy/GOx composite stoichiometry could be more reliably determined using the nitrogen isotope ratio approach in comparison to the carbon approach. We expect that this novel work will widen the stable isotope ratio mass spectrometry application in the research.

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Introduction

The polymer nanoparticles are now widely used in various experimental fields such as electronics and photonics[1-3], sensors and medicine [4], biology and biotechnology [5], also for the pollution and environmental control [6]. The first method to prepare the polymer nanoparticles was solvent evaporation [7]. By now polymers could be obtained using a number of techniques including solvent evaporation, salting-out, dialysis, supercritical fluid technology, micro-emulsion, mini-emulsion, surfactant-free emulsion, and interfacial polymerization [8]. The obtained nanoparticles could be of various chemical compositions (e.g. a mixture of inorganic and organic materials, different polymers and various biomaterials) and forms (e.g. nanospheres, tubes, rods, fibers or pellets).

Polypyrrole (PPy) has received special attention due to its biocompatibility [9, 10], rapid synthesis and high conductivity [11-13]. This polymer could be prepared by an oxidative-chemical or electrochemical polymerization of pyrrole monomers. Enzyme induced chemical polymerization is also applied for the synthesis of polypyrrole nanoparticles [14] and formed polymer has already been used for the improvement of amperometric biosensors [15]. Composite nanoparticles and/or nanocomposites consisting of enzyme glucose oxidase (GOx) based core and polypyrrole shell have been developed [16]. Such nanoparticles can potentially be

used for biomedical applications due to biocompatible polypyrrole[17] shell. Many nanostructures and nanoparticles based on glucose oxidase core and conducting polymer polypyrrole [18, 19] or polyaniline shell [20] were developed. However, neither an internal structure, nor a stoichiometry of glucose oxidase molecules and PPy of these nanoparticles are still known. Therefore, the investigations of stoichiometry evaluation are of high interest.

The isotope ratio of light stable elements in the environment is in a continuous state of flux due to mass discriminatory effects of biological, biochemical, chemical and physical processes [21]. The differences in the ratios are small (e.g. about 0.11 atom % for carbon). For these small variations, the notation in units per mil (δ; one part per 1000) has been adopted to report changes in isotopic abundance as a per mil deviation compared with an isotopic standard (Equation 1).

$$\delta_s = \left(\frac{R_S - R_{STD}}{R_{STD}}\right) * 1000\ (‰) \qquad (1)$$

where R_S is the measured isotope ratio of the heavier isotope over the lighter one (e.g. $^{13}C/^{12}C$ or $^{15}N/^{14}N$) of the sample; R_{STD} is the measured isotope ratio of the standard (e.g. VPDB (Vienna Pee Dee Belemnite) or air). Use of VPDB as standard reference has been normalized according to IAEA (International Atomic Energy Agency) guidelines for expression of δ values relative to traceable reference materials on the internationally agreed per mil scale [22]. Natural abundance light stable isotopes of hydrogen, carbon, nitrogen, oxygen and sulfur (H, C, N, O, S respectively) traditionally are used in archaeology [23], medical [24], environmental [25, 26] and paleoenvironemental reconstruction studies [27]. Substances, enriched by a heavier isotope, are used for the nutrition and metabolic balance studies. Stable isotopes have an advantage compared to the radioactive ones due to their long term stability. Isotope ratio mass spectrometers (IRMS) are used for the precise measurement of H, C, N, O, S isotopic composition. For the measurements of the heavier elements isotopic ratio inductively coupled plasma mass spectrometry (ICP-MS) is applied. For example zinc oxide (^{68}ZnO) and titanium oxide (^{46}TiO) were used as tracers in sunscreen products containing zinc oxide and titanium oxide to determine *in vivo* their dermal absorption and excretion in a human body [28].

The aim of this study was to investigate the enzymatic polymerization of pyrrole induced by glucose oxidase and to evaluate quantitatively the ratio of enzyme and monomer in the formed polypyrrole and glucose oxidase composite (PPy/GOx composite). For such kind of determination stable isotope ratio mass spectrometry was employed for the first time.

Materials and Methods

Chemicals

Phosphate buffer was made from disodium hydrogen phosphate dodecahydrate from Carl Roth Gmbh (Germany), potassium chloride from Scharlau Chemie, S.A. (Spain) and sodium dihydrogen phosphate monohydrate from Fluka (Germany) in triply distilled water. Glucose oxidase (GOx (1)) from *Aspergillus niger* (304.8 U/mg) and hydrogen peroxide were purchased from AppliChem (Germany). Glucose oxidase (GOx (2)) from *Aspergillus niger* (100-250 U/mg) was purchased from Sigma Aldrich (Germany). D-(+)-glucose from Carl Roth GmbH Co, KG (Germany) was prepared in triply distilled water at least 24 h before use in order to allow glucose to mutarotate. All the chemicals were of analytical grade if not stated otherwise. Pyrrole was purchased from Alfa Aesar GmbH & Co (Germany) and before use it was purified through Al_2O_3 column (0.4 mm diameter and 6 cm length) in order to remove all coloured components.

Polymeric PPy/GOx composite preparation

Enzymatic polymerization of polypyrrole PPy/GOx composite was performed according to the procedure presented elsewhere [14]. Briefly: the monomer was added to 2 ml reaction solution containing 0.05 M phosphate buffer pH 6.0; it was followed by the addition of the enzyme (up to 1 mg/ml final concentration) and glucose solution (up to 50 mM final concentration) at the end. The concentration of pyrrole varied from 50 to 400 mM to find out the monomer concentration influence on the polymerization rate. In order to homogenize monomer solution in buffer, it was stirred in an ultrasonic bath for 3 min. After addition of each subsequent compound the solution was mixed with a shaker for a minute. Prepared solutions were kept at 30 °C temperature in darkness. No detergents or any other stabilizers were added**.**

Sample preparation for isotope ratio mass spectrometry measurements

Polymerized samples were centrifuged at 12500 rpm, for 15 min with IEC MicroCL 17 centrifuge, Thermo Electron Corporation (USA) and supernatant was separated from the centrifugate. After centrifugation, centrifugate consisted of polypyrrole, GOx, glucose and gluconic acid, while centrifugate of remaining pyrrole, GOx, glucose and gluconic acid. Supernatant was used for the isotope ratio mass spectrometric measurements as obtained. Centrifugate was also used for the IRMS analysis as obtained or additionally washed two times with distilled water and subsequently centrifuged each time to remove the residue of glucose and gluconic acid. After additional washing obtained sediment consisting of polypyrrole and glucose oxidase was used for the stoichiometry determination by isotope ratio mass spectrometry.

Characterization of formed PPy/GOx composite

The UV-VIS spectrophotometer Lambda 25, PerkinElmer (USA) was employed for the observation of polymerization reaction. The interval between 400 and 600 nm was acquired.

The dynamic light scattering (DLS) method was used for the evaluation of PPy and GOx-based PPy/GOx composite. DLS measurements were performed with a Malvern Zetasizer Nano ZS (Germany) equipped with a 633-nm He-Ne laser and operating at 173° angle. The obtained data were analysed with Dispersion Technology Software version 6.01 from Malvern.

The high resolution field emission scanning electron microscope (SEM) SU-70 (Hitachi, Japan), and atomic force microscope (AFM) BioScope Catalyst (Bruker, USA) were used for the evaluation of PPy/GOx composite surface structure. The accelerating

voltage for the electron beam was set to 5 keV for SEM. The Peak Force Tapping mode equipped with specialised Scan Asyst tip (spring constant – 0,4 N/m, frequency 50 – 90 kHz) was used for the AFM measurements. The sample was prepared by dropping a small aliquot of polymerization solution on conventional atomically flat silicone substrate. After drying the sample was washed with copious amount of distilled water in order to remove buffer and glucose residue.

Isotope ratio mass spectrometry

Nitrogen and carbon isotope ratios in the PPy/GOx composite were measured using an elemental analyzer (EA) (Flash EA1112) coupled to IRMS (Thermo delta plus advantage) *via* ConFlo III interface. A sample was placed in a tin capsule and combusted in the elemental analyzer with the oxygen excess. For one analysis, at least 100 µg of carbon and 50 µg of nitrogen are needed. Cr_2O_3 granules in the oxidation column were used as an additional oxygen source, while copper wires in the reduction column were used for the reduction of nitrogen. Oxidation and reduction columns were operating at the 1020°C and 650°C temperature, respectively. Pure He 5.0 was used as a carrier gas, while magnesium perchlorate was used for the water removal. Gases evolved after combustion were separated by the packed column and transferred to the ConFlo III interface. This interface allowed the passing of calibration gases from the laboratory tank (N_2 and CO_2) and EA to the IRMS at different time intervals that allowed to measure nitrogen and carbon isotope ratios with high accuracy. Reference material with the known nitrogen and carbon isotopic values (caffeine IAEA 600, δ^{13}C=-27.771‰$_{VPDB,}$ δ^{15}N=1 ‰$_{air\ N2)}$[22] was used for the laboratory N_2 and CO_2 tank calibration. Analytical precision and calibration of used reference gas CO_2 to PDB ($\delta^{13}C = -31.1 \pm 0.08$‰) have been estimated by the repeated analysis of certified reference material IAEA 600, which gave an average value of δ^{13}C equal to −27.77 ‰ (certified value $\delta^{13}C = -27.77 \pm 0.04$‰) and standard deviation equal to 0.08‰. The same calibration procedure was applied for the calibration of laboratory N_2 tank ($\delta^{15}N = -2.4 \pm 0.09$‰) to air N_2. Analytical precision for the measured samples was 0.15 ‰ or better (both for the nitrogen and carbon).

Calculations by the isotope mixing model

When the isotopic composition of the initial substances –pyrrole and glucose oxidase (respectively: δ_{Py} and δ_{GOx}) and the isotopic composition of the composite material (δ_{sample}) are known, it can be expressed as:

$$\delta_{SAMPLE} = \delta_{PY} * f_1 + \delta_{GOX} * f_2 \quad (2)$$

$$f_1 + f_2 = 1 \quad (3)$$

where f_1 and f_2 are the fractions of the pyrrole and glucose oxidase in the sample, respectively. Combining equations 2 and 3 the equation 9 is obtained:

$$\delta_{SAMPLE} = \delta_{Py} * f_1 + \delta_{GOx} * (1 - f_1) \quad (5)$$

$$\delta_{SAMPLE} = \delta_{Py} * f_1 + \delta_{GOx} - \delta_{GOx} * f_1 \quad (6)$$

$$\delta_{SAMPLE} - \delta_{GOx} = \delta_{Py} * f_1 - \delta_{GOx} * f_1 \quad (7)$$

$$\delta_{SAMPLE} - \delta_{GOx} = (\delta_{Py} - \delta_{GOx}) * f_1 \quad (8)$$

$$f_1 = (\delta_{SAPMLE} - \delta_{GOx})/(\delta_{Py} - \delta_{GOx}) \quad (9)$$

The number of carbon atoms in the fraction f_1 can be calculated according to the formula:

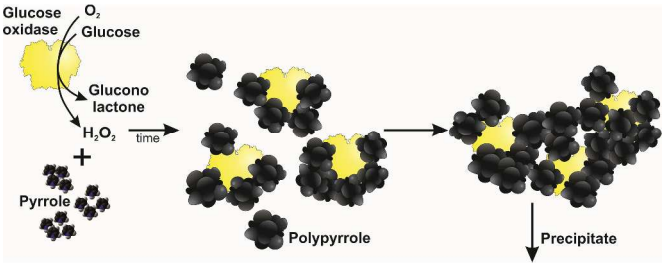
$$N_{Py} = f_1 * N_{GOx}/f_2 \quad (10)$$

where N_{GOx} is the number of the carbon atoms in the glucose oxidase.

From equation 10 the number of carbon atoms from pyrrole that are stacking on the glucose oxidase, can be calculated. The same arithmetic is valid for the calculation of the isotope ratio of nitrogen atoms.

Results and discussion

The enzymatic polymerization of pyrrole was evaluated using the DLS analyzer and UV-VIS spectroscopy. The predicted polymerization process and formation of PPy/GOx composite is presented in Scheme 1. Enzyme, glucose oxidase, in the presence of oxygen catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide. In close proximity of the enzyme the medium is more acidic due to the hydrolysis of gluconolactone to gluconic acid. Together with hydrogen peroxide, which is generated in enzymatic reaction, gluconic acid creates a favorable environment for the oxidative-polymerization of pyrrole. For this reason the encapsulation of enzyme inside the polypyrrole is highly probable. During the polymerization procedure the PPy/GOx composite from enzyme and polymer is increasing in size, due to the formation of PPy layer around enzyme. Moreover, the aggregation of PPy/GOx composite is also possible. Due to this some precipitation of agglomerates was observed during the course of polymerization. As the monomer of pyrrole is poorly soluble in water even after the ultrasonic treatment the micelles of monomer are registered with DLS (Fig 1. A, the lowest curve at 0 h). In general the GOx is soluble in water, but some hydrophobic residues are also present. If the enzyme with its hydrophobic residues attaches to the micelle of the monomer, the polymerization of such micelle is also expected. Most likely both of the processes are possible and take place in the polymerization solution.



Scheme 1 Schematic representation of pyrrole polymerization induced by hydrogen peroxide generated in catalytic reaction of glucose oxidase. GOx is surrounded by formed PPy, later agglomeration and precipitation of such PPy/GOx composite is observed.

The size variation of PPy/GOx composite, which was formed from the solution containing 100 mM of pyrrole and 1 mg/ml of GOx, was followed by DLS analyzer in the polymerization time frame from 0 h to 377 h (Fig. 1 A). At the initial stage (0 h) three particle size peaks were observed. The first peak at 10 nm was registered in all samples and it is attributed to the dissolved glucose oxidase. The other two DLS peaks could be attributed to the micelles of pyrrole, which are formed in water by ultrasound treatment during the preparation step. The relative intensity of the GOx peak at 0 h is the highest in all evaluated samples at four different concentrations, which indicates that the quantity of light scattered from dissolved GOx is more significant than that from dispersed pyrrole micelles, which were formed in the initial solution before polymerization. Later during the polymerization process the situation in the system has altered. First of all the composition and properties of materials inside the cuvette has changed. The monomer of pyrrole is more transparent in comparison with PPy and in aqueous solution pyrrole is probably assembled in small micelles; however formed polymer is of very different nature. Dark color and randomly aggregated agglomerates are expected to form during the polymerization process. In addition, no clear simultaneous growth and/or aggregation of the particles in time are observed. During the course of the reaction new polymerization centers are forming continuously and new polypyrrole PPy/GOx composites are generated. For this reason at different time intervals new and small peaks attributed to aggregated structures are observed. The DLS results illustrate that the intensity of the peak, which is representing smaller size PPy/GOx composite, is lower than that of the larger ones. This effect could be based on the fact that larger particles scatter more light. Due to all mentioned aspects the relative intensity of GOx peak is decreased in comparison to the other peaks during the course of polymerization. However, after 76 h the small increase of the GOx peak could be observed in comparison with that attributed to formed PPy/GOx composite. This change could be elucidated as the beginning of the precipitation of larger PPy/GOx composite aggregates to the bottom of cuvette because no colloid-stabilizing agents were used. For this reason the increase of relative high amount of free GOx was observed in the sample. What is more, a part of visible light is absorbed by the polymer and could influence final amount of the light, which is reaching DLS detector. Due to all the reasons mentioned above the accuracy of quantitative comparison of DLS results is limited when the peak height is evaluated. Therefore this method is better suitable for qualitative than for quantitative measurements. The principle of the DLS measurement is to register the dispersed light from the particles in the time frame and from the obtained results the size of idealized round shape particles is calculated. In this respect all the scattered light in one measurement is attributed to 100% and it is distributed between the whole range of observed DLS peaks. The intensity from the single particle depends on several reasons – the deviation of the particle shape from the ideal sphere, the ability of the particle to scatter the light and the ability to absorb it. Moreover, in the presence of four different concentrations of monomer no principle difference in the polymerization process was noticed. After the same time interval (Fig. 1B) all DLS results showed a similar tendency: the peak attributed to GOx is reduced and the size of polymer PPy/GOx composite is in the range from 300 to 700 nm. Larger PPy/GOx composite could hardly be registered due to relatively fast aggregation, which is followed by precipitation.

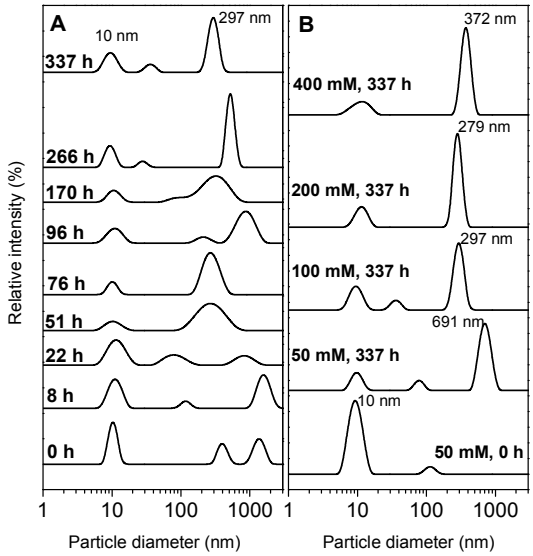


Fig. 1 DLS based evaluation of polymerization and PPy/GOx composite formation A) at 100 mM of pyrrole, in a time frame from 0 to 337 h and B) at different concentrations (50 mM, 100 mM, 200 mM and 400 mM) of pyrrole after 337 h of polymerization. For additional comparison a DLS spectrum of 50 mM pyrrole at 0 h is also presented in the B part.

The characteristic absorption peak of PPy at 460 nm [29] was registered in time during enzymatic polymer synthesis process (Fig. 2A). The absorption of polypyrrole in the solution is increasing during the time despite the fact, that some of the composites are precipitated on the bottom of the cuvette. For a better comparison, several concentrations of monomer were evaluated (Fig. 2B). As could be seen from the results (Fig. 2B), at 100 mM concentration of pyrrole the highest changes in the signal intensity were observed using UV-VIS spectroscopy. This might be due to the most optimal conditions of here exploited polymerization reaction at 100 mM of pyrrole. Thus for the next experiments of the PPy/GOx composite formation, 100 mM concentration of pyrrole was chosen.

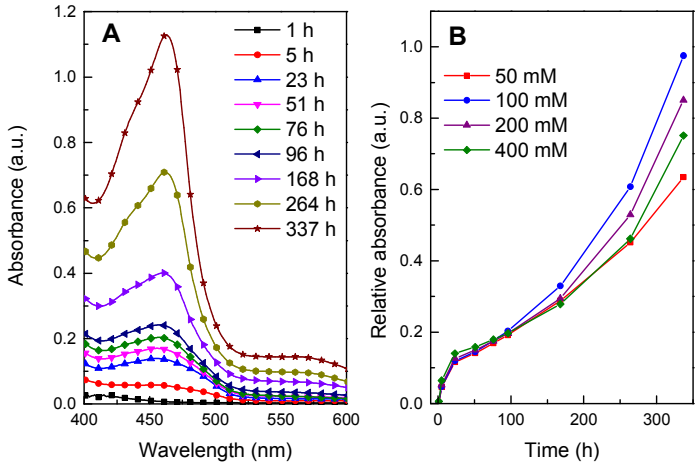


Fig. 2 Spectroscopic evaluation of polypyrrole formation during enzymatic polymerization A) in a time frame from the start until 337 h, at 100 mM pyrrole concentration and B) PPy peak intensity at 460 nm using different concentrations (50 mM, 100 mM, 200 mM and 400 mM) of pyrrole monomer. Here ordinate represents the height of the absorption peak and abscissa corresponds to the duration of polymerization process.

The obtained reaction products before the analysis with IRMS were evaluated using SEM and AFM techniques (Fig. 3). A complex and highly dense structure is presented in the SEM image. The structures seems to be aggregated however, the small detail structure is hardly visible. The higher magnification image was impossible to obtain with SEM due to sample burning. For this reason the AFM technique was employed. As could be seen from the image 3 B, the grain like structures was determined.

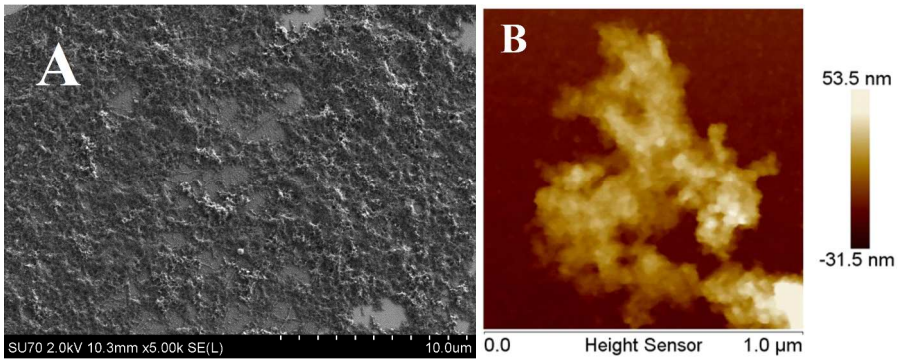


Fig. 3 PPy/GOx composite structure evaluation. A) SEM and B) AFM images of GOx/Ppy PPy/GOx composite adsorbed on silicon substrate.

During the next stage of the experiments the isotopic composition of initial materials was measured and results are presented in Table 1. Two enzymes obtained from different suppliers were examined. The first enzyme GOx (1) was obtained from Applichem and the second one – GOx (2) from Sigma Aldrich. Both of them were produced by *Aspergillus niger*. However, only for GOx (1) the isotope ratio for nitrogen and carbon was remarkably different in comparison to pyrrole or glucose. Thus, for future calculations and interpretation of results GOx (1) was selected. Calculations of the carbon isotope ratio of composite should include three components: pyrrole, enzyme and glucose, as all three constituents contain carbon in their molecular structure. Glucose and pyrrole have very similar carbon isotope ratio values and for this reason the separation of these two compartments are impossible. However glucose molecule does not have any nitrogen, hence the results calculated for nitrogen could be more precisely related to the stoichiometry of pyrrole and enzyme.

Table 1 Isotopic characterization of initial materials used in enzymatic polymerization of pyrrole: pyrrole, glucose oxidase and glucose. $\delta^{13}C$ and $\delta^{15}N$ are the isotopic composition of carbon and nitrogen respectively obtained by IRMS; C(%) theoretical and N(%) theoretical are elemental composition estimated for carbon and nitrogen respectively; C/N was calculated as the ratio of C theoretical and N theoretical.

	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C (%) theoretical	N (%) theoretical	C/N	Molar mass (g/mol)	Reference
Pyrrole	-12	1.8	71.6	20.9	3.43	67	from supplier
GOx (1)	-24	-12.5	53.4	16.9	3.16	~ 186 000[30]	and from supplier
GOx (2)	-25.6	2.2	53.4	16.9	3.16		
D-(+)- glucose	-11.2	---	---	---	---	180	from supplier

As it was mentioned before, during the polymerization reaction of pyrrole, glucose oxidase is expected to be surrounded by the formed polypyrrole chains. At present, there are no suitable analytical methods to quantify the ratio between formed PPy and entrapped GOx, but stable IRMS allows to quantify the ratio of initial materials, being involved into PPy/GOx composite. Experimental results of the IRMS measurements of the PPy/GOx composite and supernatants are presented in Table 2.

Table 2 Measured $\delta^{13}C$, $\delta^{15}N$ and C/N ratio values and calculated carbon and nitrogen approaches for the PPy/GOx composite and supernatant. PPy/GOx composite refers to the PPy and GOx aggregations analysed after centrifugation (three separate measurements are presented; symbol 'w' indicates washing procedure (2 times with distilled water) performed after centrifugation); Supernatant refers to the solution which was removed after centrifugation. Other abbreviations: f1 – calculated fraction of pyrrole; f2 – calculated fraction of GOx; number of Py molecules in the composite – estimated number of pyrrole molecules in formed PPy/GOx composite, that are surrounding the single GOx molecule; n/d (not determined) means that for the carbon approach it is not possible to calculate the number of Py molecules in the composite because $\delta^{13}C$ value for the composite was lower comparing to the GOx.

		$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C/N ratio	Carbon approach			Nitrogen approach		
					f1	f2	number of Py molecules in the composite	f1	f2	number of Py molecules in the composite
PPy/GOx composite	1w	-21.9	-5.7	---	0.17	0.83	150	0.47	0.53	710
	2w	-24.6	-5.2	3.7	n/d	n/d	n/d	0.51	0.49	830
	3w	-24.4	-2.3	3.96	n/d	n/d	n/d	0.29	0.71	1970
	4	-19.3	-5.0	18.1	0.39	0.61	460	0.52	0.48	850

Supernatant	1	-14.0	2.1	9.7	
	2	-18.5	2.6	10.2	

The highest C/N ratio was measured in the PPy/GOx composite, which was not washed with water (PPy/GOx composite 4). In this PPy/GOx composite, additional source of carbon is present, which most probably is from D-glucose used as substrate for GOx and from glucono lactone, which is the product of the enzymatic reaction. During the cleanup step most of these soluble materials were successfully washed from the PPy/GOx composite with ultrapure water. The carbon isotope ratio in the formed composite changed after the washing procedure. This change is an indicator that substances (D-glucose or glucono lactone) with the different isotope ratio were removed from the composite. As it is evident from the C/N ratio (Table 2), the liquid phase – supernatant contained a relatively high amount of carbon, the origin of which probably is residual D-glucose, unpolymerised pyrrole monomer and glucono lactone.

The enzyme to pyrrole molecule ratio was estimated using the stable isotope mass-balance equations (2-10). Using the carbon approach 150 molecules (or 460 molecules when not washed) of pyrrole were obtained belonging to one molecule of glucose oxidase. However, when nitrogen isotopes were evaluated in aggregates, 710 – 1970 of pyrrole molecules were assigned for each glucose oxidase molecule. A higher number of the pyrrole molecules (more than 4.18 times) attached to the GOx was obtained. The carbon isotope ratio of glucose and pyrrole (about -12 ‰) is similar and more positive compared to that of GOx (-24 ‰). The difference between the carbon and nitrogen approach might arise due to trapping of glucose or glucono lactone inside the PPy/GOx composite. Unfortunately, using only the carbon isotope ratio the discrimination of pyrrole, glucose, glucono lactone is impossible. On the other hand, nitrogen isotopes are absent in the glucose and later in the reaction product – glucono lactone. Moreover, as could be seen from the table 1 the nitrogen isotope ratio is different between pyrrole (1.8 ‰) and glucose oxidase (-12.5 ‰). Glucose or glucono lactone, even entrapped within the aggregate, has no influence on the final result while calculating the ratio of the pyrrole in the PPy/GOx composite using the nitrogen stable isotope ratio. We suggest that the nitrogen isotope approach based calculations of ratios in PPy/GOx composite are more accurate in comparison with the carbon based isotope approach, because in the case of nitrogen isotopic signal has no interference from carbon present in glucose or glucono lactone. It must be noted that in general precision of the elemental analyzer coupled to the IRMS is of 0.15 ‰ order. The uncertainty of estimation of carbon or nitrogen atoms in the biocomposite mostly depends on the polymerization reaction rate, because the isotope ratio measurement precision of 0.1 ‰ gives variation of 10 pyrole atoms in such type of the PPy/GOx composite. The calculation uncertainty mostly depends on the carbon and nitrogen isotope ratio measurement precision. Higher accuracy of stoichiometry determination in the PPy/GOx composite can be obtained using initial materials, which differ significantly in their isotope ratios.

Another reason of inappropriate results for the carbon isotope approach can arise due to the fact that a relatively high amount of carbon (compared with nitrogen) is present in the glucose oxidase. The enzyme molecule is composed of two subunits. Each of them is roughly composed from 2800 and 760 atoms of carbon and nitrogen respectively while pyrrole contains 4 carbon and one nitrogen atom. Thus $\delta^{13}C$ ratio is very close or ever higher to the pure GOx ratio and the model calculations do not give any results. In the future, the experiments with a relatively low GOx concentration are needed to investigate the experimental discrepancy between carbon and nitrogen isotopic approaches more precisely.

Designing isotope mixing experiments the best results can be obtained when the stable isotope ratio variation between substances of the natural isotopic composition is at highest level. For example, glucose can have $\delta^{13}C$ values from -12 ‰ to -30 ‰, because two photosynthetic pathways, commonly designated C_3 and C_4, fractionate carbon isotopes in plants to different degrees, resulting in differences of about 20 ‰ in glucose derived from these two sources. Usually C_4 plants (i.e. sugarcane) have $\delta^{13}C$ values of about -12 ‰, while $\delta^{13}C$ values for C_3 plants are in the range from -24 to -30 ‰ [31]. For this reason, firstly, the initial materials must be carefully analyzed and the best ones should be chosen, as it was shown in our experiments with the selection of enzyme. Moreover, the glucose from C_4 plants could also be used in these experiments, although the interference in calculations would still be appreciable. In this case, carbon isotope ratio values of enzyme and glucose would be similar.

Conclusions

The PPy/GOx composite obtained during enzymatic polymerization of pyrrole were investigated using the UV-VIS spectroscopy, the dynamic light scattering method and the isotope ratio mass spectrometry. The applicability of the stable carbon and nitrogen isotope ratio method for the evaluation of complex PPy/GOx composite stoichiometry was appraised. Using nitrogen isotope ratio mass spectrometry measurements approximately 710 – 1970 pyrrole monomers were estimated to surround glucose oxidase during enzymatic polymerization. However, at relatively high concentration of GOx in the solution the carbon isotope approach was not suitable for the stoichiometry determination in the PPy/GOx composite, because the stacking of GOx determines the $\delta^{13}C$ ratio of the final PPy/GOx composite. Higher accuracy of stoichiometry determination in the PPy/GOx composite can be obtained using initial materials, isotope ratios of which differ significantly.

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