COMPARATIVE DICLOFENAC DETECTION FOR CHRONIC TOXICITY LEVELS USING WATER SOLUBLE Zn-METALLOPORPHYRIN, GOLD NANOPARTICLES AND THEIR HYBRID

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Abstract

The optical detection of chronic toxicity levels of diclofenac were comparatively investigated using novel nanostructured materials, such as: a water soluble metalloporphyrin: Zn(II)-tetra-(4-N-methyl-4-pyridyl)porphyrin tetrachloride (ZnTMePyP), tailored size spherical gold nanoparticles (n-Au) of 15 - 20 nm, and the hybrid nanomaterial (ZnTMePyP/n-Au) formed between them. After spectroscopic and microscopic analyses, it can be stated that the water soluble Zn-porphyrin alone can detect diclofenac in a larger domain of concentration (2.57×10^{-5} - 14.15×10^{-5} mol/L) than the hybrid formed between the metalloporphyrin and n-Au and that the gold nanoparticles alone are not capable of diclofenac detection.

Rezumat

A fost investigată capacitatea unor noi materiale nanostructurate de a detecta prin spectroscopie UV-Vis concentrații de diclofenac din domeniul relevant toxicității cronice, utilizând o metaloporfirină solubilă în apă, și anume: tetraclorura de Zn(II)-tetra-(4-N-metil-4-piridil)porfirina (ZnTMePyP), nanoparticule sferice de aur (n-Au) cu dimensiune controlată (15 - 20 nm în diametru) precum și materialul hibrid format între ele (ZnTMePyP/n-Au). În urma analizelor spectroscopice și microscopice efectuate s-a constatat că Zn-porfirina folosită singură poate detecta un domeniu mai larg de concentrații de diclofenac (2.57×10^{-5} - 14.15×10^{-5} mol/L) decât hibridul format între ea și n-Au, precum și că nanoparticulele de aur nu au capacitate de detecție pentru diclofenac.

Keywords: diclofenac, Zn-metalloporphyrin, gold nanoparticles, gold-porphyrin hybrid, UV-Vis spectroscopy

Introduction

Diclofenac, 2-[(2,6-dichlorophenyl)-amino]-benzene-acetic acid, is a synthetic nonsteroidal anti-inflammatory analgesic drug [10], pharmaceutically formulated as different salts, usually as potassium and sodium. The drug is used to treat rheumatoid arthritis, osteoarthritis [22, 16], musculoskeletal injuries [38], ankle sprain injuries [28, 29] and is administered as post-surgery analgesic in human and veterinary medicine [23]. Many patients taking diclofenac for arthritis also need additional drugs for other chronic health problems such as hypertension [40].

Diclofenac may inhibit L-type calcium channels which participate in pain perception [54] and this is the reason why this drug is widely indicated to decrease morphine consumption after surgery in adults [43], for degenerative joint disease [35], chronic pain associated with cancer and kidney stones [26] and endodontic procedures [32].

Despite its benefic actions, diclofenac needs a co-treatment agent as a therapeutic strategy to attenuate the gastrointestinal tract complications leading to gastric ulcer disease [10, 51].

Metabolites of diclofenac are eliminated in the urine and the mean terminal elimination half-life was determined to be 88.4 hours [21].

Knowing the limiting doses that are not producing toxic or chronic drug-induced effects is a demand in drug development. Researchers found that 52% of patients receiving topical diclofenac sodium gel reported adverse events, such as: headache, abdominal pain, dyspepsia, nausea, pruritus, angina, palpitations, arrhythmia, venous thrombosis and elevation in creatinine and gastrointestinal bleeding [4, 35]. In case of hepatotoxicity, viability and drug metabolizing capacities of the hepatocytes have been assessed. Acute diclofenac toxicity (24 h) was determined to be in the range of (1×10^{-5} - 1×10^{-3} mol/L) concentrations [33].

The chronic toxicity investigated for 9 repeated doses of higher concentration (6.4×10^{-6} and 100×10^{-6} mol/L) was also tested. The toxic effects were observed starting from a very small clinically relevant concentration (6.4×10^{-6} mol/L), that generates negative influences on exometabolome, induces apoptosis in hepatocytes [17] and might induce pseudoporphyria [50].

The toxicity assay is also requested for veterinary treatments and for environmental pollution of water. Based on the fact that the serum concentrations in adult horses of up to 10 ng/mL can be attained without adverse systemic effects [7] and due to the observation that the acute toxicity value for LC50 - 48 h for *Daphnia magna* in water is 18.81 ± 7.1 mg/L [41] there is a high demand to monitor toxic concentration levels to avoid harmful effects.

The analytical methods described in literature for the determination of diclofenac in tablets and human serum, rely mostly on spectrophotometry and HPLC [12]. These methods lack in providing a valid and fast methodology for its determination in routine analysis in pharmaceutical and clinic laboratories [48].

Early spectrophotometric methods for the determination of diclofenac in tablets containing also paracetamol, in the concentration range 10×10^{-6} - 80×10^{-6} g/mL, are based on its capacity to reduce iron(III) to iron(II) when heated in aqueous solution. The ferrous ions form a coloured complex with 2,2-bipyridine having the maximum absorbance at 520 nm [2]. The drawbacks of UV-Vis spectrophotometric methods for the determination of diclofenac are the lack of sensitivity or simplicity. A more sensitive method used the basic dye methylene violet for its extractive spectrophotometric determination [46].

The need to selectively detect diclofenac in various media such as: human plasma and tap water, lead to the development of a supramolecular chemosensor based on 1,2,3-triazole [3]. The detection limit for diclofenac is 10×10^{-6} mol/L which recommends this sensor for monitoring its levels in different physiological, biological and environmental systems. A recent approach for monitoring diclofenac in spiked saliva samples [37] was based on the oxidation of diclofenac with potassium ferricyanide. Absorbance of the derivative in acetonitrile medium presented a linear calibration graph over the range of 2.5×10^{-6} - 60×10^{-6} mol/L with high precision.

In order to avoid optical interferences [20] silica nanomaterials functionalized with secondary antibodies and displaying COOH groups on the surface were used for the detection of diclofenac in water. A detection limit for DCF of 0.05×10^{-6} g/mL was reached, allowing to further use this method for analysing micropollutant pharmaceuticals in drinking and surface water samples.

Capillary zone electrophoresis was employed for the determination of diclofenac in human urine using a carbon fibre microelectrode. The limit of detection is 2.5×10^{-6} mol/L [25]. In recent years, the electrochemical and chromatographic methods [42] proved to be highly sensitive for the analysis of various drugs both in pharmaceutical products and also in human body fluids.

The square wave voltammetry method applied for the routine determination of diclofenac from urine samples, using plane pyrolytic graphite electrode, reached the lowest detection limit of 6.2×10^{-9} mol/L [18].

Other nanocomposites, consisting of $\text{Cu}(\text{OH})_2$ nanoparticles, the hydrophobic ionic liquid 1-ethyl-3-methylimidazolium hexafluorophosphate and multi-walled carbon nanotubes were used for glassy carbon electrode modification exhibiting good electrocatalytic activity for the oxidation of diclofenac in blood serum and sea water [5]. The sensor proved its validity in the range of 0.18×10^{-6} to 119×10^{-6} mol/L.

Linear sweep voltammetry (LSV) and gas chromatography (GC) coupled with mass spectrometry (MS) proved to be highly efficient for diclofenac detection in the concentration range of 5×10^{-6} - 35×10^{-6} g/mL for LSV and 0.25×10^{-6} - 5×10^{-6} g/mL for GC-MS method. Despite its fastness and precision, expensive equipment is needed [43, 55]. The use of diclofenac in the presence of morphine for the treatment of various diseases was monitored in urine samples using a 1-methyl-3-butylimidazolium chloride modified NiO/CNTs carbon paste electrode [10]. A simple and sensitive method for the determination of diclofenac was based on redox properties of Cobalt hexacyanoferrate deposited on multi-walled carbon nanotubes (MWCNTs). The diclofenac concentration range was 1.0×10^{-3} - 100.0×10^{-3} mol/L with detection limit of 0.3×10^{-3} mol/L [10].

Novel approaches are based on using nanomaterials, such as nanogold, that brought benefits for diclofenac detection due to its large surface area, enhancement of electron transfer, good biocompatibility and high conductivity [1]. A sensor for the determination of diclofenac based on gold nanoparticle/multi-walled carbon nanotube modified glassy carbon electrode was reported to give an excellent response in the range of 0.03×10^{-6} - 200×10^{-6} mol/L.

Many attempts to detect and monitor diclofenac levels in pharmaceutical preparations [44] in the presence of several substances by means of potentiometric methods were reported during time.

The system $\text{Pt}[\text{Hg}|\text{Hg}_2(\text{DCF})_2]\text{graphite}$ responds to diclofenac with Nernstian sensitivity over a wide linear range 5.0×10^{-5} to 1.0×10^{-2} mol/L at pH 6.5 - 9.0 [44].

A PVC membrane sensor based on its ion pair complex with silver for diclofenac detection demonstrated also

a Nernstian response in the range of 5.2×10^{-5} to 1.1×10^{-2} mol/L [31].

The heterocyclic compounds already demonstrated their ability to coordinate anions, so that their use to remove or detect diclofenac was tested. In order to remove diclofenac from contaminated waters calix[4]-arenes products were used as versatile receptors [11]. The porphyrins are easily implied in host guest processes and due to this property are used for potentiometric sensors formulations. The potentiometric response for Fe(III) tetraphenylporphyrin-chloride and Fe(III) tetrakis(pentafluorophenyl) porphyrin chloride based membranes to diclofenac was studied in different mediator solvents and using several ionic additives [45]. The best result was obtained by Fe(III)TPFPP-Cl ionophore in *o*-nitrophenyloctyl-ether, showing a linear response towards diclofenac concentrations between 10^{-5} and 10^{-2} mol/L.

A better performance was obtained by using as ionophores two different metalloporphyrins Mn(III) tetraphenylporphyrin chloride and manganese(III)-tetrakis(3-hydroxyphenyl)porphyrin chloride [52] for the construction of new diclofenac-selective electrodes. The best result (linear response in the concentration range 3×10^{-6} - 1×10^{-2} mol/L) was obtained for the PVC membrane based on Mn(III) tetraphenylporphyrin chloride plasticized with dioctylphthalate and having sodium tetraphenylborate as a lipophilic anionic additive.

The substitution of the toxic mercury electrodes by the more environmentally friendly porphyrin modified

electrodes is an actual trend of sustainable chemistry [39]. An amperometric method for the analysis of sodium/potassium diclofenac on a glassy carbon electrode modified with films of a tetraethenyl(II) cobalt(III)-porphyrin achieved low limits of detection (8.1×10^{-8} mol/L).

Diclofenac was also analysed with a detection limit of 1.9×10^{-5} mol/L using modified Ag colloids with thiocholine bromide, thus creating particles where the zeta potential is switched from negative to positive values [49].

Taking into consideration the unfavourable pharmacokinetic properties of drugs and the need to assess the associated risks [8], this work continues previous investigation using gold [1] and nanogold-porphyrin hybrids [14, 47] on one hand, and porphyrins [9, 15, 30] on the other hand, for biologically relevant compounds detection.

The effects generated between the noble metals nanoparticles and the porphyrins are comparatively presented in a complex study regarding diclofenac sodium detection with the purpose to help monitoring long term treated patients that might develop chronic toxicity (10^{-5} - 10^{-4} mol/L) [51]. A water soluble zinc-metalloporphyrin, Zn(II)-tetra-(4-N-methyl-4-pyridyl)porphyrin chloride (Figure 1), was used alone and compared with its hybrid with gold nanoparticles and with solely gold nanoparticles for the efficient optical detection of diclofenac.

Figure 1.

The structure of: diclofenac: 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt (a) and of 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphyrin-Zn(II) tetrachloride (b)

Materials and Methods

Reagents

The used solvents and reagents in the highest purity available (THF, DMF, acetonitrile, methanol, chloroform, zinc chloride, methyl tosilate) were purchased from Merck. Silica gel plates 60 (230 - 400 mesh, 0.040 - 0.063 mm), F₂₅₄ were purchased from Merck. Dowex resin 1X2 Cl⁻ 200 - 400 nm was acquired from Dow Chemicals, Midland, (MI).

Synthesis of Zn(II)-tetra-(4-N-methyl-4-pyridyl)-porphyrin tetrachloride (ZnTMePyP)

A mixture comprised of *meso*-tetra (4-pyridyl)-porphyrin (0.618 g, 1 mmol) and methyl-4-methylbenzene-sulfonate (0.93 g, 5 mmol) in 150 mL DMF was refluxed for 4 - 10 hours under argon atmosphere. The methylation process was monitored by TLC using silica gel plates and as eluent a mixture of acetonitrile/water (sat. KNO₃) = 8/1 (v/v) [13, 19, 36]. When methylation was complete, the mixture was

dried under vacuum, dissolved in distilled water and the impurities were extracted with chloroform. The water fraction is filtered and vacuum evaporated, giving the tetra(4-methyl-pyridyl)porphyrin tetra-tosilate. The tetra(4-methyl-pyridyl)porphyrin tosylate salt was then dissolved in 80 mL HCl acidulated H_2O (the resulting solution having the concentration of 0.1 N HCl) and 15 mL methanol. This solution is slowly drained through an ion exchange column filled with Dowex 1X2 Cl^- 200 - 400 nm resin for the substitution of tosylate anion with chloride ion, generating a more water soluble compound. The high purity porphyrin is obtained by a new vacuum evaporation. The metalation with Zn was realized applying the classical method [6, 24] with a large excess of Zn chloride.

The gold colloid (n-Au) synthesis:

The gold colloid with spherical dimensions in a narrow range (15 - 20 nm) was synthesized in an environmentally friendly manner [34] following the previous reported recipe [14].

Obtaining of the ZnTMePyP/n-Au hybrid:

The formation of the hybrid material composed from Zn(II)-tetra-(4-N-methylpyridyl)porphyrin tetrachloride and gold colloidal particles was accomplished by adding small portions of HCl acidulated Zn-porphyrin solution ($c = 10^{-5}$ mol/L, pH = 3) to 3 mL diluted gold colloid solution ($c = 4.58 \times 10^{-4}$ mol/L), under stirring, at room temperature, as follows: 20 μ L Zn-porphyrin solution up to sample 7; 100 μ L up to sample 22; 150 μ L up to sample 32. The generation of complex was monitored by UV-Vis spectroscopy (Figure 2).

Spectrophotometric detection of diclofenac by the ZnTMePyP/n-Au hybrid, bare Zn-porphyrin and n-Au alone, respectively

Diclofenac solution (20 μ L portions) having the concentration of 9.9×10^{-3} mol/L, in doubly distilled water, was successively added to 3 mL of each investigated solution: ZnTMePyP/n-Au hybrid, bare ZnTMePyP and bare gold colloid, under stirring. After 30 seconds the UV-Vis spectrum was recorded for each sample.

UV-Vis spectrometry

Spectrophotometric measurements were performed in 1 cm wide quartz cuvettes on a JASCO model V-650 spectrometer.

STEM Microscopy

STEM images were obtained using a Titan G2 80 - 200 TEM/STEM microscope (FEI Company, The Netherlands). Samples were prepared by drop-casting the gold-porphyrin hybrids with and without diclofenac from water mixtures on 200 mesh TEM copper grids coated with continuous carbon film. The images were registered at 200 kV using TEM Imaging & Analysis v. 4.7 software.

Results and Discussion

UV-Vis monitoring of the synthesis of ZnTMePyP/n-Au hybrid showed that the successive adding of acidulated solution of Zn-porphyrin to the gold colloid solution has the general effect of widening the absorption spectrum of the hybrid material (Figure 2). Thus, it can be observed that the gold colloid alone has the maximum absorption at 522 nm, whereas the hybrid material expands its absorption domain from 400 nm to 900 nm. Two isosbestic points located at 410 nm and 455 nm on either side of the Soret band of the Zn-porphyrin (436 nm), are indicative for the two intermediate species formed during the hybrid generation. The two equilibrium processes might be associated on one hand with the interaction between the negatively charged surface of the gold colloidal particles and the four N^+-CH_3 functional groups of the porphyrin and on the other hand with the formation of supramolecular structures [53]. Such supramolecular architectures are due to the strong $\pi-\pi$ intermolecular interactions of Zn-porphyrin complex, that are promoting n-Au particles to form side-by-side assemblies (Figure 4). The large flat band that is finally formed from 600 nm to 900 nm is in relation to the HOMO-LUMO levels of the zinc-porphyrin coupled to the n-Au surface plasmon [27].

Figure 2.

UV-Vis monitoring of the synthesis of ZnTMePyP/n-Au hybrid. Detail of the UV-Vis spectrum of the initial porphyrin

The STEM and TEM microscopy (Figure 3, a, b, c) of the acidified ZnTMePyP showed its preference to triangular platelet aggregation and the second image offers a frame with TEM measurements of interplanar

distances of 2.80 nm from solid crystalline state. A larger STEM image shows clearly dendritic assembling of the Zn-porphyrin triangular aggregates.

Figure 3.

The STEM (a and c) and TEM (b) microscopic images of the acidified ZnTMePyP

The STEM investigations (Figure 4) focused on the interaction between ZnTMePyP and gold nanoparticles, showed that the colloidal gold assembled by

side-by-side process on the surface of large spherical or egg-like porphyrin aggregates generated by the self-assembly of water-soluble Zn-porphyrin.

Figure 4.

STEM images of the ZnTMePyP/n-Au hybrid

The diclofenac detection using ZnTMePyP/n-Au hybrid

Diclofenac solution (20 μL portions) having the concentration of 9.9×10^{-3} mol/L, in doubly distilled water, was successively added to 3 mL of ZnTMePyP/n-Au hybrid, under stirring and the UV-Vis spectra were registered.

By increasing the diclofenac concentration (Figure 5a), the intensity of absorption of the hybrid decreased in intensity, so that in the range of 1.94×10^{-5} mol/L to 7.34×10^{-5} mol/L the intensity of absorption was linearly dependent with the diclofenac concentration (Figure 5b).

The correlation coefficient of the linear dependence was excellent and had a value of 99.61%. After a certain concentration of diclofenac was reached, the shape of the spectra and the intensity of the Soret band

increased again, a fact that insures that the process of detection is not mimed by the diluting of the solution.

The accuracy of diclofenac detection and the range of the concentrations recommend the use of this simple method for the water toxicity analysis and for low levels of chronic toxicity in patients with long treatment prescription. The AFM (Figure 6, shadow technique maps) images of the ZnTMePyP/n-Au hybrid, after treating it with diclofenac show that the material, due to diclofenac interaction, is reorganized in a uniformly manner. Triangular aggregates that are characteristic for Zn-metalloporphyrin, and that are similar in size and preserving the same orientation are built in successive layers like a tile roof, by concomitant H- and J-type aggregations. The thick of the deposited layers differs, but there is no lack of continuity. Some clearly displayed small spheres, might be the n-Au associated particles.

Figure 5.

The superposed UV-Vis spectra of the ZnTMePyP/n-Au hybrid treated with increased diclofenac concentrations (a) and the dependence between the intensity of absorption and the diclofenac concentration (b)

Figure 6.

The AFM (shadow technique maps) images of the ZnTMePyP/n-Au hybrid, after treating it with diclofenac

Figure 7.

The superposed UV-Vis spectra of the ZnTMePyP treated with increased diclofenac concentrations (a) and detail of the Q bands (b)

The diclofenac detection using solely ZnTMePyP
Respecting the same methodology that was described for the detection of diclofenac using the ZnTMePyP/n-Au hybrid, the UV-Vis spectra were presented in Figure 7 for the detection using solely ZnTMePyP. Four isosbestic points, located at 450 nm on the

Soret band and 534 nm, 577 nm and 590 nm on the Q bands of the metalloporphyrin were evidenced, proving that a complex is formed between the Zn-porphyrin and the diclofenac during the detection procedure. Many equilibrium processes are involved that might be explained by the complexity of interactions

between the Zn-porphyrin and the diclofenac molecule. Zinc atom might offer coordination space for the pharmaceutical compound, but in concurrence with the porphyrin macrocycle that can interact with the carboxylate anion of the diclofenac.

The concentration domain for which the intensity of absorption of the ZnTMePyP is linearly dependent

with the diclofenac concentration is 2.57×10^{-5} mol/L to 1.41×10^{-4} mol/L (Figure 8), proving surprisingly a larger domain of detecting capacity than that of the hybrid formed with the gold colloid. The confidence coefficient of the linear dependence was also very good, of 99.23 %.

Figure 8.

The dependence between the intensity of absorption and the diclofenac concentration

The diclofenac detection using solely n-Au

The experiment was continued, in order to inspect what results if the simple gold is treated with diclofenac. After analysing the shape of the superposed UV-Vis spectra (Figure 9), it can be concluded that the effect of diclofenac upon the absorption intensity of the gold plasmon is only that of the mixture dilution.

Figure 9.

The dependence between the intensity of absorption and the diclofenac concentration

Conclusions

Our attempts to provide some spectroscopically novel nanostructured materials that can detect chronic toxicity levels of diclofenac gave promising results. The investigation was focused on the detection capabilities of a water soluble metalloporphyrin ZnTMePyP, of tailored spherical size gold nanoparticles (15 - 20 nm) n-Au and on the hybrid nano-material ZnTMePyP/n-Au formed between them.

The generation of the hybrid was discussed and investigated by UV-Vis and microscopic techniques. Regarding the diclofenac detection, it can be stated that the water soluble Zn-porphyrin alone can detect diclofenac in a larger domain of concentration than the hybrid formed between the metalloporphyrin and that the gold nanoparticles alone are not capable of diclofenac detection. The concentration domain of diclofenac detection in a reliable fashion is 2.57×10^{-5} - 14.15×10^{-5} mol/L. The method chosen for detection, UV-Vis spectroscopy, implies neither expensive equipment, nor the lengthy preparation of the samples, thereby providing a simple and time-efficient method to assess the chronic toxicity of diclofenac in the biological fluids of patients that were long time treated with this medicine.

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