Stress-induced neoplasia detection in the lower part of gastrointestinal tract of rats using phthalocyanines

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Abstract. In this study, phthalocyanine (ZnPc, AlPc, LuPc – Pcs) compounds were employed as exogenous fluorescent markers for diagnostics of GIT adenocarcinoma in laboratorial animals (adult male rats, n = 40) after application of an experimental model of adenocarcinoma formation with metastases. The neoplastic lesions were developed under the influence of social stress and chemical stress using nitrosamines during a 9-month period of application. A significant fluorescence signal in the region of 670 – 720 nm was observed from the neoplastic lesions, which was absent in the normal mucosa. This signal is related to the fluorescence of phthalocyanines accumulated in the tumor area. The autofluorescence background covered the region of 450 – 650 nm with a maximum at 480 – 520 nm and originated mainly from protein cross-links and co-enzymes – NADH and flavins. Endogenous porphyrins fluorescence was also observed in the lesions with a maximum at 630 – 640 nm. Visually, the presence of accumulation sites of Pcs in the form of bright pink patches was observed after excitation at 405 nm, as opposed to a healthy tissue, which remained blue-violet due to the autofluorescence signal. This allows one to use the Pcs fluorescence discrimination not only in a spectroscopic mode of detection, but for imaging of the lesions investigated, which is preferable in clinical applications during endoscopic observations in humans.

1. Introduction
The gastrointestinal tract (GIT) tumors are the third most common type of all new cancer cases worldwide, including esophagus, stomach, colon, and rectum neoplasia. The EUROCARE-4 (http://www.eurocare.it/) showed that the 5-year survival rate of this type of oncological disease is about 24% of all cases in Europe. These types of neoplasia are characterized by a high risk and early appearance of metastases [1, 2].

The incidence of gastrointestinal cancer has been increasing in the last decades, so that its accurate diagnostics and effective treatment are of primary public health concern [3]. Conducting more frequent
screenings has resulted in some decrease of the GIT carcinoma mortality rate, but improvement of the diagnostic procedures is required in order to achieve a further decrease [4]. The potential of the biomedical optical techniques for a detailed minimally- or non-invasive analysis of multi-component substances, such as biological tissues, is being intensively researched in view of applications in novel clinical diagnostic modalities for differentiation of cancerous and normal tissues with high sensitivity and accuracy. Different spectroscopic modalities are being applied to increase the diagnostic accuracy for GIT cancer evaluation and to develop non-invasive real-time diagnostic tools for GIT tumors detection. One of the techniques investigated is based on fluorescence spectroscopy in a steady-state regime using intrinsic (endogenous) sources of fluorescence, or, alternatively, exogenous fluorescence using introduced contrast agents with fluorescent properties [5].

The fluorescence methods are fast, objective and provide detailed information on the biochemical and morphological status of tissues that do not depend on the qualification and experience of the medical staff. The spectral analysis is non-invasive, which makes it qualitatively different from the standard histological sampling, whereby a piece of tissue is taken. This is a traumatic procedure for the patient; moreover, in the presence of a neoplasm it can lead to complications and spreading of tumor cells through the blood to the other parts of the body, thus increasing the rate of metastasizing. The combination of endoscopic equipment with fiber-optic fluorescent probes allows clinicians to reach the internal organs, quickly differentiating different tumors in the patient's body, both by type and stage of development [5-7].

The diagnosis and differentiation of healthy and pathological tissues by means of fluorescence spectroscopy of endogenous fluorophores are based on the specific differences in the fluorescence spectrum of healthy and pathological GIT tissues. These differences arise from pathological changes in the cells and tissues during carcinogenesis. The fluorophores detected are amino acids, structural proteins, co-enzymes, endogenous porphyrins [6]. The autofluorescence diagnostics currently finds its place in the clinical practice as a “red-flag” or guiding technique in endoscopic examinations, mainly due to its limitations in image development due to the low level of the detected signal. Thus, one cannot obtain good-quality images for clinical observations of the alterations in a fluorescent mode of visualization [7].

An early detection of GIT dysplastic and neoplastic alterations using exogenous markers will enhance the diagnostic accuracy and the corresponding survival rate. The fluorescent markers, e.g., photosensitizer drugs, used in photodynamic therapy must be selective to the tumors, non-toxic, clinically approved, and relatively inexpensive [8]. The combination of fluorescence (diagnostic) and photodynamic (therapeutic) properties makes such compounds attractive candidates not only for diagnosis, but also for theranostic applications [9, 10].

The photosensitizers’ applicability as fluorescent markers depends also on the fluorescence quantum yield vs. that of producing singlet oxygen, the latter being useful in the therapeutic mode of photodynamic treatment of sensitized tumors [11]. In recent years, the most widely used photosensitizers have been those based on porphyrins, phthalocyanines and chlorines. They have their own advantages and drawbacks, as related to their chemical and optical properties. In this work, we focused our attention on the family of phthalocyanine drugs as possible markers for GIT cancer detection.

2. Methods and materials
The studies were carried out on a laboratorial model group – male white mongrel rats weighing on average 250 g. The animals were divided into two groups: a control one including ten intact animals, and an experimental group of thirty animals, where the rats were exposed to a combination of a chronic stress (overcrowding) and a chemical stress induced by a nutrition regime that included nitrates (0.2% in drinking water) and toluidine (25 µg/1 kg live weight) [12, 13]. Once the neoplastic lesions were induced, and the fluorescent examination and the spectroscopic and image analysis of the neoplasia developed were completed, the experimental animals were withdrawn from the experiment. The organs were taken for a standard histological examination carried out according to a standard histopathology procedure with the preparation of paraffin blocks and the coloring of thin sections (3-5 µm) with
hematoxylin and eosin. The histological analysis was used to prove the cancerous lesions development and their stage of growth and used as a gold standard in evaluating the spectral results obtained.

All rats underwent the procedure of exogenous fluorescence diagnostics using three different phthalocyanine compounds, namely – zinc-phthalocyanine (ZnPc), aluminium-phthalocyanine (AlPc) and lutetium-phthalocyanine (LuPc) after nine months of combined stress treatment. In each group there were ten animals for each photosensitive drug applied. The drug dose for all cases was 1.5 mg/kg and was administered i.v. 1 hour before the start of the spectroscopic measurements and image detection. The excitation light source was an AFS-405 CW LED (Polironik Ltd., Moscow, Russia) with an optical output power of 25 mW. A DinoLite digital microscope (model AM 4013 T-FWV, IDCP B.V., The Netherlands) was used for 2-D fluorescent images of the samples. An optical fiber probe with 6+1 Y-form fibers was used to deliver the excitation and emission light to and from the tissues investigated during the spectroscopic measurements. The detection in the 1-D mode was conducted using a USB4000 microspectrometer (OceanOptics Inc., Dunedin, USA, operating range 350 – 1000 nm, FWHM 1.5 nm). Ten spectra were registered and averaged per tissue area with observed exogenous fluorescence. Up to ten spectra from normal mucosa were detected and averaged as well. They were used for further comparative studies and analysis of the spectral characteristics of the lesions areas.

3. Results and discussion

Figures 1 (a), 1 (b) and 1 (c) present the fluorescence spectra obtained in vivo from stomach carcinoma of laboratorial rats with applied ZnPc, AlPc and LuPc photosensitizers.

![Fluorescent measurements of stomach cancer using exogenous](image)

**Figure 1.** Fluorescent measurements of stomach cancer using exogenous (a) zinc-phthalocyanine (b) aluminium-phthalocyanine and (c) lutetium- phthalocyanine (i.v. 1.5 mg/kg). Excitation with a narrow-band 25-mW LED in the spectral range of 400 – 420 nm.

The signals consists of autofluorescence and exogenous fluorescence parts. The typical blue-green fluorescence corresponding to the structural proteins and co-enzymes is observed for normal mucosa. In the case of tumors, the autofluorescence is weaker and re-absorption is seen in all cases related to the increased level of hemoglobin due to neovascularization of the cancerous areas. The long-wavelength peaks observed (>650 nm) correspond to the presence of ZnPc, AlPc and LuPc.
Figure 2. Comparison of the fluorescence spectra of normal mucosa, inflammatory area and carcinoma lesion of GIT – example of the presence of an endogenous photosensitizer, protoporphyrin IX (PpIX), and of exogenous Al-phthalocyanine.

In some cases, due to the selective accumulation of endogenous porphyrins we observed two signals from fluorescing photosensitizers – one peak situated at 635 nm corresponding to the protoporphyrin IX endogenously accumulated in tumor cells, and another peak at 670 – 700 nm corresponding to the exogenously applied phthalocyanine compound, which was selectively accumulated in the tumor cells as well, see figure 2.

The results are averaged for the set of animals measured sensitized with given type of phthalocyanine compound. The fluorescence intensity levels for different groups correspond to the reported fluorescence quantum yield for these compounds, namely, ~ 0.3 for ZnPc [14], 0.59 for AlPc [15] and less than 0.1 for LuPc [16].

4. Conclusions
The comparison between the values of some spectroscopic parameters in view of clinical applications of the exogenous fluorescence spectroscopy in gastrointestinal cancer diagnosis allowed us to discriminate with a good accuracy between normal and neoplastic gastrointestinal mucosa.

From the point of view of obtaining a strong fluorescence signal that can be useful in diagnostic visualization, the best results were achieved in the case of AlPc. The LuPc had the highest quantum yield and singlet oxygen generation, but sub-optimal emission properties regarding diagnostic usage. In these terms, the ZnPc compound showed moderate fluorescence properties, which were sufficient for diagnostic applications. The results obtained are foreseen to be used in the development of a system and diagnostic methodology for gastrointestinal tumor detection complementary to the existing endoscopic systems; therefore, the quantitative parameters of the fluorescence detected could be of significant importance in the development of appropriate diagnostic algorithms.

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