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CONTENTS

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NATIONAL SCIENTIFIC PROGRAMMES WITH EUROPEAN DIMENSIONS

BIOTECHNOLOGICAL PRODUCTION OF VALUABLE PLANT PHARMACEUTICALS: ANTICANCER AGENTS

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Abstract

Many anticancer compounds are still derived from plants since the chemical synthesis of the chiral molecules is not economic. Often, the respective plants are grown in plantations as when they are harvested from nature, conservation problems may arise. Retail prices and demands are relatively high. Since the natural supply is limited, several research groups have explored the possibility of employing plant cell or organ in vitro cultures for the biotechnological production of these compounds as an alternative. We have developed conventional cell and hairy root cultures of many rare medicinal plant species, mainly spread in Bulgaria and the Balkans, for optimization of the production of anticancer compounds. Emphasis has focused on designing appropriate bioreactor technologies, suitable to culture delicate and sensitive plant cells or hairy roots. This article summarizes some of our results concerning the production of certain anticancer compounds from tissue cultures of rare medicinal Astragalus and Linum plants. Various problems involved and possible ways to overcome using biotechnological approaches are discussed on the basis of examples from our own research. Recently, the interest of international pharmaceutical industries has been directed more and more to biotechnology. We believe that cell cultures of Astragalus and Linum plants as a source of biologically active anticancer compounds can play a role in this respect.

INTRODUCTION

In the coming decades, several new enabling technologies will be required to develop the next generation of advanced plant-based pharmaceuticals. With modern biotechnology it has become possible to use plant cells for the production of specific pharmaceuticals. Many anticancer compounds are still derived from plants since the chemical synthesis of the chiral molecules is not economic. Often the respective plants are grown in plantations as when they are harvested from nature, conservation problems may arise. Retail prices and demands are relatively high. Since the natural supply is limited, several research groups have explored the possibility of employing plant cell or organ *in vitro* cultures for the biotechnological production of these compounds as alternative. Using the right culture medium and appropriate phytohormones it is possible to establish in vitro cultures of almost every plant species. Starting from callus tissue, cell suspension cultures can be established that can even be grown in large bioreactors. Moreover, biotechnological production of these plant products is a more environmentally friendly way than is currently occurring.

STUDIES ON PRODUCTION OF IMPORTANT SECONDARY METABOLITES IN THE AUTHOR'S LABORATORY BY IN VITRO CULTURES

In Bulgaria research in the area of plant tissue culture technology for production of pharmaceutical substances is now concentrated in Faculty of Pharmacy, Department of Pharmacog-

nosy, Medical University of Sofia. Here the unique for Bulgaria Laboratory for pharmaceutical products – "Pharmaceutical Biotechnology" is located. Faculty of Pharmacy-Sofia is the only one in the country to offer higher education on pharmaceutical biotechnologies for students on Pharmacy. Our research group is engaged in the production of pharmacologically active substances from plant origin in *in vitro* cultures. Work in this area has led to the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, lignans, phenols, flavonoids, amino acids, etc. This technology offers an alternative production system for high-value compounds in cultivated cells, the chemical synthesis of which is not economically feasible and which therefore still have to be isolated from plants. A rational engineering of secondary metabolism to increase the contents of these low molecular weight compounds requires a thorough knowledge of the whole biosynthetic pathway and a detailed understanding of the regulatory mechanisms controlling the flux. Such information is not yet available for the vast majority of secondary metabolites, including anticancer plant compounds, explaining why only limited success has been obtained up to now.

The collection of *in vitro* cultures of different plant species consists of about 400 selected strains or clones (Fig. 1). The Laboratory has all facilities needed for aseptically successful work with plant cells *in vitro*. In the earlier period many new unique cultures of medicinal plants were established, both conventional cell and hairy roots, and especially methodical problems were studied. In our laboratory we focus on the production of some important pharmaceuticals in plant cell cultures and have successfully established cell cultures for production of anticancer agents. Despite its not long tradition the Laboratory served as a training site for many undergraduate and PhD students from different universities.

I am very grateful to many colleagues (both on the Faculty of Pharmacy, Medical University of Sofia and University of Dusseldorf, Germany) as well as many postdoctoral and graduate students for their multiple contributions to this collaborative research. Funding by grant D002-128/2008 (I. Ionkova) by the Ministry of Education and Science, Sofia, Bulgaria, is gratefully acknowledged.

RESEARCH FOCUS AND CAPABILITY

Tissue culture: The team has extensive experience in various tissue culture technologies including embriogenesis, genetic transformation, regeneration of transgenic plants, development of air lift bioreactor system for cultivation of hairy roots, optimisation of bacterial strains and transformation methodology, investigation of roots morphology. A methodology was development for the physiological selection of desirable genotypes for a variety of species. Selection methodology is generated by collecting data over a wide range of environmental conditions including light intensity, temperature, abiotic stress.

Natural Product Biosynthesis: The work group has experience with production of secondary metabolits (alkaloids, saponins, flavonoids, polysaccharides, sterols, phenol acids etc.) in intact plants, conventional and transformed cultures; activation of alkaloid biosynthesis in hairy roots trough double transformation by 4 different Agrobacterim rhizogenes strains; induction of saponin biosynthesis using end-product inhibition.

BIOTECHNOLOGICAL APPROACHES FOR THE PRODUCTION OF POTENTIAL ANTICANCER LEADS

Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. About 60% of anti-tumor and anti-infection drugs are of natural origin. Moreover, of the 252 drugs that are considered essential by the World Health Organisation, 11% are obtained exclusively from plants (WHO, 1992). In the coming decades, several new enabling technologies will be required to develop the next generation of advanced plant-based pharmaceuticals. With modern biotechnology it has become possible to use plant cells for the production of specific pharmaceuticals. Using the right culture medium and appropriate phytohormones it is possible to establish in vitro cultures of almost every plant species. Starting from callus tissue, cell suspension cultures can be established that can even be grown

in large bioreactors. Moreover, biotechnological production of these plant products is a more environmentally friendly way than is currently occurring.

Saponins and lignans are important classes of natural products produced by different *Astragalus* and *Linum* species. Nowadays, saponins and podophyllotoxins are the most important of these compounds from the point of view of medicine due to their anticancer and immunomodulation activities. Chemical synthesis is possible, but not economic. Therefore, these compounds are still extracted from plants collected from the wild and there is great demand to find alternative sources for these drugs. Such an alternative source could be plant cell and organ cultures.

Three strategies have been followed to affect accumulation:

1. Enhancement of the saponin and lignan biosynthesis in cell lines.
2. Compartmentalization of the products.
3. Breakdown of the products.

The actual production will be the result of biosynthesis minus breakdown (catabolism and chemical breakdown) of the product, provided that storage of the product is possible, thus avoiding autointoxication of the producing cells. We study these three points parallel in some more detail using some of the results of our own research as a basis.

In recent years advances in research on the *Astragalus* species have been made, due to their anticancer constituents and medicinal application as an immunostimulant or as anticancer drugs [1, 6]. Currently much of the pharmacological research on *Astragalus* is focused on its immune-stimulating and anticancer saponins, polysaccharides and flavonoids useful in treating immune deficiency conditions. It increases the number of stem cells in bone marrow and lymph tissue and encourages their development into active immune cells. It appears to help trigger immune cells from a "resting" state into heightened activity. It also enhances the body's production of immunoglobulin and stimulates macrophages. *Astragalus* can help activate T-cells and natural killer (NK) cells. Additionally, the flavonoids, saponins, and polysaccharides found in *Astragalus* root help minimize free radical damage to membranes.

Our research was focused on the use of plant tissue cultures of *Astragalus* plants for improvement of natural compound production. Now we have established cell lines of various plant species of interest in our laboratory [6]. In a screening program a large number of cell lines from different species of *Astragalus* are set up, derived preferably from high-producing plants. Since the root is mostly the site of saponin, and polysaccharide biosynthesis, in vitro root cultures from different *Astragalus* species have been able to produce large amounts of secondary metabolites (Table 1).

Antitumor activity will undoubtedly continue to be the most clinically relevant property of lignans [2, 3, 4]. Due to these biological activities, lignans, and especially cyclolignans, have been the objective of numerous studies focused to prepare better and safer anticancer drugs. The production of anticancer compounds, such as lignan podophyllotoxin (PTOX), by plant in vitro cultures from different *Linum* species in our Laboratory is presented in Table 2. Cell cultures of different Linum species of section Syllinum are shown to produce considerable amounts of lignans, mainly 6-methoxypodophyllotoxin (MPTOX). Although both PTOX and MPTOX have comparable cytotoxic activity, due to the different substitution in position 6, MPTOX is not used for the production of anticancer drugs [5].

Recently cultures of transgenic organs are exploited widely, particularly hairy root cultures. These cultures are obtained by genetic transformation of plant tissues with the pathogenic soil bacterium Agrobacterium rhizogenes. The main advantages of hairy root cultures are fast growth in hormone-free media, genetic and biochemical stability and high productivity for long time of culturing. Moreover, hairy root cultures are able to produce metabolites, which normally occur not only in roots, but in stems, leaves and/or flowers of intact plants. Hairy root cultures may be also used for transformed plants production. On the basis of results of our investigations the production of some important plant secondary metabolites (ariltetraline lignans, saponins, polysaccharides, etc.) by hairy root cul-

tures of several *Linum* and *Astragalus* species was performed.

Based on the exciting results in production of medicinal compounds reported above using cell in vitro cultures, we have successfully established a lot of cell cultures - selected strains or clones from rare medicinal plants for the production of anticancer compounds at our research laboratory on Pharmaceutical biotechnology.

SCALE–UP OF PLANT CELL CULTURES

Since plant cells produce unique pharmaceuticals, which can be harnessed, they need to be produced in large-scale bioreactors. Selection of the best performing cell line, its maintenance and stabilization are necessary prerequisites for its production in bioreactors and subsequent scale-up of the cultivation process to the industrial level. Scale-up of growth and product yield depends on a multitude of factors, such as growth medium, conditions of cultivation, inoculum, type of reactor and processing conditions. The composition of the growth medium, elicitors and precursors, etc. can markedly influence the production. Cell/tissue types such as cell suspension cultures, immobilized cells and hairy roots have been very ideal for scale-up. Configuration of bioreactors used for microbial cells cannot always be utilized directly for plant cells, owing to distinctive features, which are not favorable for plant cell cultivation. Plant cells are less stable in productivity, highly shear sensitive, exhibit low oxygen requirements slow growth (doubling time 25 - 100 h) and often occur as cell clumps of 2-4 mm diameter. Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology with several advantages for mass cultivation of plant cells:

- It gives better control for scale up of cell suspension cultures under defined parameters for the production of bioactive compounds;
- Constant regulation of conditions at various stages of bioreactor operation is possible;
- Handling of culture such as inoculation or harvest is easy and saves time;
- Nutrient uptake is enhanced by submerged culture conditions which stimulate multiplication rate and higher yield of bioactive compounds; and
- Since the biosynthetic efficiency of populations varies, for this purpose a high yielding variety should be selected as a starting material. The fundamental requirement in all this is a good yield of the compound, and reduced cost compared to the natural synthesis by the plants.

A bioreactor facility (2-litre capacity) was set up for up-scaling of cell culture production of podophyllotoxin, saponins and other therapeutic agents in our laboratory. The dynamic unstructured model of variable volume fed-batch fermentation process for ariltetralin lignan production in suspension cultures of *Linum tauricum* (Linaceae) was created. Our approach to the investigation includes the following main procedures: description of the process by generalized stoichiometric equations; preliminary data processing and calculation of specific rates for main kinetic variables; identification of the specific rates taking into account the dissolved oxygen tension; establishment and optimisation of dynamic model of the process; simulation researches [7]. MATLAB is used as a research environment. Some statistical investigations are realized by STATGRAPHICS package [8-11].

The fed-batch cultivation of *L. tauricum* suspension cultures in a 2 litre stirred tank bioreactor (Fig. 2), using optimised medium leads to substantial increase (8-10 times higher than production in batch cultivation) of the levels of both lignans [1]. The maximum biomass reaching 22.3 $g.L^{-1}$ dry weight. An improvement in the lignan accumulation from 2.5 $mg.L^{-1}$ and 2.36 $mg.L^{-1}$ for 6MPTOX (6-methoxy-podophyllotoxin) and 4'DM-6MPTOX (4'-demethyl-6-methoxypodophylotoxin), to 25.35 $mg.L^{-1}$ and 19.17 $mg.L^{-1}$ respectively in a cell culture of *L. tauricum* for 21 days was achieved.

All in vitro cell lines (extracts and isolated compounds) were tested for cytotoxic properties with potential anti–cancer activity.

CONCLUSION

Today several distinct chemicals derived from plants are important drugs currently used in one or more countries in the world. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The evolving commercial importance of secondary metabolites has in recent years re-

sulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. Plant biotechnology has the potential to generate valuable products such as safe, cheap and effective plant-based pharmaceuticals or plants with environmental advantages. Plants are highly efficient natural refineries and large quantities of anticancer products can be produced in small areas safely and relatively cheaply. We believe that cell cultures of *Astragalus* and *Linum* plants as a source of biologically active anticancer compounds can play a role in this respect.

Acknowledgements

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Table 1. Production of secondary metabolites from in vitro cultures of *Astragalus* species

Astragalus Species	In vitro culture	Compounds isolated
A. hamosus	Callus, suspension hairy roots	Saponins, Soyasapogenol B, β-sitosterol, Astragalin, Rutin, Isorhamnetin-3-O-glycoside phenol acids
A. boeticus	Callus, suspension hairy roots	Saponins, Soyasapogenol B, β-sitosterol,Flavonoids, Phenol acids
A. missouriensis	Callus, suspension hairy roots	Isoquercitrin, Quercitrin, Rutin, Hyperoside, Phenol acids, Saponins
A. edulis	Callus	Quercetin, Kaempferol, Isorhamnetin, Saponins
A. gummifer	Callus Root cultures hairy roots	Polysaccharides Polysaccharides Polysaccharides
A. membranaceus	hairy roots Air lift bioreactor	Polysaccharides Cycloastragenol, Astragenol, Soyasapogenol B, β- sitosterol, Stigmasterol, Campesterol, Astragalosides I-III, new astragaloside
A. mongholicus	hairy roots Air lift bioreactor	Astragalosides I-III, β- sitosterol, Stigmasterol, Campesterol, Polysaccharides
A. glycypyllos	hairy roots	Cycloastragenol, Astragenol, Soyasapogenol B, Cycloartane saponins
A. englerianus *A. monspessulanus*	hairy roots hairy roots	Cycloartane saponins Cycloartane saponins
A. brachycera	hairy roots	Cycloartane saponins Polysaccharides, Sterols

A. canadensis	hairy roots	Cycloartane saponins Cycloastragenol, Astragenol, Polysaccharides
A. falcatus	hairy roots	Cycloartane saponins Polysaccharides
A. oxyglotis	hairy roots	Cycloartane saponins Polysaccharides
A. sulcatus	hairy roots Air lift bioreactor	Cycloartane saponins Polysaccharides, Sterols, Swensonine

Table 2. Production of secondary metabolites from in vitro cultures of *Linum* species

Linum Species	**In vitro culture**	**Compounds isolated**
Linum album	Suspension	PTOX, 6MPTOX, DPTOX, Pinoresinol, Matairesinol, Lariciresinol, β-peltatin, α-peltatin
Linum altaicum	Cell cultures	Justicidin B Isojusticidin B
Linum austriacum	Callus, Suspension, Root, Hairy root	Justicidin B, Isojusticidin B
Linum austriacum ssp. *euxinum*	Cell cultures	Justicidin B, Iisojusticidin B
Linum africanum	Callus, Suspension	PTOX, DPTOX
Linum campanulatum	Callus, Suspension	Justicidin B
Linum cariense	Suspension	6MPTOX 5'-demethoxy-6-methoxypodophyllotoxin, and the corresponding 8'-epimers 6-methoxypicropodophyllin, 5'-demethoxy-6-methoxypicropodophyllin
Linum bulgricum	Callus, Suspension	PTOX, 6MPTOX
Linum elegans	Callus, Suspension	6MPTOX
Linum flavum	Root	6MPTOX
Linum flavum	Suspension	6MPTOX
Linum flavum	Suspension, Root like tissue	6MPTOX, 5'-demethoxy-6-methoxy-PTOX
Linum flavum	Hairy roots	6MPTOX
Linum flavum	Hairy roots	Coniferin
Linum leonii	Callus	Justicidin B
Linum leonii	Hairy roots	Justicidin B
Linum lewisii	Cell cultures	Justicidin B, Isojusticidin B
Linum linearifolium	Callus, Suspension Air lift bioreactor	PTOX, 6MPTOX
Linum mucronatum ssp. *armenum*	Shoot, Suspension	6MPTOX, PTOX
Linum narbonese	Callus	Justicidin B
Linum nodiflorum	Suspension	6MPTOX
Linum nodiflorum	Suspension	6-MPTOX, DPTOX, PTOX
Linum persicum	Callus, Suspension	PTOX, 6MPTOX, peltatin
Linum tauricum	Callus, Suspension Shoots, Hairy roots Air lift bioreactor	6MPTOX 4'-demethyl-6MPTOX

Abbreviations: PTOX – podophyllotoxin; 6MPTOX – 6-methoxypodophyllotoxin; DPTOX – deoxypodophyllotoxin; 4'DM-6MPTOX –4'-demethyl-6-MPTOX.

Fig. 1. Collection of *in vitro* cultures of different plant species, producing anticancer compounds

Fig. 2. Fed-batch cultivation of *Linum tauricum* suspension cultures in a 2 litre stirred tank bioreactor

БИОТЕХНОЛОГИЧНА ПРОДУКЦИЯ НА ЗНАЧИМИ ЛЕКАРСТВА ОТ РАСТИТЕЛЕН ПРОИЗХОД: ПРОТИВОТУМОРНИ АГЕНТИ

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Резюме

Много противотуморни продукти все още се получават от растения, тъй като химичният синтез на хиралните молекули не е икономически изгоден. Често съответните растения се отглеждат в плантации, тъй като прекомерното им събиране от естествени находища може да предизвика проблеми, свързани с опазването им. Цените и търсенето са сравнително високи. Тъй като природните източници са ограничени, редица изследователски групи проучват възможността за използване на in vitro

растителни клетъчни и органови култури за биотехнологично производство на тези субстанции като алтернатива. Разработени са конвенционални клетъчни и коренови култури от редки растителни видове, разпространени предимно в България и на Балканите, за оптимизиране на производството на противотуморни съединения. Проучванията са насочени към създаването на подходящи технологии за биореакторното култивиране на тези чувствителни клетки и коренови култури. Представени са някои от резултатите по отношение продукцията на противотуморни съединения чрез ин витро култури на редки лекарствени Astragalus и Linum видове. Отделните проблеми и възможните начини за преодоляването им чрез използване на биотехнологични подходи се обсъждат чрез примери от нашите собствени изследвания. В последно време интересът на международната фармацевтична индустрия е насочен все повече и повече към фармацевтичните биотехнологии. Екипът е убеден, че ин витро култури от Astragalus и Linum видове, създадени в нашата лаборатория, като източник на противотуморни съединения, могат да играят роля в това отношение.

SENSORY AND COGNITIVE PROCESSES IN PERCEPTION OF FIRST AND SECOND ORDER VISUAL STIMULI. INVESTIGATIONS IN HEALTHY PEOPLE AND PATIENTS WITH TYPE 2 DIABETES

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Abstract

Human visual system is the basic source of information about the surrounding world. Probably due to this fact about 70% of the human brain cortex is directly or indirectly engaged with processing the information obtained visually. This processing is very complicated, and on the one hand gives opportunity for recognition of visual objects form regardless of differences in such their aspects as size, bias, color, location, and on the other hand – for quite precise assessment of these differences. In order to realize these tasks visual system extracts proper features from the images, as at the very first stage of this process it changes from point-by-point description (at retinal level) to more summarized quasi Fourier-transformation of the images (at cortical level). This is effectuated by orientation- and spatial-frequency (SF) selective mechanisms, organized in the so-called "modules". The first task of the present project is assessment of the spatial-summation properties of the mechanisms in these "modules" by studying the effect of the width and length of stimuli-gratings with different SF on the threshold and on the reaction time for their detection, as well as on the amplitude and the latency of the visual evoked responses (VER). In accordance with the above-mentioned "module" model it was expected that the summation should be more effective for the stimulus length rather than for the width and to be proportional to the stimulus spatial period, but not of the same angular value. The second task of the project is to seek new evidence that one or two processing stages are involved in grating discrimination by orientation and SF. For the purpose the orientation- and SF-difference between the stimuli will be varied within a wide range, as well as VER at different tasks – choice sensomotor, binary sensomotor and mental tasks will be recorded. It is expected that the orientation- and SF-difference of stimuli would not influence considerably the early waves of VER, but it would prolong substantially late waves as a consequence of the above-mentioned second stage of visual information processing in discrimination tasks. These experiments will be also carried out with patients with diabetes type 2 and we expect in this case longer latency of the late VER waves. Since visual system extracts contours determined

not only as a spatial gradient of the brightness (I-st order contours) but also as a spatial gradient of the random noise contrast (II-nd order contours), we will seek analogies between mechanisms extracting one or another type of contours. For the purpose we will study again a well-known illusion in which the contours of the perceived image created by two superimposed gratings with different orientation but the same SF, could coincide or not with the real grating orientation as a function of the SF and the contrast. In our case we will use predominantly II-nd order gratings. As far as the existence of this illusion is considered as evidence that the outputs of different orientation- and SF-selective mechanisms combine their outputs in the process of contour extracting, and in a neuro-physiological aspect this is assumed as synchronization of mechanism neural responses – all this makes important to study the behavior of the VER, too. In this case we expect to observe higher amplitude of some, most probably, late waves of VER. We also plan to carry out these experiments with diabetics, assuming weaker effect of the so-called "combining" of the selective mechanisms.

The proposed research is mainly of theoretical interest but some deviations of the measured parameters – the detection threshold, the reaction time and the amplitude and latency of VER could be also tested as diagnostic tools.

INTRODUCTION

The studies in this field of the visual physiology and psychophysiology in Bulgaria date from the 60-ties of the past century. Their start was made in the Institute of Physiology, BAS (today Institute of Neurobiology), BAS and in the Department of Physiology, Medical University, Sofia.

Both the Departments of the Institute of Neurobiology, "Sensory neurobiology" and "Cognitive psychophysiology" have a rich experience in the investigations of evoked potentials. In the second Department this is the basic research method. In the first department, the initial processes of description and coding of information from visual objects are of main interest. In the second department the accent is on the higher cognitive processes of perception. Moreover, in the present project we plan to implement some of the investigations together with the Clinic of Endocrinology, Medical University – Sofia, with diabetic patients (type 2) in order to seek deviations in the standard psychophysical characteristics – contrast threshold, reaction time, as well as in the amplitude and latency of the VER waves. The use of VER and the visual event related potentials (VERP) in practice to assess diabetic patients is episodic, despite the fact that such deviations in some VER waves are available in these patients. Thus, collaboration between all the three groups in the research team seems to be a quite natural approach.

Processing of visual information in human brain is extremely complicated and effective and as a result, the visual system solves problems far above the capabilities of any powerful technical system for image processing and recognition. On the one hand, the visual system is able to attach to one and the same category (form) images with very different physical characteristics. For example, let's imagine a cat, which could be upright, laid down, little, big, black, white, located in different parts of the visual field, observed under different angle, etc. However in all cases we combine these too different images with one only category "cat", extracting from the images some invariant to the above-listed transformation features. This doesn't necessarily mean that information about different aspects of images is ignored by the visual system. The practice shows that together with the invariant recognition of the image form we are able to distinguish images also by size, orientation, location, color, etc. Furthermore, it is well known that form recognition on the one hand, and discrimination by size, orientation, location, color on the other, are independent processes. Several experiments have shown that different brain structures are involved in the process of form recognition (temporal lobe) and image variants discrimination (parietal lobe).

These unique capabilities of the visual system put forward the question about the codes employed by the visual system and the underlying mechanisms. It is well known that at retinal level image description is based on point-by-point principle, i.e. the spatial relief of excitation of the retinal output (ganglion cells) approximately

corresponds to the spatial relief of illumination onto photoreceptors, created by the observed image. However, this point-by-point principle is not preserved at the primary visual cortex level. There, the receptive fields are with prolonged form (Hubel, Wiesel, 1959). Due to this reason these receptive fields are orientation selective. In addition, because of the different widths of their excitatory and inhibitory zones, the receptive fields of the primary visual cortex exhibit also selectivity to the stimulus spatial frequency (SF). This was demonstrated both by psychophysical data of Campbell, Robson, 1968 and several other authors, as well as by electrophysiological data of Glezer, 1978 and De Valois, Tootell, 1983. One of the most convincing evidence that orientation- and SF-selective mechanisms exist in the visual system is the fact that when the stimuli differ by orientation more than 15º and by SF more than 1 octave they could be discriminated at the detection threshold. However, when these differences are smaller, the discrimination is possible at contrasts higher than the threshold value. Moreover, discrimination, in this case, requires more time. Vassilev et al., 1982; Zlatkova, Vassilev and Mitov, 2000 suggest that the visual system compares activity of mechanisms responding to both test stimuli during the second stage of analysis.

The receptive fields which cover the same part of the visual field and are tuned to different orientations and SF are assumed to form the so-called "neuronal module". The receptive fields of separate modules cover different parts of the visual field and have very different sizes, respectively very different values of the lowest preferable SF. Thus, a set of "modules" performs a local quasi Fourier-transformation of the presented images.

A question arises: Which are the advantages of the local quasi Fourier-analysis over the point-by point image description? First of all, invariant to the size description of images in the visual system could be realized. It is well known that one and the same object observed from different distances or the same objects but with different size create very different images onto the retina. In this case, the ratio between the SFs and the amplitudes of the separate harmonics in their SF spectrum retains the same. In this way, "modules" with different sizes would respond similarly to presentation of identical objects of different size.

The second advantage of the local quasi Fourier-transformation in the visual system is related to another fundamental problem of form recognition – separation of visual objects from the background. Assuming a process of local quasi Fourier-transformation in the visual system and as well as a difference in the SF spectrum of the visual object and the background, such a problem might be solved if SFs related to the background are rejected.

The third advantage of the local quasi Fourier-transformation in the visual system is related to the image contour extraction, as far as they seem to be the most informative part of them. The first hypothesis about this process is that contours are extracted by specialized "detectors" of edges or lines as the simple receptive fields in the primary visual cortex play this role. In several contemporary models, the contour extraction is considered to be performed not in one, but in two stages: a) processing by orientation- and SF-selective mechanisms; b) combining the outputs of these mechanisms in order to determine the location and the type of the local features (edges, lines, etc.). This follows from the fact that spatial phases of separate SF-components are very close to each other at the places of contours, as well as the I-st and II-nd derivation of light distribution have a maximum.

Several evidences exist in support of the mentioned two-stage model. One of the most convincing is described by Meese, Georgeson, who present to their subjects two superimposed sinusoidal gratings of equal or close SF, one of which with orientation of 45º and the other – with orientation of 135º. When the contrast of the gratings was relatively low, the subjects observed a picture, corresponding to the light distribution in the visual field – dark or light squares the sides of which were orientated in the direction of the gratings - 45º and 135º. At higher grating contrast, a chess board with squares orientated toward 0º and 90º was observed. This lack of correspondence between the light distribution and the perceived image has an

explanation. First of all, it shows again that visual perception is an active process in which the brain reconstructs the image of the world on the basis of a limited number of features, extracted from the image at an earlier stage and using special own rules. If we assume that these features are the image SF-components, we should observe exactly a "chess board" with squares orientated to 0º and 90º because such an image has contrast energy maximums at 45º and 135º, correspondently, as the stimulation has been.

When discussing the image contour extraction in the visual system, we have had in mind contours as a light spatial gradient in the visual field. However, human observers are also able to perceive contours which are represented by spatial gradient of the contrast. In this case, the visual field consists of randomly distributed light and dark points (visual noise), as the contrast between them was of different values in different parts of the field but with the same mean brightness. When the spatial gradient of the contrast is high enough, contours might be observed and different images might be synthesized by them. Such images are known in literature as the II-nd order stimuli, unlike the images constituted by light-gradient-contours, known as I-st order stimuli. The investigations have shown that II-nd order stimuli are processed not only by the mechanisms responding to I-st order stimuli, but also by specialized mechanisms. The fact that cross-adaptation between I-st and II-nd order stimuli was not observed, whereas the adaptation between stimuli of the same type is substantial, supports this assertion. In addition, moving I-st order gratings with proper SF induces optokinetic nistagmus, whereas the II-nd order gratings do not induce the nistagmus (Harris and Smith, 1992). Furthermore, the perception of the I-st and the II-nd order stimuli is impaired selectively by lesions in areas V2/V3 and MT, respectively (Vaina, Cowey and Kennedy, 1999).

The interest to the spatio-temporal organization of the visual system and to the manner it codes different aspects of visual objects is not due to theoretical reasons only. However, the results of this research could find some practical application. It is well known that perception is impaired when some neurological and other diseases are in the case. Some psychophysical characteristics – contrast threshold, reaction time, etc, as well as characteristics of VER might be used for evaluation of the patient condition. Investigation of one type of evoked responses - the VERPs (i.e. potentials registered under conditions when the subjects are not passive observers only, but they have some task, for instance stimulus discrimination, counting) is applied as an adequate and non-invasive method for studying the information processing – sensomotor and cognitive one at central brain level both in healthy persons and patients with neurological and other diseases. (Polich J., Herbst KL. 2000; Robertson C., Empson J., 1999; Montirosso R. et al., 2002; Vieregge P., Verleger R. et al. 1994; Verleger R. et al., 2003; Philipova D. et al. 1997; Philipova D. 1998; Philipova D. et al. 2004, etc.). It is widely accepted in literature that early P1, N1, P2 components are related to the early information processing, automatic, related to the sensory analysis after the stimulus, while late N2 and P3 components reflect the late cognitive processes. The components latency reflects the time for information processing, whereas the amplitude reflects the level of activation of the brain structures. (Kok A., 1997). The research of the visual and cognitive information processing at a central brain level in diabetics is of substantial importance since the retina cells are extremely susceptible to the change of the blood glucose level and the disease often affects the periphery as well as central nervous systems. To study neurophysiological changes at central brain level Kurita et al., 1996 investigated the auditory P300 in 60 insulin independent patients. Patients with diabetes showed prolonged P3 latency compared with the controls. A trend between the long latency and retinopathy was found, while the periphery neuropathy, blood glucose level and the duration of the disease did not correlate with the prolonged P3 latency. These findings suggests that pathological neuro-physiological mechanisms at central brain level in diabetes could be assessed by P3 latency and that microangiopathy and metabolite disorders during the preceding 4-8 weeks period could contribute partially for these changes. Mc Crimon et al. 1996 study the effect of the insulin induces control-

led low blood sugar on the early stages of the visual information processing and contrast sensitivity in 20 healthy subjects. They found that hypoglycaemia causes significant disorder of the cognitive processing, assessed by digit symbol task and trail taking B task. Low blood sugar evokes significant deterioration of the visual information processing speed – the time for analysis, visual discrimination and sensory-motor reaction. Substantial deterioration of the contrast susceptibility is monitored in hypoglycaemia patients, whereas the visual clearance and stereoscopic vision do not change.

Dey et al., 1997 study cognitive functions at relatively young (less than 55 years) insulin independent diabetics with disease duration from 5 to 18 (mean 3.2) years and respective healthy control group. Neuro-psychometric tests, as Mini mental status examination, Neurobehavioral cognitive status examination and P300 latency determination were applied. The researchers found prolonged P3 latency in patients compared with the healthy controls. Duration of the diabetes and diabetic polyneuropathy did not correlate with any of the parameters for assessments of the cognitive functions. The results show that moderate deterioration of the cognitive processes could be available in comparatively young diabetics.

Smid et al., 1997 investigate cognitive processes in hypoglycaemia patients by ERPs in a task for selective attention and sensory-motor choice reaction. The onset of the lateralized potential and reaction time are delayed during hypoglycaemia. Results suggest that the stimulus selection and motor response selection are prolonged, stimulus selection quickly restores after the restoration of the euglycemia, but the response selection doesn't restore. Slow shift in the cortical potential with wide frontal distribution which appears during low blood sugar is discussed in connection with the frontal mechanisms, included in the control of subordinate, modally specific mechanisms for selection. Homorka et al., 2000, found beta band reduction in patients with hypoglycaemia, while patients without pronounced hypoglycaemia showed only delta band reduction (1.3–3.5 Hz). Patients with often low blood sugar medical history showed significantly lowed vigilance when are not in hypoglycemic period compared with the group with lack of such periods in the anamnesis. Hissa et al., 2002 study interconnection between clinical findings, availability of retinopathy and preceding episodes of hypoglycaemia and P300 in 44 patients with insulin independent diabetes without cognitive changes. Patients are clinically tested also with Folstein Mini – Mental test for assessment of cognitive functions. The preceding low blood sugar periods are specified through anamnestical data and symptoms of the hypoglycaemia are scaled from 0 to 15 points. It is found that the latency of P300 is significantly prolonged for diabetics compared with the healthy controls and it depends on the age but not on the metabolic changes, disease duration and availability of retinopathy or preceding episodes of low blood sugar.

The research of visual information processing with diabetes patients has not only theoretical meaning but could have clinical importance. Since a large number of decisions have to be taken in conditions of limited time for perception and sensory analysis and low visual contrast (for example driving), investigation of visual perception and cognitive processing in diabetics has important practical application.

SCIENTIFIC TASKS

In the present project we plan investigations in the following directions:

Task 1. New psychophysical and electrophysiological evidence for the "module" organization of orientation- and SF-selective mechanisms in the human visual system will be searched. For this purpose several groups of investigations will be carried out:

1.1. Investigations of the spatial-summation characteristics of the visual system for detection of I-st and II-nd order stimuli-gratings. In these experiments the stimuli will be presented in 2-D Gaussian windows, spatial constants of which along the stimulus length and width will be varied independently.

– The threshold contrast for detecting of gratings as a function of their length and width will be investigated.

– Reaction time for grating detecting as a function of their length and width will be inves-

tigated.

– The dependences of the amplitude and the latency of the VER as a function of the grating length and width will be studied.

– Possible changes in the amplitude and latency of the VER to I-st and II-nd order stimuli-gratings recorded with patients with diabetes type 2 will be studied.

Task 2. Electrophysiological evidence whether visual information processing is performed in one or two stages when the subject task is orientation- and SF-discrimination of I-st and II-nd order stimuli-gratings with large or small difference between them. For this purpose the following studies will be carried out:

2.1. VER will be recorded with different subject tasks - choice sensomotor task and binary sensomotor task. The orientation- and SF-difference between the stimuli will be varied within a wide range - 5° ÷ 90° in orientation and 0,2 ÷ 2 octaves in SF.

2.2. Possible changes in the amplitude and the latency in visual event related potentials (VERPs) will be searched when patients with diabetes type 2 serve as subjects and their experimental tasks will be again coarse and fine discrimination.

Task 3. Investigation of I-st and II-nd order contour extraction and comparison of the underlying mechanisms. For the purpose the well-known illusion will be studied (Meese, Georgeson, 1996). In this illusion the contours of the perceived image created by two superimposed gratings with different orientation but the same SF, coincide or not with the real grating orientations as a function of the SF and the contrast.

3.1. Studying conditions (grating contrast and SF) under which the above-mentioned illusion is observed. For the purpose two superimposed sinusoidal gratings of I-st or II-nd order will be presented, one with orientation of 45° and the other – with 135°.

3.2. VER recording with stimuli employed in 3.1. i.e. to stimuli consisting of two superimposed gratings with different orientation but the same SF. The stimulus SF as well as the contrast will be varied according to the data obtained in 3.1., so that this illusion to be observed under some conditions and not observed – under other conditions.

Comparison of conditions under which the illusion is observed between normal subjects and patients with diabetes type 2.

EXPECTED EFFECT AND RESULTS

Having in mind the above-discussed "module" model, we expect to observe differences in the spatial summation along the stimulus length and width for each employed SF. Furthermore, we expect that critical values of the length and width up to which the summation effect is observed at different SFs, will not be of the same angular value, but will be proportional to the grating spatial period of the corresponding stimulus.

We expect the early waves of VER concerned with the image description in the visual system to be independent on the orientation- and SF-difference, whereas the late waves related to the decision process evoked by the discriminated task to show prolonged latency at small difference in orientation and SF. In this way possible prolongation of the latency of late waves at small differences would be an electrophysiological correlate of the above-suggested second stage of information processing. As it was suggested, this stage was related to comparison of activity of mechanisms with adjacent orientation- and SF-tuning curves, activated in a different extent by the two discriminated stimuli.

Data will be obtained allowing examining whether contour extraction in II-nd order stimuli is based on the same principles as contour extraction in I-st order stimuli – combining of output signals of orientation- and SF-selective mechanisms. If this proves to be the case, conditions under which this combining is effectuated might be determined. We also expect to find a VER-correlate of the "outputs combining" expressed as changes in the late VER-waves.

Data will be also obtained related to sensomotor and cognitive information processing in patients with diabetes type 2 in dependence on the type of stimulation – luminance or contrast defined (I-st or II-nd order). This, possibly, might allow verifying some deviations in perception characteristics – contrast threshold, reaction time, and the amplitude and latency of the VER as clinical tests.

Cooperation between the teams from the different research units will guarantee interdisciplinary approach to solve an important for the all society problem related to the quality of life and work.

PROJECT MANAGEMENT

• In the Institute of Neurobiology, BAS

Assoc. Prof. Dimitar Mitov, PhD as a principal investigator of the project will coordinate implementation of individual tasks included in the project and will manage the spending of the financial resources. Moreover, he will both conduct and participate in the planned psycho-physiological and electrophysiological experiments, and in preparing of articles and reports, their publication and presentation at scientific forums.

Prof. A. Vassilev, MD, DSc will take part as a consultant and as a researcher in psycho-physiological and electrophysiological investigations planned in the project. He will also take part in literature monitoring, preparing articles and reports.

Assist. Prof. Tcvetalin Totev will elaborate apparatus and software for visual stimulation, employed when VER is recorded. He will also take part in most psycho-physiological and electrophysiological experiments, data processing, preparing the data for publication and their presentation at scientific forums.

Assist. Prof. Milena Michaylova, PhD will participate in the VER studies, data processing, preparing the data for publication and their presentation at scientific forums.

Research fellow Kalina Racheva and **research fellow Ivan Christov** will participate in the psycho-physical experiments, data processing, preparing the data for publication and their presentation at scientific forums.

Assoc. Prof. Dolja Phillipova, MD, PhD will participate in the project as a principal investigator and researcher in the electrophysiological studies of the ERPs, in processing of electrophysiological data, writing reports and articles and their presentation at scientific forums.

Research fellow Stilliyan Georgiev and **research fellow Hristina Hristova** will participate in the electrophysiological investigations, as the latter two will be responsible for the data storage and their statistical processing.

Assist. Prof. Juliana Dushanova, PhD and **Assist. Prof. Gloria Nikolova** will participate in software development (data processing software), as well as in the computer processing of the electrophysiological parameters, by methods for averaging, subtraction. Power spectrum of the potentials will be evaluated.

For study of the neuro-physiological mechanisms and their eventual disorders in diabetics, alpha, beta, delta, theta, gamma bands are investigated, as well as the processes of synchronization and desynchronization after the stimulus, related to the sensory analysis and cognitive processing of information.

• In the clinic of Endocrinology of Medical University – Sofia

Assoc. Prof. Z. Kamenov, Assoc. Prof. V. Christov, V. Karamfilova, MD. Their mutual tasks include participation in the clinical processing of the cases – anamnesis, status, laboratory examination and differential-diagnostic determination. This clinical activity will be realized both in the Clinic and in the ambulatory practice of each of the specialists. Their engagement is summarizing of the endocrinological aspects of the project in the final scientific product. Apart from these joint tasks, the specialists from the endocrinological group will also have individual tasks:

Assoc. Prof. Zdravko Kamenov – Head of the Project Department of Endocrinology. He will be responsible for the clinical part of the project.

Assoc. Prof. Vladimir Christov - Head of the Clinic of Endocrinology will be responsible for the clinical part of the project.

Vera Karamfilova, MD will coordinate clinical processing of the cases in the endocrinology team and will direct them toward the next stage of the research in BAS.

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СЕНЗОРНИ И КОГНИТИВНИ ПРОЦЕСИ ПРИ ВЪЗПРИЯТИЕТО НА ЯРКОСТНО- И КОНТРАСТНО-ДЕФИНИРАНИ ЗРИТЕЛНИ СТИМУЛИ. ИЗСЛЕДВАНИЯ ВЪРХУ ЗДРАВИ И БОЛНИ СЪС ЗАХАРЕН ДИАБЕТ ТИП 2

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Резюме

Зрителната система на човека е основният източник на информация за обкръжаващия ни свят. Може би поради това около 70 % от кората на главния мозък на човека е ангажирана пряко или косвено с преработка на зрителна информация. Тази преработка позволява, от една страна, да се разпознава формата на зрителните обекти, независимо от различията в такива техни аспекти като размер, наклон, цвят, местоположение, а от друга страна – тези различия да бъдат оценявани добре. За да изпълнява тези задачи зрителната система извлича от изображенията подходящи признаци, като още на първия етап на този процес преминава от поточково (на ретинално ниво) към по-обобщено квази Фурие-описание на изображенията (на корово ниво). Това става с помощта на ориентационно- и пространствено-честотно (ПЧ-но)-избирателни механизми, организирани в т.нар. "модули".

Първата задача на настоящия проект е да се оценят пространствено-сумационните свойства на механизмите в тези "модули", като се изследва ефектът на ширината и дължината на стимули-решетки с различна ПЧ върху прага и времето на реакция за откриването им, както и върху амплитудата и латентността на зрителните предизвикани потенциали (ЗПП). Очаква се, съгласно споменатия "модулен" модел, сумацията по дължина да се окаже по-ефективна, отколкото по ширина, и в зависимост от ПЧ на стимулите да е пропорционална на техния пространствен период.

Втората задача на проекта е свързана с търсенето на нови доказателства за наличието на един или два етапа при различаването на стимулите по ориентация и ПЧ, като за целта разликата между предлаганите за различаване стимули се варира в широк диапазон и се регистрират ЗПП при различни задачи – двигателна по избор, бинарна двигателна и ментална задачи. Очаква се степента на различимост на стимулите да не повлиява съществено ранните вълни на ЗПП, но да удължава съществено късните вълни, като израз на споменатия по-горе втори етап на преработка на зрителната информация. Тези експерименти ще се проведат и с пациенти със захарен диабет тип 2, като се очаква удължаването на латентността на късните вълни на ЗПП да е още по-съществено удължено.

Тъй като зрителната система възприема контури, които са дефинирани не само като пространствен градиент на яркостта (контури от I-ви порядък), но и като пространствен градиент на контраста (контури от II-ри порядък), ще се търсят аналогии между механизмите, извличащи единия и другия тип контури. За целта ще се изследва отново една известна зрителна илюзия, при която контурите на възприетата картина, съставена от две решетки с различна ориентация, могат да съвпадат или да не съвпадат с ориентацията на съставните решетки в зависимост от тяхната ПЧ и контраст. В случая, обаче, ще се използват решетки от II-ри порядък. Доколкото възникването на тази илюзия се смята като доказателство, че при извличането на контурите на изображенията в зрителната система се осъществява "комбиниране" на изходите на ориентационно- и ПЧ-но-избирателни механизми, а в неврофизиологичен аспект това се изразява в синхронизиране на активността на тези механизми, това прави интересно да се проследи и поведението на ЗПП. Очаква се в този случай да се наблюдава нарастване на амплитудата на някои, най-вероятно по-късни вълни на ЗПП. Планира се тези експери-

менти също да бъдат проведени и с пациенти, страдащи от захарен диабет, с предположението за по-слаб ефект на т.нар. "комбиниране" на избирателни механизми.

Планираните изследвания имат преди всичко теоретична стойност, но някои отклонения от измерените показатели – праг за откриване, време на реакция, амплитуда и латентност на ЗПП - биха могли да имат и диагностична стойност.

RAPANA THOMASIANA HEMOCYANIN AS ADJUVANT FOR CONVENTIONAL VACCINES

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Abstract

Killed viral vaccines and bacterial toxoids are weakly immunogenic. Numerous compounds are under evaluation as immunological adjuvants and peptide-carriers to improve the immune response. The hemocyanins, giant extracellular copper proteins in the blood of many mollusks, are widely used as immune stimulants. In the present study we investigated the adjuvant properties of hemocyanin isolated from marine gastropod Rapana thomasiana. An immunization with Influenza vaccine or tetanus toxoid combined with Rapana thomasiana hemocyanin (RtH) and Keyhole limpet hemocyanin (KLH) in mice induced an anti-influenza cytotoxic response lasting at least 5 months and an antibody response to viral proteins. The IgG antibody response to the tetanus toxoid (TT) combined with RtH or KLH was comparable to the response of the toxoid in complete Freund's adjuvant. The results obtained demonstrate that the hemocyanins are acceptable as potential bio-adjuvants for subunit vaccines.

INTRODUCTION

Many of the vaccines that are in use today consist of mainly killed viruses and microbial agents, or, alternatively - viral subunits or bacterial toxoids. The desired immune response to vaccines is the production of antibodies and/or generation of cytotoxic T-cells, and this is enhanced by adding certain substances to the vaccines. Previously used vaccines made from live or killed whole organisms were effective, but suffered from high reactogenicity. As vaccine manufacturers developed safer, less reactogenic subunit vaccines, they found that with lower reactogenicity came reduced vaccine effectiveness. Adjuvants are vaccine additives that enhance the elicited levels of antibodies and specific T lymphocytes. According to their chemical nature, adjuvants are a highly heterogeneous group of compounds with only one thing in common: their ability to enhance the immune response. The mode of action of adjuvants is formation of an antigen depot with slow release at the site of inoculation, presentation of antigen to immunocompetent cells and production of various cytokines [1]. In conventional vaccines adjuvants are used to elicit an early, high and long-lasting immune response. The newly developed purified subunit or synthetic vaccines using biosynthetic, recombinant and other modern technologies are poor immunogens and require adjuvants to evoke the immune response [2-4]. There are several types of adjuvants; today the most common adjuvants for human use are aluminium hydroxide, aluminium phosphate and calcium phosphate. However, there are a number of other adjuvants based on oil emulsions, products from bacteria, endotoxins, paraffinic and vegetable oils. Alum has been widely used in human

vaccines for 70 years, but the molecular mechanism of its action and the target cells are still unknown. It has been proposed that absorption to it increase antigen availability at injection site allowing an efficient uptake by antigen-presenting cells (APCs) [5]. Alum could also increase antigen uptake by dendritic cells in vitro, further supporting an antigen delivery function [6].

The desire for new and improved adjuvants stems not only from the need to make existing inactivated vaccines more potent, but also to gain features such as antigen-spreading ability, more rapid seroprotection, stimulation of T-cell immunity, and longer-lasting protective immunity. Safety and tolerability are critical regulatory issues confronting the new adjuvants, and pose the greatest barrier to new adjuvant approvals. Therefore, the benefits of incorporating any adjuvant into vaccines must be balanced against any increased reactogenicity or risk of adverse reactions. Unfortunately, in most cases, increased adjuvant potency is associated with increased reactogenicity and toxicity. The best example for this is complete Freund's adjuvant (CFA). While it remains the gold standard in terms of adjuvant potency, its extreme reactogenicity and toxicity precludes its use in human vaccines.

The hemocyanins (Hcs) are a multigene family of giant extracellular copper proteins which serve as oxygen carriers in the blood of many mollusks [7, 8]. Their large molecular size (4 to 8 MDa), xenogenic character and carbohydrate content have been implicated in inducing strong immune response in mammals. Hcs have been applied as hapten carriers, as adjuvants in immunocompetent tests and as experimental antigens in studies of the immune system. Keyhole limpet hemocyanin isolated from marine gastropod *Megathura crenulata* is a powerful immunogen and therefore has been widely used as a carrier for peptides inducing antibodies with specificity for the native proteins from which they were derived [1, 9-14]. Recently, Hc obtained from the Chilean gastropod *Concholepas concholepas* was reported to possess adjuvant immunostimulatory effect [15-18].

In our previous studies on the hemocyanin isolated from *Rapana thomasiana* – a marine gastropod living along the west coast of the Black Sea – we demonstrated its high immunogenicity as a single model antigen and also its properties as a strong protein carrier for viral peptides from Influenza hemagglutinin [19]. These results suggested a potential role of RtH as an acceptable compound needed for adjuvanicity of standard vaccines without covalent binding to the antigen.

The aim of the present work was to investigate the adjuvant properties of RtH and KLH in an experimental murine model and to use them as non-conjugative carriers of viral or bacterial proteins.

MATERIALS AND METHODS

Hemocyanins preparation

Living marine snails *Rapana thomasiana* were caught near Bulgarian coast of the Black Sea (Varna) and stored in sea water. The hemolymph was collected by bleeding through several diagonal slits made on the foot of the mollusc and filtered through gauze. Phenylmethanesulphonyl fluoride (PMSF) was added (1mM) to the crude material to avoid possible proteolysis of the hemolymph. Hemocytes and other cells were removed by centrifugation at 5000 x *g* for 30 min at 4°C. The hemolymph was concentrated by the ultrafiltration system Tangential Ultrafiltration ProVario-3, Pall-Filtron, Dreieich, Germany. Native RtH was purified from the concentrated hemolymph using ammonium sulfate precipitation method developed by Idakieva et al. [20]. The purity of the isolated Hc was controlled by gel chromatography, SDS- and native PAGE, and transmission electron microscopy (TEM) as described previously [21]. Hc solution was passed once through a purification column to remove endotoxin contaminations (Detoxigel column, Pierce). The level of the remaining endotoxin was determinated by Limilus Amebocyte Lysate coatest gel (LAL) (Chromogenix AB, Molndal, Sweden). The final purified and sterile Hc solution contained 0.48 EU/mg protein and was used for further study of its adjuvant properties.

KLH was purchased from Calbiochem (Darmstadt, Germany).

Construction of conjugated molecules Bovine serum albumin – influenza peptide (BSA–IP)

The hemagglutinin intersubunit peptide IP

(containing T and B cell epitopes) from the influenza virus strain A/PR/8/34 was used to make the construct. The synthesis of Ac-(coding region HA317-341)-NH-$(CH_2)_6$-NH_2 was carried out using a Fmoc-based manual solid phase peptide synthesis protocols on 2-Cl-Trt resin. The peptides Ac-(coding region HA317-341)-Ahx-K-NH_2 were purchased from Caslo Laboratory (Lyngby, Denmark) and were purified (≥ 98 % purity) by HPLC [22].

The covalent coupling of the BSA (Sigma-Aldrich, Taufkirchen, Germany) to the peptides was performed by the classical EDC (1-ethyl-3(3'-dimethylaminopropyl) carbodiimide.HCl, Fluka AG, Buchs, Switzerland) cross-linking technique using a spacer (Ahx-K-NH_2) in the C-end of the peptides [23]. The BSA (in concentration 0.1 mg/ml in 0.1M sodium phosphate buffer, pH 6.0) was mixed with a 20-fold molar excess of the peptide (dissolved in 10% (v/v) N, N-dimethylformamide (Sigma-Aldrich)) in the same buffer to 0.02 mg/ml final concentration. The reaction was started by addition of carbodiimide at 60-fold molar excess over the BSA and the mixture was stirred overnight at 4°C, dialyzed against PBS and concentrated by ultrafiltration (XM10). The construct BSA–IP was used for coating of plates in ELISA measuring the level of anti-flu antibodies formation.

Animals

Balb/c mice were obtained from Iffa-Credo, L'Arbresle, France (Charles River Company) and bred under specific-pathogen-free conditions in our animal facility. Female mice aged 8 to 10 weeks were used for immunization.

Immunization protocols

The protocols used were approved by the Animal Care Commission at the Institute of Microbiology in accordance with the International laws. Control groups of Balb/c mice (6 to 8 mice each) were injected i.p. with PBS only or with commercial anti-flu vaccine Influvac (2007/2008, Solvay Pharmaceuticals, The Netherlands) alone or with the same vaccine emulsified in an equal volume of Freund's complete adjuvant (CFA, Sigma). Further in the text the commercial vaccine is abbreviated as Vac or when administered in CFA as Vac+CFA. The vaccine dose contained 15 μg influenza hemagglutinin (HA) per mouse. Two other groups of mice were immunized with Vac combined to RtH or KLH (100 μg per mouse). Two more groups were treated with RtH or KLH alone. Mice were boosted 21 days later with the same doses of PBS, Vac (without adjuvant), Vac+KLH, Vac+RtH, RtH or KLH as described above. The reimmunization of animals treated with Vac emulsified in CFA was done with Vac emulsified in an incomplete Freund's adjuvant (IFA). The last treatment was administered 14 days later under the same schedule.

Other groups of mice were administered with 20 μg per mouse of tetanus toxoid (from Bulbio, Sofia, Bulgaria) alone or with tetanus toxoid emulsified in an equal volume of CFA, or with the same toxoid combined to RtH or KLH (100 μg per mouse). Second and third immunizations were performed under the same schedule as described above. The mice were bled before each immunization and after the last treatment. Collected sera were kept frozen at −70°C before testing for antibodies and cytokines.

Enzyme linked immunosorbent assay (ELISA) for anti–flu and anti–TT IgG and IgM antibodies

BSA-IP or TT diluted to 20 μg/ml in coating buffer ($NaHCO_3$, pH 9.6) was used for coating of microplates (Nunc, Roskilde, Denmark) by incubation overnight at 4°C. After washing with PBS/0.05% Tween 20 and blocking with 1% BSA, serum samples diluted 1:100 for measuring of IgG or IgM antibodies were added and incubated for 1h at room temperature. The plates were then washed and incubated for 1h at room temperature with alkaline phosphatase-labelled goat anti-mouse IgG or IgM (Pharmingen BD, San Diego, USA). After washing, Sigma 104 phosphatase substrate was added and the absorbance was measured at 405 nm. The obtained ELISA results were presented as relative units (RU), corresponding to the titer of anti-IP or anti-TT standard antibodies used for ELISA.

Cytokine detection

IL4 and IFN-γ levels were measured in mouse sera using commercial ELISA kits (BD Biosciences, USA)

Cytotoxity assay

The influenza A/Aichi/2/68 (H3N2) strain (The Collection of the Stephan Angeloff Insti-

tute of Microbiology, Sofia, Bulgaria) was grown on MDCK cells and after pelleting, the virions were concentrated by ultracentrifugation and suspended in PBS.

Confluent 3T3 (mouse embryo fibroblasts) cell monolayer cultured in DMEM with 5% FCS was incubated with 10^2/ml virus for 18h at 37°C. Then cells were washed, trypsinized and transferred to the wells of a 96-well tissue culture plate (2.10^5 cells/ ml). Freshly isolated spleen cells from immunized mice were used as effector cells in a non-radioactive cytotoxic assay at a ratio between target and effector cells 1:40. After incubation for 4h at 37°C the cells were centrifuged and the lactate dehydrogenase (LDH) concentration in the supernatants was determined by the CytoTox assay (Promega, USA) according to the manifacturer's instructions. The percentage of specific lysis was calculated as follows: specific lysis (%) = (experimental release-spontaneous release/(maximum release-spontaneous release) x 100. Maximal or spontaneous release was obtained by incubating of the target cells with 1% Triton or with medium only.

Statistical analysis

Values in the figures correspond to mean ±SD. All ELISA, cytokine and cytotoxity samples were triplicated. The unpaired Student *t*-test was used to determine differences between each two groups. The two-tailed Mann-Whitney U test was used when appropriate. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Adjuvant activity of RtH and KLH for anti–flu IgG and IgM antibodies formation

Blood samples were collected by retro-orbital puncture and mouse sera were prepared and tested for IgM and IgG anti-IP antibodies by ELISA. The Vac+KLH groups have produced high levels of IgM antibodies after the second immunization. In the group vaccinated with Vac+CFA and Vac+RtH IgM antibody formation started after the last immunization and serum IgM levels further reached the level registered in the group injected with Vac+KLH (Fig. 1, lower panel). Induction of IgG anti-flu antibodies was not observed in the groups immunized with Vac, Vac+CFA and Vac+RtH, but an increase in anti-IP titers was observed in the Vac+KLH treated group (Fig. 1, upper panel).

Adjuvant activity of RtH and KLH for anti–TT IgG and IgM antibodies formation

Testing of the sera for specific anti-TT IgG and IgM antibodies by ELISA showed that the groups immunized with TT, TT+KLH and TT+RtH produced high levels of IgG antibodies even after the second immunization (week 5) (Fig. 2, upper panel). RtH showed better adjuvant property than KLH at this point, similar to the antibody response to TT+CFA. After the third immunization TT+RtH and TT+KLH reached the maximal values obtained by TT+CFA. The group immunized with TT alone also showed high levels of anti-TT IgG antibodies after first and second booster.

For all immunized groups IgG class switching was observed. In the group treated with TT+KLH and TT+CFA high levels of IgM antibodies were developed even after booster vaccinations (Fig. 2, lower panel).

Cytokine detection

IL4 and IFN-γ levels were measured in mice sera using ELISA kits. To investigate whether the anti-hemocyanin response corresponds to the Th1/Th2 type of immune response we examined the cytokine levels after immunization with bacterial or viral proteins with RtH or KLH. We observed a high correlation between the IL4 and IFN-γ levels obtained after immunization with Vac+KLH and Vac+RtH (Fig. 3). In both cases the cytokine production measured was lower than the levels obtained after treatment with the control groups.

Vaccination with TT did not result in an increased production of IL4 and IFN-γ in any group except in the TT+KLH treated group where an increase of IL4 synthesis was observed (Fig. 4). However, IL4 and IFN-γ levels were modestly decreased after treatment with TT+RtH.

Generation of cytotoxic cells

We examined the CTL activity of freshly isolated spleen cells from all animals against influenza virus-infected 3T3 cells three months after the last immunization. Immunization with RtH or KLH alone induced a low cytotoxic activity, while the effects of Vac and Vac+CFA were moderate. The effector cells obtained from the Vac+KLH group lysed 7.5 % of the infected cells

(Fig. 5). The cytotoxic effect in the Vac+RtH immunized group was significantly stronger than the effect in other treated groups.

DISCUSSION

Adjuvants are substances which are added to vaccines to boost immune response, but the major unsolved challenge in their development is how to generate a potent adjuvant effect while avoiding reactogenicity or toxicity. The development of recombinant DNA technology presents a new way to design vaccines that no longer have side effects. The goal of vaccine development is to produce multivalent vaccines with built-in adjuvanicity, and the engineering approach may satisfy that need. Such a hybrid DNA molecule was constructed by us, encoding a T- and B-cell epitope-containing influenza hemagglutinin peptide and a single-chain (scFv) antibody fragment binding to mouse complement receptors 1 and 2. A single immunization with a plasmid containing the described construct induced a strong anti-influenza cytotoxic response lasting for more than 6 months [24, 25].

The use of Hcs from different sources as carriers of haptens and peptides for production of monoclonal antibodies and as carriers of vaccines against infectious diseases is generally accepted. Despite the increasing use of sophisticated small antigenic fragments containing defined B and T cell epitopes, distinct adjuvants and vaccine preparations are desirable. We have tested RtH and KLH as non-conjugated adjuvant for standard vaccine preparations – commercial tetanus toxoid and flu vaccine.

Side effects of the KLH have been studied a lot due to its large use as bio-adjuvant and immunological modulator for different types of vaccines, including bacterial, viral and anti-cancer ones in animal models and humans. In their study concerning immunotoxicity Roth et al. have shown that only the levels of KLH specific Abs have been elevated in the group, immunized with KLH (50 μg/rat in aluminium hydroxide) compared to the control non-treated animals, and there was no detectable difference between them in the following parameters: number CD4+ cells and lymphocyte count; development of germinal centers in Axillary lymph nodes and macrophage accumulation and granuloma formation/capsule in the site of the injection [26]. Boelens et al. examine the antibody response to KLH in healthy and trauma patients and did not find either in the patients or in the healthy controls anti KLH antibodies of the IgE-isotype [27].

In previous experiments we have used higher doses of RtH for immunization (250 μg/mouse) and we observed the animals for six months [19]. There weren't any side effects, IgE synthesis or life span changes detected (data not shown).

The usual adjuvant quantity used for immunization is much higher then we have used in our experiments. In standard vaccines $Al(OH)_3$ is 170 – 200 μg per dose, but many authors used even higher quantities to obtain adjuvant properties. Using huge amounts of adjuvants could lead to severe reactions, IgE synthesis and significant alteration in the cytokine profile [28, 29]. In the present research we have used very low doses of both hemocyanins – 100 μg per mouse. The cytokine profile does not show any indication for anaphylactic reactions and supports the conclusion for low risk of side effects.

In our previous experiments we have used various flu vaccines in different models [24]. In general, conventional vaccines provoked high antibody titers when administered in CFA. In this experiment the small quantity of Vac combined with CFA resulted in much lower antibody levels than in previous studies [24]. Using the RtH as a protein carrier of a T- and B-cell epitope-containing influenza hemagglutinin peptide leads to generation of high anti-influenza IgG antibody levels [19]. In contrast, repeated immunization with an influenza vaccine without covalent conjugation to RtH did not lead to a significant antibody production. The present results show high levels of IgG and IgM antibodies in the animals immunized with Vac combined with KLH (Fig. 1).

A similar immunostimulatory effect was observed by both Hcs that differ in their origin and structure. We compared the serum levels of IL4 and IFN-gamma characterizing Th1/Th2 responses. Both Hcs combined with Vac resulted in the reduction of IL4 and IFN-gamma values after vaccinations (Fig. 3).

Vaccination with Hcs combined with Vac appears to be very effective at inducing long-lived

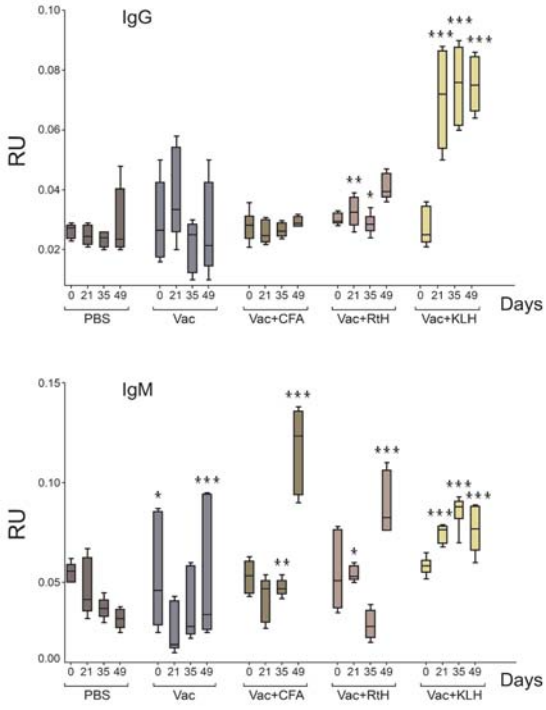
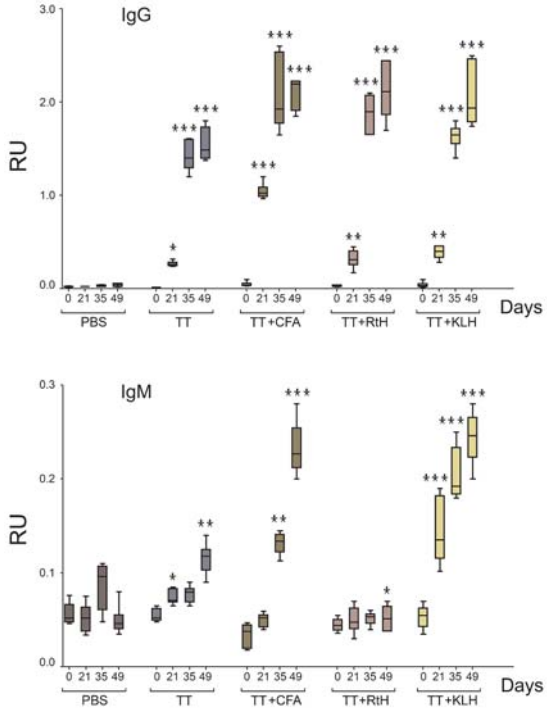


Fig. 1.



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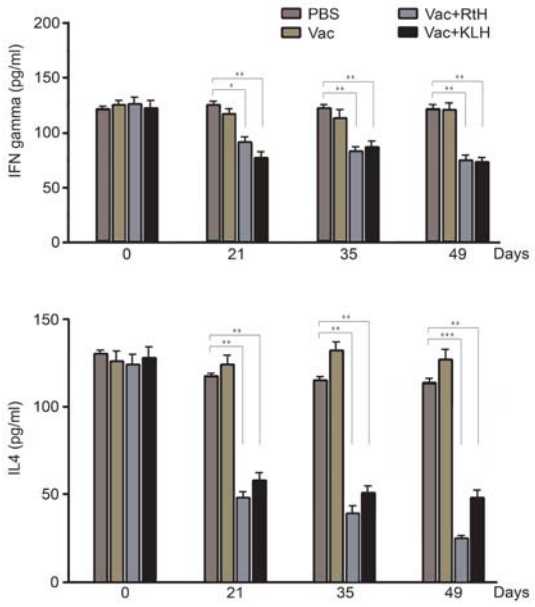

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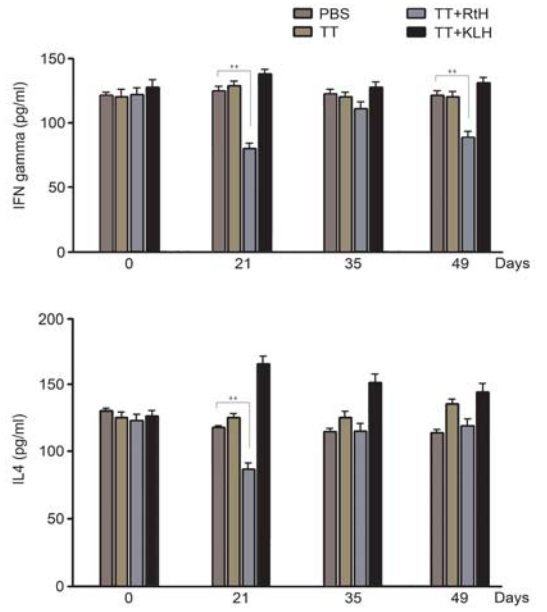

Fig. 4.

cytotoxic memory. Our data show that high CTL anti-influenza activity was present 5 months after the first immunization with Vac+RtH or KLH (Fig. 5). These results are in accordance with data showing that immunization with DNA vaccine coding influenza-peptide generates CD8+ cytotoxic T lymphocytes persisting for at least 6 months [24]. The CTL response after the administration of Vac+RtH was much stronger than that observed after immunization with the Vac alone. Exogenous antigens can prime some CD8+ cytotoxic T cells when administered in CFA [30-32]. In our hands the conventional vaccine in CFA induced weak anti-influenza cytotoxic immunity. Hcs used as hapten-carriers stimulate major histocompatibility complex class I CD8 and also class II CD4 T-cell responses [16, 33]. These data support the results obtained from a CTL activity after immunization with Vac-RtH and Vac+KLH.

More powerful antibody production is achieved after repeated administration of a non-

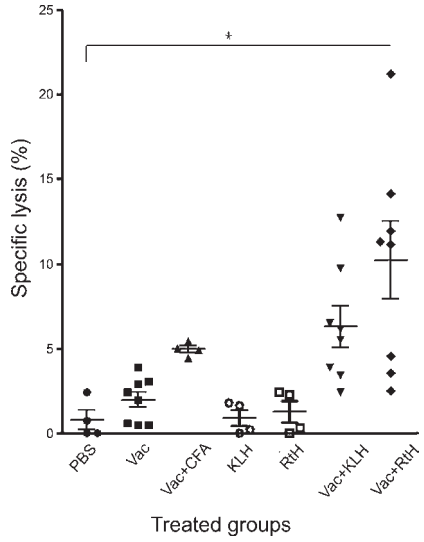

Fig. 5.

conjugate TT with RtH or KLH (Fig. 2). Our results showed that immunization with a tetanus toxoid without adjuvant leads to a significant antibody production and it might be concluded that the effect was due to the self-adjuvant properties as a result of the big size of the toxoid molecule. In some cases tetanus toxoid is even used as a protein carrier for peptides derived from influenza hemagglutinine [1]. Vaccination with TT+RtH and TT+KLH appears to be effective at inducing a humoral response. High IgG anti-TT titers were found after the first booster and following the second one they reached the levels obtained after administration of TT+CFA. Production of IL4 and IFN-gamma was reduced after the first immunization in the group treated with TT+RtH and that became normalized before third immunization (Fig. 4). It could be explained with the limited protein quantity immunized in the animals and the different mechanism of adjuvanicity enhanced by hemocyanins.

CONCLUSION

In general, immunization with Hcs as adjuvants leads to immune response without any dramatic effects as a local or general inflammation, storm cytokine production and other connected side reactions. These extremely foreign for mammals substances stimulate the immune system for generation of CTL and antibody formation and their protein origin make them acceptable as a potential bio-adjuvants for subunit vaccines. The results obtained demonstrate that RtH and KLH are able to induce a humoral and CTL immune response when they are used as adjuvants of non-conjugated bacterial and viral proteins.

Legend to the figures

Fig. 1. Serum anti-IP antibody titers in mice injected i.p. with PBS or with 15 μg of commercial Influenza vaccine alone (Vac) or vaccine in CFA (Vac+CFA). Two other groups of animals were immunized with Vac (15 μg per mouse) combined to 100 μg RtH or KLH. Mice were boosted 21 days later and once again 14 days later with the same doses. The animals were bled before each and 15 days after the last immunization and the sera analyses were performed by ELISA. The results are presented as Relative Units (RU), calculated using standard polyclonal IgG and IgM antibodies against IP. The titers obtained from the standard's dilutions were used to create a curve from 0.01 to 0.2 RU. The data are represented as mean ± SEM from individual sera collected (n=6-8). The experiments were repeated twice. *P* values are calculated using the Mann-Whitney U test (*$P<0.05$; **$P<0.01$; ***$P<0.005$), in comparison to PBS treated controls.

Fig. 2. Anti-TT IgG and IgM levels induced by TT immunization with or without adjuvant. The mice were injected i.p. with PBS or with 20 μg of TT alone or TT in CFA (TT+CFA). Two other groups of mice were immunized with TT (20 μg per mouse) combined to 100 μg RtH or KLH. The treated groups were boosted 21 days later and once again 14 days later with the same doses. The animals were bled before each and 15 days after the last immunization. The results are presented as Relative Units (RU), calculated using standard polyclonal IgG and IgM antibodies against TT. The titers obtained from the standard's dilutions were used to create a curve from 0.01 to 0.5 RU for IgM measurement and from 0.1 to 5.0 RU for IgG determination. The data are represented as mean ± SEM from individual sera collected (n=6-8). The experiments were repeated twice. *P* values are calculated using the Mann-Whitney U test (*$P<0.05$; **$P<0.01$; ***$P<0.005$), in comparison to PBS treated controls.

Fig. 3. Cytokine profile of mice vaccinated with

Influenza Vac. The animals were immunized as described in the legend to Fig. 1. Serum levels of IL4 and IFN-gamma were measured by sandwich ELISA using commercial cytokine assays. The data represent mean ± SD from individual sera collected. *P* values are calculated using the Mann-Whitney U test (**P*<0.05; ***P*<0.01; ****P*<0.005), in comparison to PBS treated controls.

Fig. 4. Cytokine production after administration of TT to the experimental animals. The mice were immunized as described in the legend to Fig. 2. Serum levels of IL4 and IFN-gamma were measured by sandwich ELISA using commercial cytokine assays. The data represent mean ± SD from individual sera collected. *P* values are calculated using the Mann-Whitney U test (**P*<0.05; ***P*<0.01; ****P*<0.005), in comparison to PBS treated controls.

Fig. 5. CTL activity of splenocytes isolated at day 150 after the first immunization of all test-groups. 3T3 cells pulsed with the influenza virus were cultured with effector spleen cells from individual mice at a ratio 1:40 for 4h at 37^0C. LDH concentration in the supernatants was determined in triplicates by a commercial CytoTox assay. Each icon indicates the result obtained from individual mice as a part of the treated group. Results are expressed as the mean value ± SD comparing immunized and PBS only-treated mice. **p*<0.05; Student t-test. The data are representative of at least 3 independent experiments.

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ХЕМОЦИАНИН ОТ RAPANA THOMASIANA КАТО АДЮВАНТ ЗА СТАНДАРТНИ ВАКСИНИ

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Резюме

Ваксините получени от убити вируси, както и тези, съдържащи бактериални токсоиди са слабо имуногенни. Множество химични и биологични агенти се използват в качеството си на потенциални адюванти и протеини-носители в състава на редица ваксини с цел да бъде усилен имунният отговор. Хемоцианините са група големи мед-съдържащи белтъци, изолирани от хемолимфата на молюски, които намират широко приложение като имуностимулатори. В настоящата работа изследваме адювантните качества на хемоцианин, изолиран от морски охлюв Rapana thomasiana (черноморска рапана). Имунизацията на мишки със субединична грипна ваксина или тетаничен токсоид, комбинирани с хемоцианин от молюските Rapana thomasiana (RtH) или Megathura crenulata (KLH) доведе до индуциране на цитотоксичен имунен отговор продължаващ най-малко 5 месеца, както и до генериране на хуморален имунен отговор срещу вирусните белтъци. Антитяловият отговор към тетаничния токсоид (TT), имунизиран заедно с RtH или KLH беше съпоставим с този, индуциран при прилагането на токсоида с пълен адювант на Фройнд. Получените резултати демонстрират, че хемоцианините могат да бъдат приложени като потенциални био-адюванти за субединични вирусни и бактериални ваксини.

CHOLESTEROL–LOWERING PROPERTIES OF LACTOBACILLI

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Abstract

The aim of this study was to evaluate the cholesterol-reducing properties of Lactobacillus strains. A large number of lactic acid bacteria with intestinal origin were collected from the feces of healthy volunteers and assessed for their properties to reduce the cholesterol content in a laboratory growth medium. The uptake of cholesterol occurred when the Lactobacillus strains were growing in the presence of bile salts and it was dependant from the ability of the bile tolerance of the strains. In parallel with total cholesterol-lowering activity the bile-salt-hydrolyzing properties of the strains were evaluated. The best cholesterol-reducing strain was L. brevis 5/11, which showed very high of both total cholesterol-lowering activity and bile-salt hydrolase activity. The probiotic strain L. gasseri 4/13, which has excellent adhesion level, demonstrated very high cholesterol-reducing effect, too. As a result of this work several strains with cholesterol-lowering effect were selected and one of them (L. gasseri 4/13) possessed other probiotic effects and was chosen for further clinical experiments.

INTRODUCTION

Lactic acid bacteria (LAB) are normal components of the intestinal microflora in both humans and animals and have been associated with various health-promoting properties. For this reason, there has been much interest in developing food products containing these bacteria as dietary adjuncts (6). Several studies have indicated that consumption of certain cultured dairy products resulted in reduction of serum cholesterol. Consumption of yogurt also has been shown to decrease serum cholesterol levels in humans (4). Grunewald (3) observed a significant decrease in serum cholesterol in rats fed with milk fermented with *L. acidophilus*. One beneficial effect that has been suggested to result from human consumption of LAB is a reduction in serum cholesterol levels, as suggested by the results of several human and animal studies, reviewed by Pereira and Gibson (7). This effect can partially be ascribed to an enzymatic deconjugation of bile acids (5).

In the GI tracts of humans and animals, most intestinal bacteria encounter significant amounts of bile salts, which are continuously present via enterohepatic circulation (9). Bile salts are synthesized mainly from cholesterol, conjugated with glycine or taurine in the liver, stored in the gall bladder, and released into the duodenum in response to the ingestion of fatty food. In addition to their function in the intestine as natural emulsifiers, bile salts possess some detergent-like antimicrobial properties. Some bacterial species have developed mechanisms to resist the detergent action of bile salts and have evolved to transform bile salts biochemically. Among the biochemical modifications of bile salts that are exhibited by many GI microorganisms, hydrolysis of the conjugated bile salts is considered the primary metabolic activity because bile salts need to be deconjugated before further sterol transformations take place (1). The enzyme responsible, bile salt hydrolase (BSH) (EC 3.5.1.24).

Other way to get to decrease of cholesterol content is the absorption of cholesterol by cell-wall components of bacteria. This effect would be helpful for the reducing of cholesterol in intestinal tract.

In the present work, the in vitro BSH activity of a number of LAB strains was studied in view of their potential cholesterol-lowering effects through enhanced BSH activity. Direct cholesterol-reducing effect was assessed, too.

MATERIALS AND METHODS

Bacterial strains

Intestinal and dairy lactobacilli were isolated after plating of 0.1-ml of respective dilutions of fecal or milk/cheese homogenates on MRS agar (Merck, Darmstadt, Germany). The plates were in-

cubated at 37°C for 3 days under anaerobic conditions (10% CO_2, 80% N_2, 10% H_2). The single colonies were purified trice and the species belonging and strain identity were determined by help of species-specific PCR, Amplified Ribosomal DNA Restriction Analysis, sequencing of hyper variable rDNA V6-V8 regions, and Pulsed field gel electrophoresis, according to Dimitrov et al. (2).

Screening of cultures for BSH activity

Qualitative BSH activity of the cultures was evaluated using the procedure described by du Toit et al. (11). Sterile filter disks were impregnated in an overnight culture of the test strain and placed on MRS agar plates supplemented with 0.5% (wt/vol) taurodeoxycholic acid sodium salt (TDCA; Sigma) and 0.37 g of CaCl2 (Merck)/liter. The plates were incubated anaerobically at 37°C for 72 h, after which the diameters of the precipitation zones were measured. MRS agar plates without supplementation were used as controls. Each strain was tested in triplicate. The three strains that displayed the largest precipitation zones were selected for further study.

BSH assay

A modification of the high-performance liquid chromatography (HPLC) method described by De Smet et al. (1) was used to determine quantitative BSH activity. A reverse-phase Ultrasphere ODS column (Hichrom Ltd.) (80 A°, 5 µm, 150 by 4.6 mm) was used. The HPLC system consisted of an SHIMADZU series apparatus equipped with an autosampler and on-line vacuum degasser. Free and conjugated bile acids were eluted with a linear gradient of methanol aqueous buffer at a flow rate of 1.0 ml/min. The solvents used were solvent A, which consisted of a mixture of 65% methanol in 0.03 M sodium acetate adjusted to pH 4.3 with phosphoric acid, and HPLC-grade methanol (solvent B). The elution program was as follows: isocratic elution was performed with 15% solvent B and 85% solvent A for 8 min and then a 17-min linear gradient to 85% solvent B, and the mobile phase composition was finally maintained at 85% solvent B for further 5 min. The detector was set at 210 nm, and chromatography was performed at room temperature. The injection quantity was 10 µl. All bile acids used as standards (conjugated and free) had a purity of 97% or more and were purchased from Sigma.

Bile salt extracts

To recover bile salts from the MRS broth cultures, cells were removed by centrifugation (6,000 *g* for 10 min at 5°C). The method of De Smet et al. (1) was modified to recover bile salts from the spent broth. Samples (1 ml) of the supernatants were acidified through the addition of 10 µl of 6 N HCl to stop BSH activity. Lithocholic acid was used as an internal standard and added to a final concentration of 8 mM. Isopropanol (4 ml) was used to extract the bile salts (1:4 [vol/vol]). The samples were then mixed for 60 min at 420 rpm and centrifuged at 1,000 *g* for 10 min. The isopropanol layer was transferred to a clean test tube and evaporated under an N2 flow at 37°C. After complete isopropanol removal, the bile salt extract was redissolved in 800 µl of methanol and filtered through a 0.45-µm-pore-size polysulfone HPLC filter (Whatman). Prior to injection in the HPLC filter, samples were stored at -20°C.

Quantitative BSH activity

BSH activity of the cultures was determined using the HPLC procedure described above. Strains were compared for their abilities to deconjugate bile acids during growth. Dilution bottles of about 100 ml capacity were filled with 70 ml volumes of MRS broth, autoclaved at 121°C for 15 min, and cooled to around 50°C. The MRS broth was then supplemented with a filter-sterilized solution of sodium taurocholate (TCA) and sodium glycocholate (GCA) to give a final concentration of 1 mM for each bile acid. Overnight MRS broth cultures of the strains undergoing testing were inoculated (1% [vol/vol]) into the medium, and the mixtures were incubated anaerobically at 37°C for 24 h. Samples were taken aseptically at various time intervals during the incubation period. Growth was monitored through absorbance at 650 nm, and bacterial enumeration was determined by plate count on MRS agar (after inoculation and at the end of the 24-h incubation). Samples were also analyzed for pH and bile acids. The experiment was performed in triplicate for each strain, and uninoculated MRS broth supplemented with TCA and GCA was used as a control. The BSH enzymatic activity was expressed as nanomoles of GCA and TCA deconjugated

per minute. The strain with the highest BSH activity and simultaneously the highest adhesion properties and probability of survival through the upper gastrointestinal tract was chosen for further studies.

Measurement of cholesterol uptake

A freshly prepared MRS broth cultures of the LAB strains were inoculated (1%) into 10 ml of sterile MRS broth containing 100 mg/l cholesterol and the desired concentration of oxgall (Difco). The tubes were incubated anaerobically for 24 h at 37°C in a GasPak hydrogen-carbon dioxide anaerobic system (BBL Microbiology Systems). Cells were removed from the broth by centrifugation for 10 min at 12,000 x g and 4°C. The cell pellet was resuspended in a volume of distilled water equal to that of the original broth culture. The ophthalaldehyde method for measuring cholesterol described by Rudel and Morris (8) was used to determine the amount of cholesterol in the resuspended cells and spent broth. Uninoculated sterile broth was also analyzed in some experiments. Since some modifications with respect to sample volume and reagent volumes were used, the procedure is described here for convenience. The sample (0.5 ml) was placed into a clean test tube (duplicates for each sample). Three milliliters of 95% ethanol were added to each tube, followed by 2 ml of 50% potassium hydroxide. The contents of all tubes were mixed thoroughly after addition of each component. Tubes were heated for 10 min in a 60°C water bath, and after cooling, 5 ml of hexane was dispensed into each tube. After mixing thoroughly with a Vortex for 20 s, 3 ml of distilled water was added, and the mixing was repeated. Tubes were allowed to stand for 15 min at room temperature to permit phase separation. Then 2.5 ml of the hexane layer was transferred into a clean test tube. The hexane was evaporated from each tube at 60°C under the flow of nitrogen. o-Phthalaldehyde reagent (4 ml) was added to each tube. The reagent contained 0.5 mg of o-phthalaldehyde (Sigma Chemical Co.) per ml of glacial acetic acid. The tubes were allowed to stand at room temperature for 10 min, and then 2ml of concentrated sulfuric acid were pipetted slowly down the inside of each tube. The contents of each tube were immediately mixed thoroughly on the Vortex mixer as described previously. After standing at room temperature for additional 10 min, the A550 was read against a reagent blank. The A550 was compared with a standard curve to determine the concentration of cholesterol. Results were expressed as micrograms of cholesterol per milliliter. The same procedure was used for the standard curve, except the following amounts of cholesterol (99% standard for chromatography; Sigma) were assayed in place of the samples: 0, 10, 20, 30, 40, and 50, ug. The A550 values were plotted against micrograms of cholesterol.

RESULTS AND DISCUSSION

Results of the screening for the ability to deconjugate bile salts were obtained from 53 strains of the genera *Lactobacillus*. An overview of the presence of BSH in Lactobacillus strains is given on Table 1. Under the name *Lactobacillus*, a heterogenous group of lactic acid bacteria is assimilated, which can be subdivided according to phylogenetic position. They live in a wide variety of environments like the intestines, milk products, fermented plant material, vagina, and mouth. From all *Lactobacillus* strains tested 32% were BSH positive (17 out of 53). All of the strains that belong to intestinal species as *L. gasseri* and *L. acidophilus* have BSH activity. Species with various habitats, such as *L. plantarum*, *L. casei* and *L. rhamnosus* have less BSH activity which is concentrated mainly in the strains with intestinal origin. Strains of typical dairy species like *L. bulgaricus* and *L. helveticus* have either no BSH activity or a very low incidence. All these findings suggest that BSH activity in lactic acid bacteria is strongly correlated with natural habitat. Strains with BSH activity come from an intestinal environment in which they are exposed to bile salts. However, the fact that not all strains with intestinal origin have BSH shows that some bacteria without BSH can survive in this environment.

The results from BSH assay for different Lactobacillus strains demonstrated the highest BSH are given on Table 2. BSH activity is expressed as the average amount (in micromoles) of cholic acid (CA) formed per 10^{10} CFU per min during the 24-h incubation period. Results are shown as mean ± standard deviations (n = 3).

Table 1. Bile salt hydrolase (BSH) activity in various species of *Lactobacillus*

Species	number of strains	intestinal origin	from other sources	BSH positive from feces	BSH positive others
L. delbrueckii subsp. bulgaricus	10	0	10	0	0
L. helveticus	8	0	8	0	0
L. casei	7	3	4	2	1
L. rhamnosus	7	4	3	3	1
L. plantarum	11	5	6	3	1
L. acidophilus	2	1	1	1	1
L. gasseri	9	9	0	9	0
L. brevis	2	1	1	1	1
L. fermentum	5	4	1	3	0

Table 2. BSH activity of the *Lactobacillus* strains with highest activity

Strain	Amount of CA, µmol	Standard deviation
L. brevis 5/11	37.7	± 2.0
L. fermentum 11/2	30.9	± 2.3
L. gasseri 4/13	27.9	± 3.2
L. gasseri 3/11	26.0	± 2.0
L. acidophilus 21	25.5	± 1.9
L. fermentum 9/2	23.9	± 3.4
L. gasseri 3/11	17.0	± 3.2
L. rhamnosus 3/1	15.1	± 1.1
L. fermentum 7/1	12.3	± 0.9
L. plantarum F12	9.5	± 0.9

Conjugated bile salt analysis of the culture at regular time intervals showed a marked decrease in the concentration of GCA and TCA during the early stationary phase of growth, in this case after 24 h of incubation.

The amount of bile in the fermentation medium influenced both the growth of the strain and the amount of cholesterol assimilated. Indeed, as the amount of oxgall in the medium increased, the growth rate of the strain decreased significantly ($P < 0.05$). The percentage of cholesterol reduction in the culture medium seemed to be constant and small at oxgall concentrations lower than 0.5%. The amount of cholesterol measured in the supernatant fluids in the medium with 2% oxgall was significantly lower than for the other oxgall concentrations tested ($P < 0.001$). This resulted in a significantly higher percentage of cholesterol reduction.

Results from the screening of cultures for cholesterol uptake are shown in Table 3. Cholesterol concentrations in the cell suspensions (absorbed cholesterol) ranged from 4.1 µg/ml for strain *L. bulgaricus* b5 to 79.6 µg/ml for strain *L. brevis* 5/11. Based on the concentrations of cholesterol in the control and spent broths, minimal amounts of cholesterol were removed from the broth by cultures of strains *L. bulgaricus* and *L. helveticus*. The strains *L. brevis* 5/11, *L. gasseri* 4/13, and *L. acidophilus* 21 caused the best reductions in the amount of cholesterol in the broth portion during the 24-h growth period. Some strains LAB were much more active than others with regard to the uptake of cholesterol.

Table 3. Assimilation of cholesterol by *Lactobacillus* strains during anaerobic growth in MRS broth containing 100 mg/ l cholesterol and oxgall

Strain	Cholesterol in the spent broth, µg/ ml	Cholesterol in the cell suspension, µg/ ml
L. brevis 5/11	21.0	79.6
L. fermentum 11/2	30.2	69.0
L. gasseri 4/13	27.5	72.1
L. gasseri 3/11	39.1	61.2
L. acidophilus 21	30.1	69.2
L. fermentum 9/2	44.8	55.0
L. gasseri 3/11	46.9	53.2
L. rhamnosus 3/1	58.8	41.2
L. fermentum 7/1	62.8	37.5
L. plantarum F12	62.4	37.5

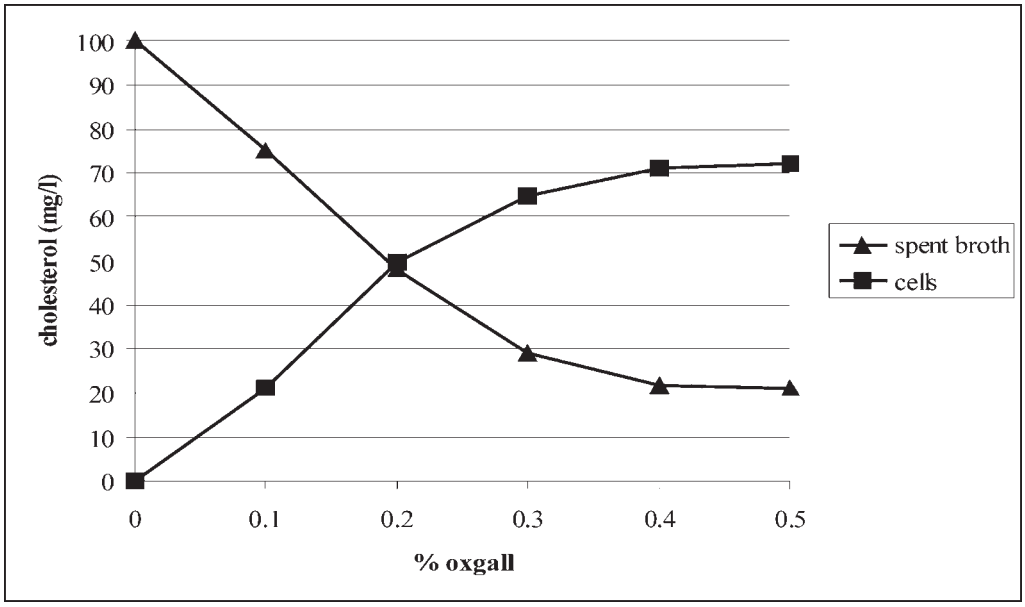

Fig. 1. Influence of oxgall on the uptake of cholesterol by L. gasseri 4/13.

For the probiotic strain *L. gasseri* 4/13, previously demonstrated excellent adhesive properties to epithelial cells and survivability in intestinal tract, the correlation between the cholesterol uptake and oxgall concentration was evaluated. The results are presented on Figure 1.

When the strain *L. gasseri* 4/13 was grown anaerobically, the amount in the broth decreased, whereas the amount in the cells increased. The amount of bile in the growth medium influenced the assimilation of cholesterol by *L. gasseri* 4/13 (Fig. 1). The amounts of cholesterol in the spent broth and in the resuspended cells after a 24-h growth period are plotted against the oxgall concentration (each value is the average from three trials). As the amount of oxgall was increased in the growth medium, the amount of cholesterol detected in the spent broth decreased. This was associated with increases in the amounts of cholesterol in the resuspended cells. Cholesterol was not removed from the broth during the growth of the lactobacilli, nor did appreciable amounts appear in the cells until more than 0.1% oxgall was present in the growth medium. The amount

of uptake appeared to level off at oxgall concentrations greater than 0.4%.

CONCLUSIONS

In summary, this study provided evidence for the considerable cholesterol-lowering properties of some *Lactobacillus* ssp. strains with intestinal origin. There was a correlation between BSH activity of the strains and the ability to reduce the cholesterol content in-vitro. High level BSH activity is detected in *Lactobacillus* strains with intestinal origin in contrast to LAB strains from other sources. The probiotic strain *L. gasseri* 4/13, which showed survivability in intestinal tract and excellent adhesive properties to epithelium, demonstrated cholesterol-lowering properties, too.

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ХОЛЕСТЕРОЛ–РЕДУЦИРАЩИ СВОЙСТВА НА ЛАКТОБАЦИЛИ

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Резюме

Целта на настоящото изследване е да се изпитат холестерол-редуциращите свойства на щамове лактобацили. Голям брой щамове млечнокисели бактерии с интестинален произход бяха колекционирани от фекалии на клинично здрави доброволци и бяха изпитани за редуциране нивото на холестерола в лабораторни условия. Редуциране нивото на холестерола се наблюдава, когато щамовете лактобацили се развиват в присъствие на жлъчни соли и е в зависимост от жлъчно-солевия толеранс. Освен общата холестерол-редуцираща активност, бе изследвана и хидролизиращата активност спрямо жлъчните соли. Щамът с най-силна холестерол-редуцираща активност е L. brevis 5/11, който демонстрира както значимо понижаване нивото на холестерола, така и висока хидролизиращата активност спрямо жлъчните соли. Пробиотичният щам L. gasseri 4/13, който притежава значими адхезивни свойства, показва и висок холестерол-редуциращ ефект. Като резултат от това изследване бяха селектирани няколко щама с висок холестерол-редуциращ ефект и един от тях (L. gasseri 4/13), притежаващ и други пробиотични свойства, бе избран за бъдещи клинични изпитания.

INNOVATIVE MICROBIOLOGICAL FUEL CELLS AS GREEN POWER SOURCES

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Abstract

Electricity generation from renewable sources and effective waste treatment are key challenges for the sustainable development. Microbiological (Microbial or Bio-) Fuel Cells provide an elegant solution by linking both tasks. Biofuel cells, which can directly generate electricity from biodegradable substances, have rapidly gained increasing research attention. Widely available fuel sources and moderate operational conditions make them promising in renewable energy generation, wastewater treatment, as power sources for remote or implantable devices, etc. The aim of this project is a verification of possibilities for conversion of bioenergy into electricity by development and testing of biofuel cells, utilizing whole microorganisms as biocatalysts. The implementation of the project is based on interdisciplinary investigations, requiring cooperation of specialists in biochemistry, microbiology, electrochemistry and engineering. Complex microbiological and biochemical analyses are carried out for preliminary selection of proper microorganisms (prokaryotes and eukaryotes), which electrochemical behavior is further analyzed in biofuel cells. Various traditionally used and novel synthesized materials are examined as potential electrodes. The selection of appropriate mediator is based on cytotoxicity tests as well as on electrochemical measurements. An optimization of the microorganisms-mediator-electrode system as well as the whole biofuel cell performance in regards to improvement of its output characteristics (cell voltage, generated current and power density) is also carried out. The expected results from realization of the project are: development of a biofuel cell, based on the use of whole microorganisms; development of a set of methods for testing separate components and the whole fuel cell system; construction of a lab stand for testing and demonstrations; interdisciplinary training of students and specialists; transfer of knowledge and know-how to specialized enterprises. In more global aspect, the expected future economical and ecological effect is related both to green electricity production and waste water treatment technologies

INTRODUCTION

Every year the global energy demand increases. While petroleum products currently supply most of this demand, the increasing difficulty of sustainable supply and the associated problems with the pollution and global warming are acting as a major impetus for searching of alternative renewable energy technologies. Fuel cells offer a possible solution of this problem. Although conventional fuel cells generally use either hydrogen or methanol, some cells have been developed which run on other fuels such as hydrocarbons [1, 2]. Hydrogen is gaseous and this gives rise to storage and transportation problems. Moreover, many of the alternative fuels that could be used within fuel cells are still dependent on petroleum products and therefore offer few advantages. It is clear that approaches by which common waste materials and the chemical energy locked within them could be utilized would offer many benefits. For example,

if a molecule of glucose is oxidized completely to CO_2 (usually with atmospheric O_2 providing the oxidant), there are 24 electrons available for current generation. Furthermore, if the glucose is produced by photosynthesis, then the process is carbon neutral, which clearly offers environmental benefits.

More recently, there has been an upsurge in research in biofuel cells. Factors driving this research comprise the increasing problems of supply and pollution that concern the use of fossil fuels and the special medical application offered by the design of small devices implantable within the body, such as pacemakers. In many situations an ideal power supply would be a fuel cell that is capable of running on the compounds such as sugars found *in vivo*.

Biofuel cells represent an innovative technology for simultaneous electricity generation and organic waste purification. The principle is based on the direct conversion of the biochemical energy of living cells into electrical energy. Utilization of entire microorganisms oxidizing the biodegradable organic matter, the operation at ambient temperatures, and the use of neutral electrolytes and inexpensive carbon-type electrodes are the biggest advantages of biofuel cells over chemical fuel cells.

There are a number of reviews on bioelectrochemistry that include some coverage of work on biofuel cells including those by Aston and Turner [3], Bennetto [4], Katz et al. [5], Barton et al. [6], Shukla et al. [7] and Rabaey and Verstraete [8]. In 2006, the journal "Biosensors and Bioelectronics" published a special issue devoted exclusively to biofuel cells, including several research papers and an extensive review of the field [9]. Based on accomplished results, numerous important trends for further R&D activities are pointed out in this review. Parts of them are objectives of the current project.

Although lots of experimental results have been recently reported in the scientific literature, there are no clear methods and systemized criteria for selection of microorganisms and conditions for their effective use concerning energy production into fuel cells, yet. By realization of the present project, the working team is aiming at development of objective criteria and set of methods for selection of electrogenic microorganisms and their implementation in microbial fuel cells (MFCs). A key point for the improvement of MFC efficiency is connected with the clarifying of the electron transfer mechanism, which is also an objective of the project team. Thus, implementation of the project is in tune with the modern trends of the R&D in the field of innovative biofuel cell technology.

PROJECT TEAM

The research group working in the field of biofuel cells in Bulgaria has been established in the last 3 years by the leader and members of the project team. In spite of the short term, comparable results with those reported from leading teams in the field have been obtained. Innovative ideas and results, concerning the use of eukariotic microorganisms as biocatalysts in biofuel cell have been presented [10].

The R&D in the field of biofuel cells is based on interdisciplinary investigations,requiring cooperation of specialists in biochemistry, microbiology, electrochemistry and engineering. The work team of the current project is composed of scientists from four institutions, possessing serious theoretical and practical experience in the abovementioned fields.

The project leader Assoc. Prof. Dr. Mario Mitov has gathered experience as a leader, coordinator and contractor in project implementation on an institutional, national and international level. Along with coordination of activities between separate research units Assoc. Prof. Mitov is leading the research work of the group from the Chemistry Department at the South-West University, Blagoevgrad. The long-term experience in characterization of novel nanostructured materials, hydrogen-absorbing ones, batteries and fuel cells, accompanied by the received international awards for fuel cells demonstration training models, helped in organizing and gathering the young research team for this project.

The second research unit in the project is from the Biochemistry and Microbiology Department of the University of Plovdiv with team leader Assist. Prof. Dr.rer.nat. Yolina Hubenova. The rich research experience and laboratory biochemical practice of Dr. Hubenova includes a number of specializations abroad, the latest of

which was in the Rhein University Friedrich-Wilhelm – Bonn, Germany (5 years). The team members have accumulated extensive experience in yeast investigations, bacterial cultures, in composting of waste plant biomass, isolation of enthomopatogenic strains, determination of antimicrobial and fungicide activity, prebiotic activity, etc.

The "Electrochemistry of Biocatalysts and Metal-air Systems" Department at the Institute of Electrochemistry and Energy Systems – BAS, Sofia, led by Assoc. Prof. Dr. Anastasia Kaisheva has piled up more than 30 years of broad practical experience in the metal-air systems field. A series of Zn-air batteries with alkaline electrolyte have been constructed, developed and introduced to practice by this research unit. Air gas-diffusion electrodes for operation in salt electrolytes, Al-air batteries, Mg-air cells and enzyme electrodes for application in biosensors have also been developed in the department.

The last unit is the team from the Institute of Physical Chemistry "Acad. Rostislav Kaishev"– BAS, Sofia, led by Assoc. Prof. Dr. Rashko Rashkov. This research group has quite serious experience in the electrochemical deposition of multicomponent nanostructured materials upon various supports and their characterization by electrochemical and other methods in view of use as catalysts and electrocatalysts of various reactions. Successful cooperation of the team with foreign research institutes is expected to support the implementation of the project.

PROJECT OBJECTIVES

The theme of the project is consistent with the strategic aims and priorities for development of new competitive scientific products and technologies, targeted at solving global energy and ecological problems. The leading tendency is directed to substitution of the traditional hydrocarbon fuels with alternative renewable energy sources. Significant part of the research is orientated to development of high-effective ecologically friendly energy sources. In this connection, new material and catalyst systems as well as new methods for analysis and electrochemical studies are developed.

The aim of the project is verification of possibilities for conversion of bioenergy into electricity by development and testing of biofuel cells, utilizing whole microorganisms as biocatalysts.

To achieve the project goal, the following main tasks are going to be fulfilled:

***Task 1*. Selection of appropriate cell culture based on genetic, biochemical, microbiological, cytological and enzymatic criteria.**

Our previous investigations [10, 11] show that there is a connection between the log-phase of growth of cell culture, the content of the nutritious medium and the electrochemical characteristics of the biofuel cell. These findings determine the necessity for developing and application of a set of methods for preliminary selection, based on the following criteria:

- Genetic – selection of special microorganism strains, which possess an increased expression of main enzymes from the glycolysis and from different stages of the carbohydrate metabolic pathways;
- Cytological – include cell fractionation aimed to prove the origin of electrons, which reach the anode as well as analysis for proving the fractionation rate;
- Biochemical – biochemical analysis of main biopolymer groups, characterizing the level of the metabolism within time – carbohydrates, phosphates, proteins, lipids for each investigated strain;
- Microbiological – selection of cell strains, cell cultivation in media compatible with electrochemical processes, viability and preservation of the cultures;
- Cyto-biochemical – determination of cytotoxicity and inhibition degree of various mediators on different cell cultures;
- Enzymological – determination of specific activity of the leading enzymes.

By application of the described criteria and corresponding analysis, appropriate prokaryote and eukaryote microorganisms will be selected and the optimal conditions for their cultivation and use as a biological microreactor into fuel cell will be defined.

***Task 2*. Investigation of the selected cell strains behaviour into experimental electrochemical cell.**

Biofuel cells are sophisticated multicomponent systems, in which besides a determined

biocomponent – whole living microorganisms or isolated enzymes, there are specific functional components such as electrodes, electrolytes, separators, etc., indispensable for implementation of the entire electrochemical process.

Microorganisms offer some major advantages over enzymes in that they can catalyze a more thorough oxidation of many biofuels and can be less susceptible to poisoning and loss of activity under normal operating conditions making them a popular choice for use in biofuel cells. Several reviews on this subject have been published [7,8]. Furthermore the whole living cells, especially microorganisms, are cheaper than isolated enzymes.

Frequently, however, the optimal conditions for cell growth and development do not correspond to the requirements for effective flow of electrochemical process as well as for realization of a fuel cell with exact exploitation characteristics.

One major drawback, however, is that it can be extremely difficult to utilize the electrons generated by the reaction occurring inside the cell. One possible solution is via the use of mediators; however, the compounds chosen for this purpose must satisfy a number of criteria. Firstly they must be capable of being transported across the cell membranes of the microorganisms and they must also be non-toxic.

The potential for exploiting the full gamut of reactions that a microorganism is capable of was realized by work, which utilized glucose and *E. Coli* and provided an electrical yield close to the theoretical maximum of 24 faradays per mole of glucose [12]. Other microbial fuel cells were found to be capable of generating currents of up to 2A [4].

We have recently proved the possibility for introducing eukaryotes (*Saccharomyces cerevisiae* and *Candida melibiosica* yeast cells) instead of prokaryotes as a microreactor into biofuel cells [10, 11].

The choice of the most appropriate cultures and microorganisms strains for realization of the final aim of the project includes investigations joined to verification of the behaviour of the selected strains (on the base of the indicated in task 1 criteria) under conditions of the electrochemical system by minimal variation of the rest of the components.

Appropriate experimental electrochemical cell, considered with the required specific conditions for cell strains cultivation will be selected.

***Task 3*. Examination of different electrodes, electrolytes, mediators aimed to optimize the effectiveness of the system microorganisms–mediator–electrode.**

A key role for realization of high effective biofuel cell falls on the optimization of the working system biocomponent-mediator-electrode.

Taking into account that the generated by biofuel cells current densities are relatively small (in the range of $\mu A/cm^2$) the used electrodes have to be with well-developed surface and high conductivity. In already published investigations [9,13], various carbon materials – carbon fibres, cloth and others are used. By different modifications of the electrodes in some cases an improvement of the electrochemical characteristics – cell voltage, current density, power [9] has been achieved.

For implementation of this part of the task, various in content and morphology nanomodified carbon electrodes will be obtained and investigated in view of improving the electron transfer kinetics, and as a result – the whole electrochemical behaviour of the anode half cell. Based on the comparative analysis of the obtained results an optimal anode for the biofuel cell will be selected.

For an effective electron transfer from the living cells to the anode the choice of an appropriate mediator is of crucial importance. The mediator selection is based on the following requirements: a potential just different enough from the potential of that of a microorganism to facilitate electron transfer while maintaining a high (electrochemical) cell potential, a high diffusion coefficient in the supporting electrolyte and through the cell membrane, rapid electron transfer from living organisms to the electrode, suitability for repeated redox cycles, non-cytotoxicity and good profiles of absorption/adsorption/desorption processes at the organism, electrode and other fuel cell surfaces so that it remains in the solution and available for the process.

The choice of an optimal mediator will be performed by examination of already described in the literature substances [8, 9], as well as of new ones. After analysis for non-cytotoxicity to the living cells, the influence of the respective mediator on the electrochemical characteristics of the system will be traced out.

In order to decrease the ohmic losses, respectively overpotential, it is necessarily also to optimize the anolyte constituent part. The varying of conditions (supporting electrolyte, pH, and temperature) requires additional investigations about the selected microorganisms conduct in a medium different from those for optimal cell culturing.

***Task 4*. Development of different constructions of microbiological fuel cell and selection of the optimal one.**

A fuel cell represents a device, which directly transforms energy, and as a consequence it can theoretically produce energy as long as the electrodes are supplied with fuel and oxidant. In reality some factors such as corrosion, improper functioning, the life cycle of the system separate components and others limit the life cycle of the fuel cells. In this regard, besides the precise selection of the electrode materials, the construction of the fuel cell significantly influences its electrochemical characteristics and general operation. This applies specifically for systems operating with liquid electrolyte. The existence of two half-cells that separately work steadily is not always enough to state that a combination of them will lead to the construction of an electrochemical power source with sound characteristics. An "incompatibility" between the two electrodes has been observed in some systems. It is expressed by migration of active substances from one of the electrodes to the other through the electrolyte and the separator. Another frequently encountered problem in fuel cells is the "cross-over" of fuel from the anode to the cathode area, which hampers the efficiency of the system. The geometry of the surface not only of the electrodes, but also of the cell itself could have a significant impact upon the operation of the fuel cell.

Lots of the efforts in fuel cell technology development are directed to reducing the size of separate components and to making constructions lighter. Extensive work is done to elaborate and improve the structure of the electrodes and the electrolyte phase, together with the construction of the system. A serious boost in this direction comes from achievements in the development of polymer materials [14, 15].

The peculiarities connected to the work with biocomponents require very careful selection of all materials from which separate components of the cell will be prepared. Contemporary engineer plastics offer a wide range of possibilities. The devices utilizing them display high quality, good dimensional stability, good mechanical strength, perfect ware resilience, sound current isolation properties, etc. Transparent polyester and primarily Plexiglas materials are highly appropriate for construction of commercial or demonstration fuel cells.

The general construction will be elaborated subsequent to the selection of materials. A laboratory biofuel cell concordant to the specific work conditions (pH, temperature, etc.) of the biocomponents will be designed. A good solution will be if the construction enables operation in a jet-injection mode. Thus constant flowing of electrolyte, fuel and electrochemical processes products will be achieved. This system allows also maintaining of a relatively constant pH value in the electrolyte.

***Task 5*. Optimization of the biofuel cell operational characteristics.**

Optimization of microbiological fuel cells is important in order to extract the maximum performance from these systems. The performed investigations indicate that the power output of biofuel cells can be affected by a number of different factors [16].

Both in the anode and cathode compartments losses occur due to overpotentials. Although small current densities flow through the electrode surface, these losses need consideration. To decrease the activation overpotential, catalysts need to be added to the electrode, or a suitable mediator is needed to transfer the electrons from the cathode to oxygen. Generally, Pt at contents up to 45% w/w is used as an electrocatalyst [17]. The use of Pt enables an open air cathode, decreasing aeration cost, but

the investment cost is considerable. Another option is adding $K_3[Fe(CN)_6]$ to the liquid catholyte [18], which is aerated. Less expensive catalysts are currently being developed, which may render the open air cathodes more feasible in terms of capex.

Another factor that lowers efficiency is diffusion of oxygen into the anode chamber. Replacing the separator membrane with a salt bridge was found to have a detrimental effect, probably due to an increase in the internal cell resistance.

The selection of a membrane separating anode and cathode represents a choice between two opposing requirements:

- High selectivity: the higher the selectivity for protons, the better the biofuel cell will operate and the lower the resistance of the membrane.
- High stability: membranes need to be robust in a colloidal and nutrient rich environment, which the bacterial suspension generally is.

Nafion™ has been widely used as proton exchange membrane (PEM) for fuel cells and MFCs [19-21], and has the large advantage of being very selective for protons. However, this membrane contains sulfonic acid groups that are binding with ammonia present in the bacterial solution. Hence, at this moment, this membrane type scores high for selectivity but low for stability.

A second approach is the use of a more general cation exchange membrane (CEM), such as Ultrex™. This type of membrane has a larger resistance and is less selective but generally shows larger stability. These membranes have been reported to perform adequately for over three months [22].

In accordance with the pointed considerations, various commercial and modified proton-exchanging membranes will be tested as potential separators for effective dividing of anode from cathode compartment.

The performance of biofuel cells has been shown to be affected by magnetic fields. Immobilized on the anode surface enzymes such as glucose oxidase or lactose dehydrogenase as well as cathodes with cytochrome c/cytochrome oxidase were analysed and the influence of the magnetic field on the activity of biological component was proven [23]. A pronounced enhancement of the bio-electrocatalytic process is observed for both anodic systems, although a far smaller effect was observed for the cathode system. This enhancement was shown to be due to a magneto-hydrodynamic effect, which facilitates enhanced mass transport.

Similar studies carried out with redox reaction Fe^{3+}/Fe^{2+} interdigitated dot electrodes has shown that applying of magnetic field enhances the current from 45 to 57 μA [24].

For a good operation of a microbiological fuel cell, both protons and electrons need to migrate from the anode to the cathode at the highest possible rate. Diffusion is not sufficient to reach acceptable levels of current and cell potential. Moreover, lacking proton transport could decrease the pH of the anode to undesired levels for bacteria. Therefore, turbulent conditions need to be introduced to the anode and the cathode. Stirring or circulation of the electrolyte can be applied to solve this problem. Both methods, however, cost energy and deplete the overall efficiency of the microbiological fuel cell system.

At the final stage of optimization the influence of the hydrodynamic conditions on the operational characteristics of the developed biofuel cell will also be studied.

Problems of lifetime, stability and power density also need to be addressed, although possible benefits of this technology are likely to drive continuing research. We need to improve our knowledge of biocatalysis, electron processes at surfaces, biological and other material stability to realize this vision.

***Task 6*. Development of a demonstration model of a microbiological fuel cell for training and popularization of the innovative technology for green energy production.**

Aiming at popularization of fuel cell technology, different research teams work on development of various fuel cell systems, by which the potentials of these novel power sources for mobile as well as stationary applications are demonstrated. One of the major functions of similar systems is their use in different educational programs with a view to make the young generation familiar with the operational principles and advantages of fuel cell technology.

Considering the hydrogen economy and fuel cells as the most perspective alternative of currently used energy sources and taking into account the prognosis and trends for their wider use in the next 5 to 10 years, the leading countries make large investments in the development of various demonstration prototypes as well as realization of pilot projects by using them.

Based on the achieved results, development of a demonstration model of biofuel cell is planned. The model will be used for training of students in the two partner universities as well as for further research activities. The performance of this demo model on specialized exhibitions will contribute to wider popularization of this innovative technology for green energy production with a view of its implementation into practice.

EXPECTED RESULTS AND IMPACT

The expected major results from implementation of this project are as follows:

• Developed, systematized methodology for selection of appropriate cell strains in view of their application in microbiological fuel cell.

• Developed experimental electrochemical cell for optimization of the microorganisms-mediator-electrode system.

• Selected biocomponent-mediator-electrode system for application in microbiological fuel cell.

• Selected construction for a laboratory biofuel cell.

• Chosen appropriate components (electrodes, separators, electrolytes, mediators, biocomponents) for realization of a demonstration biofuel cell.

• Developed demonstration model of a microbiological fuel cell.

The developed end product will be used for popularization of the innovative technology of biofuel cells by various demonstration forms, as well as for training of students, graduate students and PhD ones.

Besides the scientific and practical results benefits in some other aspects are also expected:

• Forming of beneficial environment for young scientists

Parallel with solving significant scientific tasks, participation of young researchers in the project is one of the major priorities. Besides training and introducing young people (students, postgraduate and PhD ones) to current problems and actual state of the investigations, they will also master the main methods in an interdisciplinary applied sphere, which will widen up their scientific horizons and will boost the finding of innovative technical solutions and projects. Parallel to this the material base of the participating in the overall project units will be renovated and modernized. Mastering of specialized technical equipment will enhance the quality of both current and future investigations and will raise the level of our laboratories even closer to European standards and criteria for development of modern labs in Bulgaria. In order to maintain the high scientific level of the team, exchange of know-how among project participants is planned. It includes specializations and joint work not only among the units (various specialized labs of the participating institutions, such as microbiological, biochemical, electrochemical and physicochemical ones), but also with foreign labs occupying leading positions in our thematic field. In order to keep track of the actual state-of-the-art of the scientific theme participation of our representatives in Bulgarian and international scientific forums is planned. It will provide not only beneficial interdisciplinary environment for discussions, but we will be able to present our new scientific accomplishments.

• Potential for future development of the research team implementing the project

Implementation of the project will significantly increase the qualification of both the research team as a whole and every individual participant. Interdisciplinary character of the investigations will undoubtedly enrich the theoretical knowledge and practical skills of all participants in various scientific spheres. Periodic discussions and micro-symposia which will accompany the solving of the tasks will lead to generation of ideas based on know-how in many scientific fields. These ideas will serve as grounds for future scientific investigations and participation of the team in oncoming national and international projects.

The large volume of the research work requires participation of highly specialized young researchers, which will provide the opportunity

for preparation of PhD theses in all major research fields of our work sub-groups. What is more, the interdisciplinarity of the project will improve the quality of preparation of new PhD theses as well as the investigations intensity and distribution of scientific achievements and will provide opportunities for new habilitation works of the post-docs participating in the project. The fruitful collaboration between our highly specialized departments opens up a huge potential for knowledge transfer, which will lead to more integrated application of the results obtained by each department. Integration of the achievements for the separate elements of the biofuel cell will result in a better end product with increased due to the detailed analyses in all scientific aspects reliability. Solving of the current problems gives opportunity for their practical application and achievement of new scientific ideas and developments. With participation in future joint projects not only the interdisciplinarity of the work team will grow but relations to business and economic organizations interested in the introduction of new technologies in their products and/or services will be achieved.

• Potential for knowledge transfer and results applicability

Timely incorporation of the obtained results in specialized lecture courses will make publicly known the globally sensitive issues related to new energy sources. We also envisage amelioration and updating of the education material, including the development of new lab exercises in both universities – in Blagoevgrad and in Plovdiv, which will encompass the newest achievements from the project realization. This will renovate and update the education process. In a larger context it will lead to attracting interest of future specialists to the specific project subjects and will increase their ecological awareness.

Our scientific achievements will be distributed via development of a demonstration prototype and a stand for demonstrations and lab tests. Participation of engineers in the project will result in new developments including construction of fuel cell models with an impact to future industrial application.

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ИНОВАТИВНИ МИКРОБИОЛОГИЧНИ ГОРИВНИ ЕЛЕМЕНТИ КАТО ИЗТОЧНИЦИ НА ЕКОЛОГИЧНО ЧИСТА ЕНЕРГИЯ

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Резюме

Производството на електрическа енергия от възобновими източници и ефективното очистване на отпадъци са две от водещите направления за осигуряване на устойчиво развитие. Микробиологичните (Микробиални или Био-) горивни елементи предлагат елегантно решение и на двата проблема. Биогоривните елементи, които са директни преобразователи на химичната енергия на биоразградими субстрати в електрическа енергия, привличат нарастващо внимание сред изследователите. Широко достъпните суровини и умерените операционни условия ги нареждат сред перспективните технологии за генериране на енергия от възобновими източници, пречистване на отпадни води, като токозахранващи източници на устройства за имплантиране или такива, работещи в отдалечени райони и др. Целта на настоящия проект е изследване на възможности за превръщане на биоенергия в електричество чрез разработване и тестване на биогоривни елементи, използващи цели микроорганизми като биокатализатори. Изпълнението на проекта се базира на интердисциплинарни изследвания, изискващи кооперирането на специалисти от областта на биохимията, микробиологията, електрохимията и инженерните науки. Чрез комплексен микробиологичен и биохимичен анализ се прави предварителен подбор на подходящи микроорганизми (прокариоти и еукариоти), чието електрохимично поведение се анализира в биогоривни елементи. Различни традиционно използвани и нови материали се изследват като потенциални електроди. Изборът на подходящи медиатори се основава на цитотоксични тестове, както и на електрохимични измервания. Ще бъде извършена и оптимизация на системата микроорганизми-медиатор-електрод, както и на цялостния биогоривен елемент с оглед подобряване на неговите работни характеристики (напрежение на клетката, генерирана плътност на тока и мощността). Очакваните резултати от реализирането на проекта са: разработване на биогоривен елемент, основан на използване на цели микроорганизми; разработване на цялостна методология за тестване на отделните компоненти и горивния елемент като цяло; конструиране на лабораторен стенд за изпитания и демонстрации; интердисциплинарно обучение на студенти и специалисти; трансфер на знания и ноу-хау към специализирани предприятия. В по-глобален аспект, очакваният икономически и екологичен ефект за в бъдеще се отнася към технологиите за екологосъобразно производство на електроенергия и пречистване на отпадни води.

COMPUTER MODELING OF ELECTROMAGNETIC FIELDS IN BIOLOGICAL STRUCTURES

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Abstract

Computer modeling of electromagnetic fields distributed in biological structures is of paramount importance for investigations of processes and phenomena during medical diagnosis and therapy. Here a complete process methodology of electromagnetic model building is presented. The process contains a method for automatic 3D model building, electromagnetic properties measurement system and electromagnetic field computational algorithm based on Finite element method (FEM). Recent achievements of the FEM give possibilities to model fully three-dimensional, nonlinear, inhomogeneous, or anisotropy multi-tissue, and multi-joint systems of biological structures. Implementation of the developed methodology to investigation of the electromagnetic field distributions in human body tissues during magnetic stimulation demonstrates capabilities to solve such complex problems.

INTRODUCTION

Interaction of external electromagnetic fields with the human body has gained increasing attention throughout the past years. Electromagnetic phenomena are used day by day as examination and therapeutic tools in various medical applications. On the other hand, owing to the increasing number of electromagnetic and wireless devices in daily surroundings possible adverse health effects of electromagnetic fields could appear.

Up-to-date development of electromagnetic devices, bioinformatics, biomaterials, measurement systems, information technologies, etc. give possibilities for optimal investigations of fields, processes and phenomena in biological structures during their interactions with electromagnetic devices in order to realize precise medical diagnosis and therapy.

Modeling and optimization of fields and processes during energy interaction between electromagnetic device with nonlinear, inhomogeneous and complex biological structures lead to producing of high quality and energy safe medical electromagnetic devices and to realizing of precise medical diagnosis and therapy[1-6].

In this paper the interaction of low-frequency electromagnetic fields with different organs and parts of the human body is analyzed using field modeling, simulations and visualization. Computer modeling of electromagnetic fields distributed in biological tissues is of paramount importance for investigations of processes and phenomena during medical diagnosis and therapy. Finite element method (FEM) is a powerful numerical method capable to solve such complex problems, taking into account all characteristics and special features. Recent achievements of the FEM give possibilities to model fully three-dimensional, nonlinear, inhomogeneous, or anisotropy multi-tissue, and multi-joint systems of biological structures.

In order to achieve accurate field distribution results realistic 3D geometry models are developed. The data from modern medical imaging techniques, such as CT, MRI, Ultrasound, have been used for building of precise three-dimentional high-resolition anatomical model of the human body. Recently different techniques have been utilized to convert automatically 3D image data into 3D FEM models or into numerical meshes suitable for FEM analysis [7-10]. Due to the complicated structure of the human body, consisting of organs with varying dimensions and geometrically complicated shapes, all exhibiting different electrical properties, the major task from the

simulations point of view is to achieve a very fine spatial resolution in the computations. To solve this problem we developed a method for automatic 3D model building of various parts and organs of the human body suitable for FEM modeling. The geometry models are built by image slices data acquired by CT. Finally, the geometry models present the human anatomy as mesh structures. Mesh is built satisfying the specific FEM criteria for achieving good solution accuracy.

For computer modeling of electromagnetic fields distributions in human body the electromagnetic properties of live tissue have to be incorporated into 3D geometrical models of human organs. Bioimpedance spectroscopy labels the measurement of the complex-valued resistance of biological tissue at various frequencies. It is used as a low-cost, easy to use and non-invasive approach to estimate various body parameters. Because of the capacitive properties of the cell and other body membranes, the measured body impedance is higher at lower frequencies [11-18]. For precise determination of electromagnetic properties a concept of computerized measurement system is proposed. Specific electromagnetic material properties are introduced for each tissue of the developed model.

FEM formulation using magnetic vector potential and scalar electric potential (A-V,A) is applied for modeling of electromagnetic field distributions in generated 3D models of human organs. The analysis is made by ANSYS software [19].

The computer modeling of electromagnetic processes, fields and phenomena is implemented for field distribution investigation during magnetic stimulation [21-24].

3D MODEL BUILDING

Method for 3D model building of the human parts and organs is developed. The structure of the method is shown in Fig. 1. As an input a sequence of 2D slices acquired by visual diagnostic equipment (ultrasound, CT or MRI) are used. Application of image processing techniques essentially enhances the image properties. The basic steps of 3D model building method are data acquisition, segmentation, element decomposition, volume generation and property association. Acquired 2D slices are collected in a 3D image stack. This stack is used for generation of 3D voxel Data Base (DB). All image and data processing is done over that 3D voxel DB.

Segmentation of 3D image DB is the process

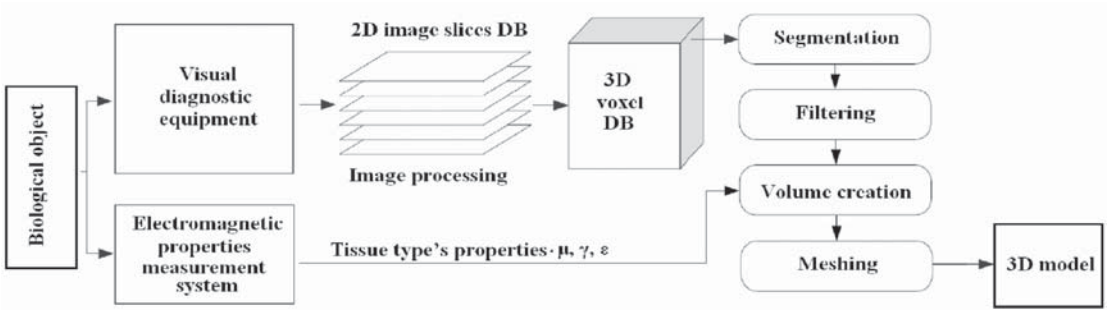


Fig. 1. Structure of the method for model building

of tissues and their boundaries identifications. This process is quite time-consuming, especially when large data sets are used. In such cases automation of segmentation procedures is required. Here organs segmentation is made semi-automatically by recognizing their contours in the 3D voxel DB. Tissue segmentation is made by voxel intensity histogram shown in Fig. 2. Each tissue type is associated by its intensity level depending on the used diagnostic equipment.

Segmentation filtering procedure is applied for tissue separation. Intensity histogram, shown in Fig. 2, is used for separation where uncommon voxels are excluded from organ database. As output of that stage voxel databases are separated for each tissue type. After that each voxel is decomposed in five tetrahedrons as shown in Fig. 4. These tetrahedrons form the initial mesh representing the solid anatomy geometry. For

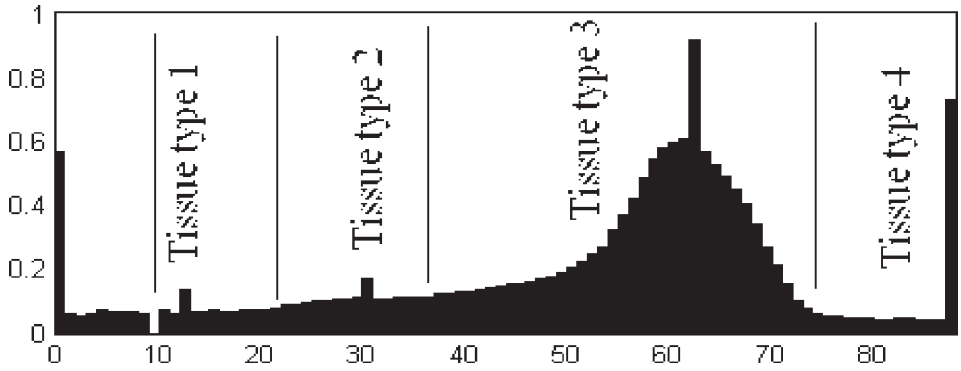


Fig. 2. Voxel intensity histogram for tissue segmentation

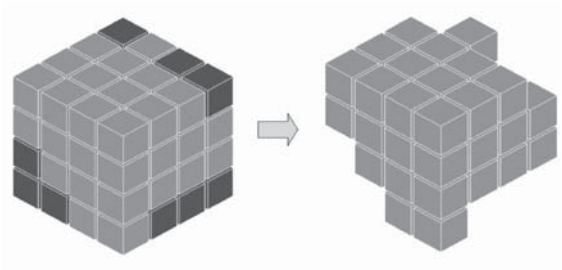


Fig. 3. Slices stack filtration

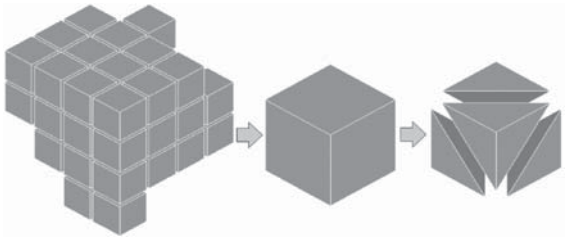


Fig. 4. Voxels decomposition process

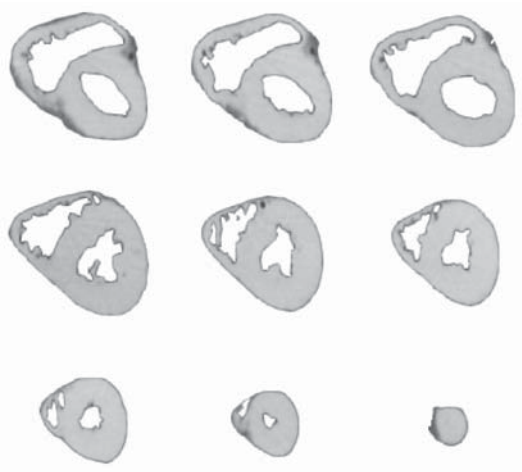


Fig. 5. 2D slice sequence of the human heart

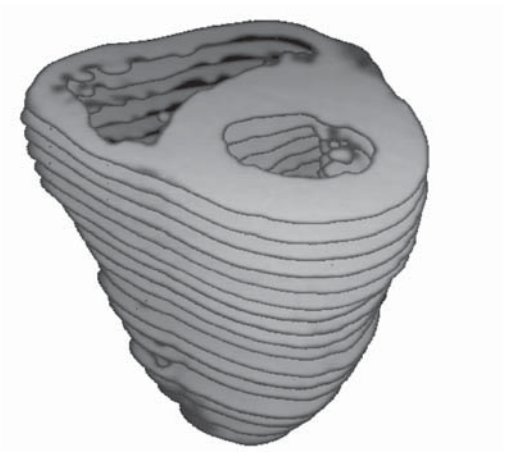


Fig. 6. 3D slices stack assembly of the human heart

volume creation the tetrahedrons are glued together. Outer tetrahedrons could be deleted for smooth model surface. In that stage we separate the solid volumes representing the different tissue types. Solid volumes are collected together in common coordinate system and elements numbering.

The generated geometry model is imported in FEM software where it is treated as a volume object. Imported volumes are associated with corresponding electromagnetic material properties.

For method demonstration a 3D model of a part of a cardiac muscle is built. The sequence of 2D slices is acquired by ultrasound scanner. In image segmentation the heart muscle position and external boundaries are pointed. Heart tissue is filtered by its image intensity. Stack with 22 slices is used. The distance between slices is 5 mm. Part of the slice stack is shown in Fig. 5. Three-dimensional slice stack is shown in Fig. 6. In Fig. 7 and Fig. 8 the reconstructed volume and meshed volume of the human heart are given, respectively.

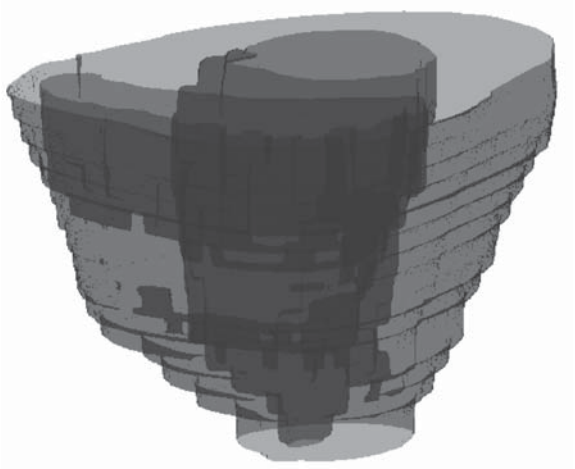


Fig. 7. Reconstructed volume of the human heart

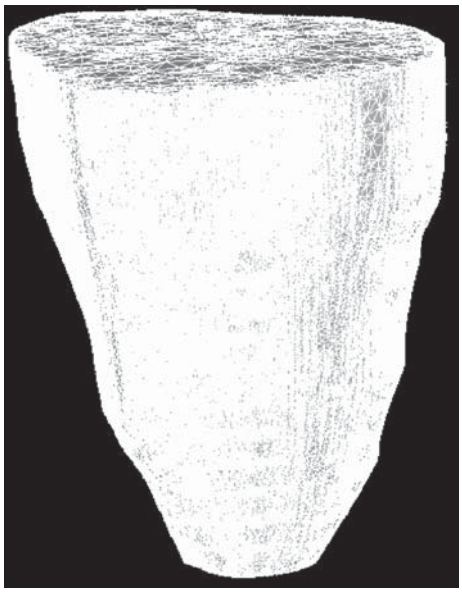


Fig. 8. Meshed volume of the human heart

ELECTROMAGNETIC PROPERTIES OF BIOLOGYCAL STRUCTURES

Electromagnetic properties of human tissues, such as electric permittivity - ε, magnetic permeability - μ and electric conductivity – σ, are of paramount importance for computer modeling of electromagnetic fields in the human body. These properties are acquired by the developed measurement system.

Computerized measurement system

The computerized measurement system is set up taking into account all requirements and conditions for measurement of electromagnetic properties of human body tissues as frequency coverage, measurement range, measurement accuracy, ease of operation, etc. Considering measurement accuracy and ease of operation, the auto balancing bridge method is the best for measurements up to 110 MHz. This method is commonly used in modern low frequency impedance measurement instruments. Its operational frequency range has been extended up to 110 MHz and Agilent 4294A precision impedance analyzer is used [20]. The proposed system is multi-frequency bioimpedance measurement system. The developed experimental computer system can interface with output devices acquiring flexible testing process.

The measurement system architecture and scheme are presented in Fig. 9 and Fig. 10. The

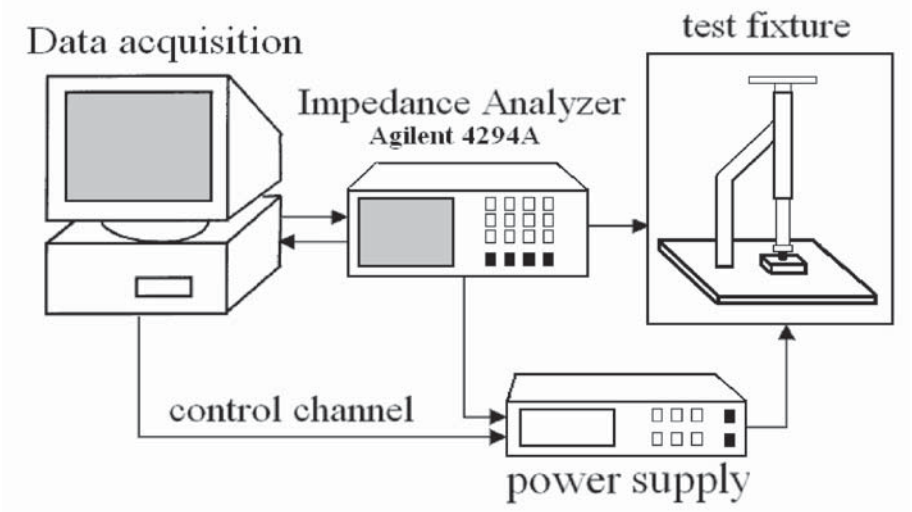


Fig. 9. Scheme of the impedance measurement system

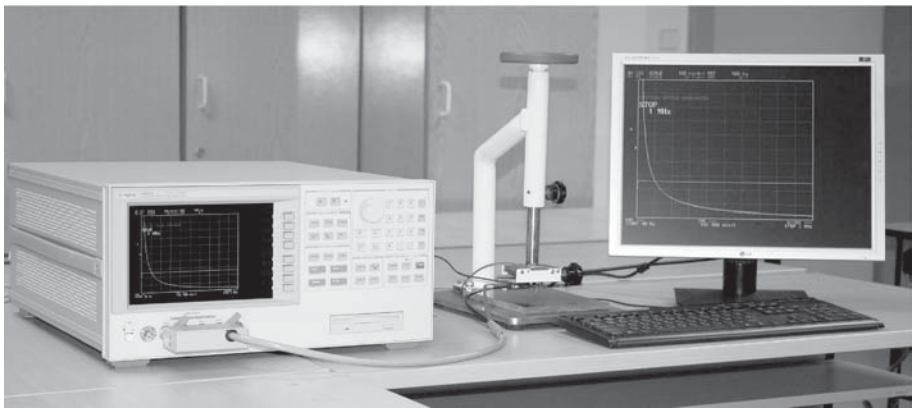


Fig. 10. Measurement system outlook

system contains: impedance analyzer, measurement test fixtures, personal computer and additional power supply block. The heart of the system is Agilent Technologies 4294A [20] precision impedance analyzer with frequency range 40 Hz - 110MHz, impedance range 10mΩ - 100MΩ. The test signal level range is 5mV to 1V rms or 200μA to 20mA rms, DC bias range is 0V to ±40V or 0mA to ±100mA, accuracy ±0.08%.

The test fixture plays an important role in impedance measurement both mechanically and electrically. The quality of the fixture determines the limit of the total measurement quality. The contact terminals of the test fixtures is 4-terminal that are suited to different applications. Additional power supply is used to realize specific test requirements.

Impedance of human tissue

The components of the human body impedance are capacitive X (reactance), and resistive R (simply called resistance). The capacitance arises from cell membranes, and the resistance from extra- and intracellular fluid.

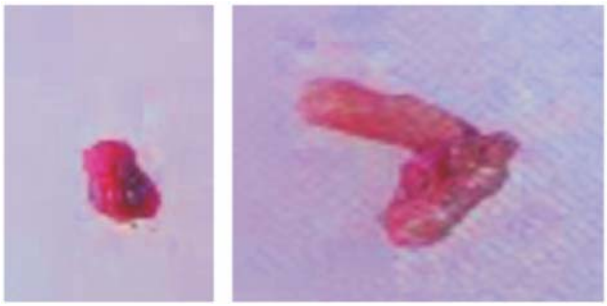
Impedance measurements were performed on cancer human tissue samples. For each tissue type a pair of samples are acquired, one for the tumor and another for normal tissue. Probes of many cancer types are investigated. Same results are presented to demonstrate the acquired typical bio-impedance data for breast, ovary and rectum cancer. The digital photos of samples are presented on Fig. Fig. 11-13.

Samples are shaped as simple cylinder with cross section $S=3.14mm^2$ *and* $l=8mm$. Electric impedance of each tissue type is measured with the proposed measurement system. Impedance amplitude and angle are stored and visualized for broad frequency range from 100Hz to 10MHz. Impedance amplitude and angle of rectum tissue samples are shown in Fig. 14. Normal tissue conductivity is better then tumor tissue in low frequency range, up to 10 kHz. Impedance amplitude and angle of ovary tissue samples are shown in Fig. 15. Here the tumor tissue is with better conductivity in the whole frequency range. Impedance amplitude and angle of breast tissue samples are shown in Fig. 16; here the normal tissue is with better conductivity in the whole frequency range. Impedance character of all samples is capacitive. Tumor and normal tissues can be distinguished on their impedance frequency spectrums.

Electrical conductivity y and electrical permittivity e due to current flow or polarization induced by an electric field in matter under investigation are measured. These properties are determined by measurement of bioimpedance using known shape and sizes of samples. An approach for reconstruction of electromagnetic properties σ, ε and μ is developed by applying sinusoidal fields.

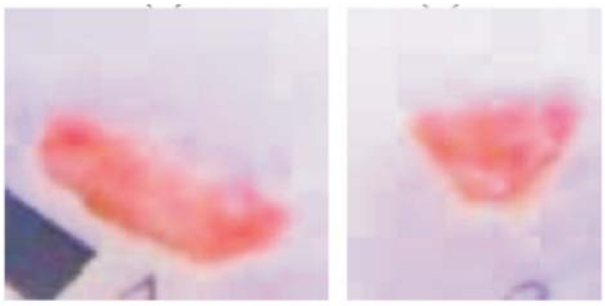
The relative dielectric permittivity ε and electric conductivity σ for the rectum tissue samples are reconstructed by the proposed approach and shown in Fig. 17.

Infrared (IR) thermography is a rapid, efficient and full-field technique used for medical diagnosis. Thermal images acquired by modern thermo-vision camera of the normal and tumor tissues for the rectum, ovary and breast are shown in Fig.Fig. 20-22.



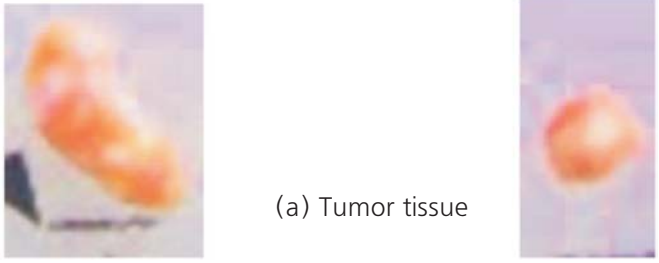
(a) Normal tissue (b) tumor tissue

Fig. 11. Rectum samples



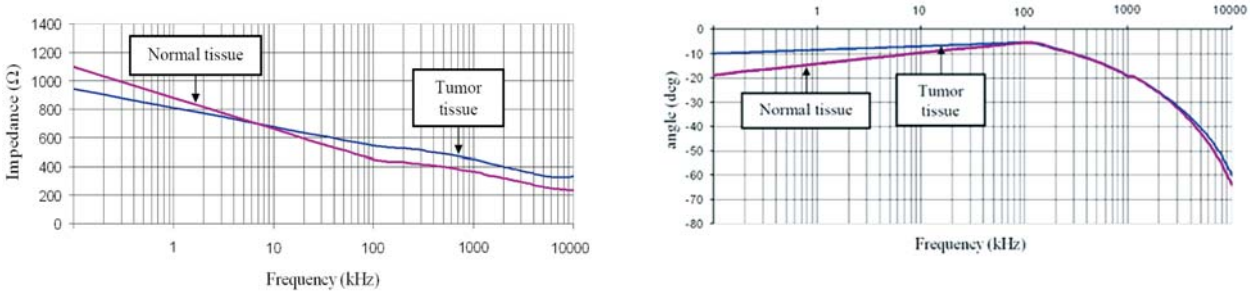
(a) Tumor tissue (b) normal tissue

Fig. 12. Breast samples



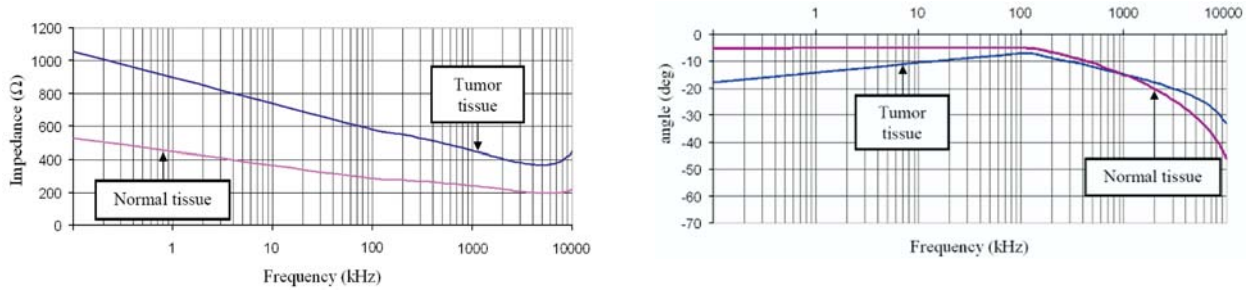
(a) Tumor tissue (b) normal tissue

Fig. 13. Ovary samples



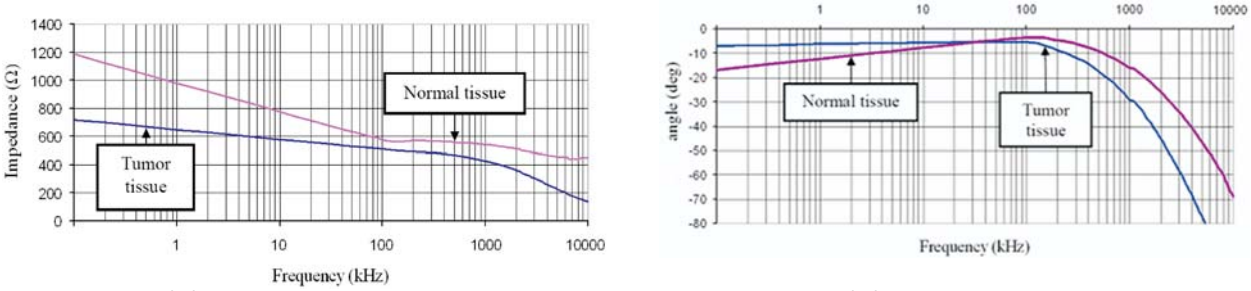
(a) impedance amplitude (b) impedance angle

Fig. 14. Impedance amplitude and angle of rectum tissue samples



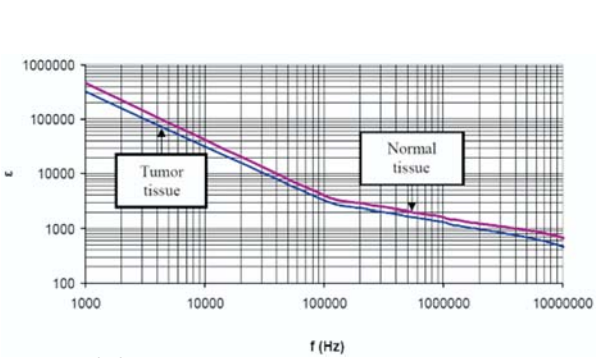
(a) impedance amplitude (b) impedance angle

Fig. 15. Impedance amplitude and angle of ovary tissue samples

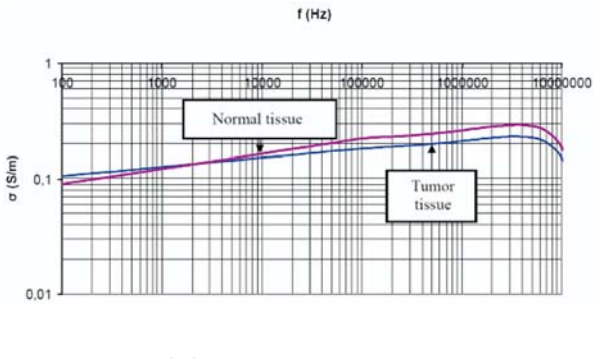


(a) impedance amplitude (b) impedance angle

Fig. 16. Impedance amplitude and angle in breast tissue samples

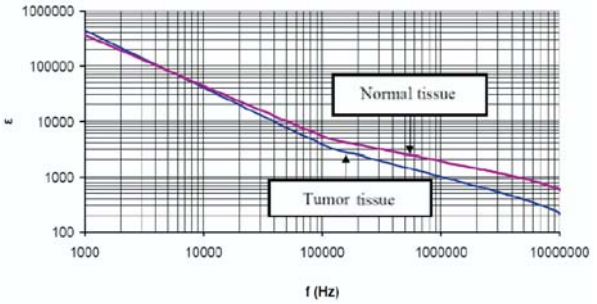


(a) Relative dielectric permittivity ε

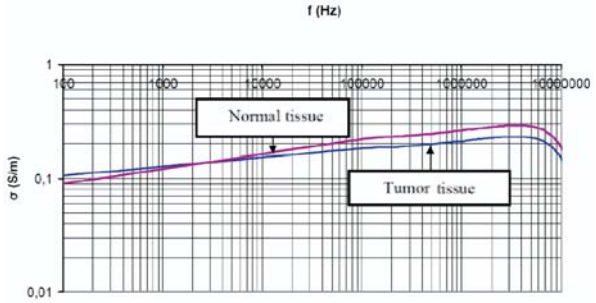


(b) Electric conductivity σ

Fig. 17. Rectum tissue samples

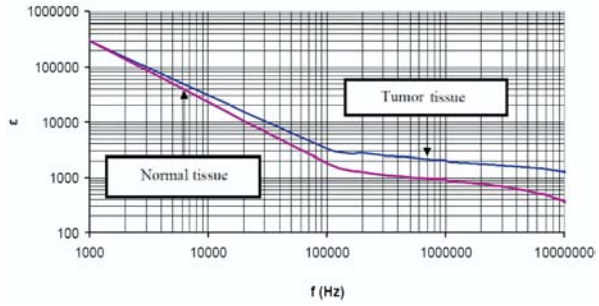


(a) Relative dielectric permittivity ε

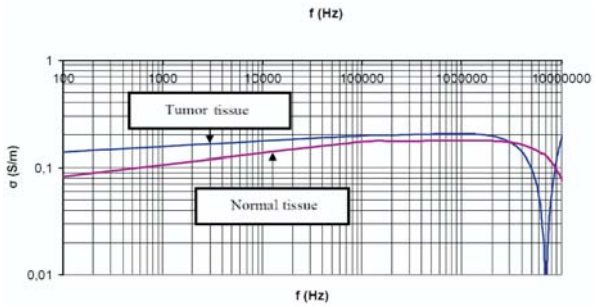


(b) Electric conductivity σ

Fig. 18. Ovary tissue samples

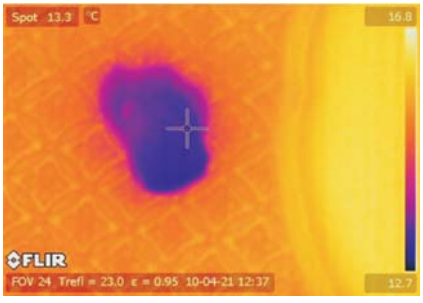


(a) Relative dielectric permittivity ε

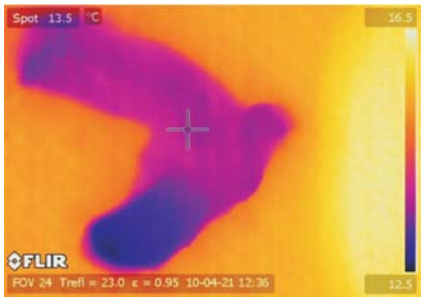


(b) Electric conductivity σ

Fig. 19. Breast tissue samples

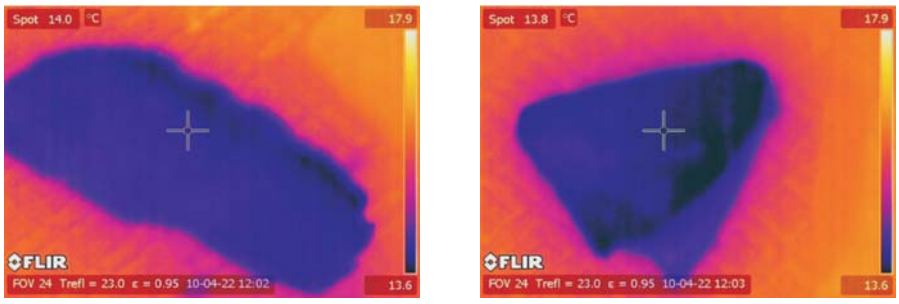


(a) Normal tissue



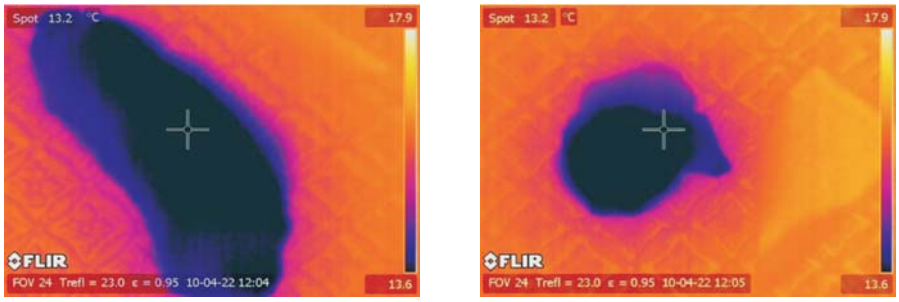
(b) Tumor tissue

Fig. 20. Infrared thermography of rectum samples



(a) Tumor tissue (b) Normal tissue

Fig. 21. Infrared thermography of ovary samples



(a) Tumor tissue (b) Normal tissue

Fig. 22. Infrared thermography of breast samples

ELECTROMAGNETIC FIELD MODELING

Electromagnetic field distribution inside the conductive tissue region depends on the time varying magnetic flux density. The activation source is the electric field **E** induced in the tissues and expressed by using Faraday's law

$$\nabla\times\mathbf{E}=-\frac{\partial\mathbf{B}}{\partial t} \quad (1)$$

where **B** is the magnetic flux density, $\mathbf{B}=\mu\mathbf{H}$, μ is magnetic permeability, **H** is the magnetic field intensity.

According to $\nabla\mathbf{B}=0$ and introducing magnetic vector potential **A** by $\mathbf{B}=\nabla\times\mathbf{A}$ from (1) is obtained

$$\nabla\times(\mathbf{E}-\frac{\partial\mathbf{A}}{\partial t})=0 \quad (2)$$

Using electric scalar potential V_ε, (2) can also be expressed as

$$\mathbf{E}=-\frac{\partial\mathbf{A}}{\partial t}-\nabla V_\varepsilon \quad (3)$$

The induced current density $\mathbf{J}_e$ satisfies the Ohm's law is

$$\mathbf{J}_e=\sigma\mathbf{E}=-\sigma(\frac{\partial\mathbf{A}}{\partial t}+\nabla V_\varepsilon) \quad (4)$$

According to Ampere law can be written

$$\nabla\times\mathbf{H}=\mathbf{J}-\sigma\mathbf{E} \quad (5)$$

The governing equation for magnetic vector potential-electric scalar potential (A-V, A) formulation is

$$\nabla\times\frac{1}{\mu}\nabla\times\mathbf{A}=\mathbf{J}-\sigma(\frac{\partial\mathbf{A}}{\partial t}+\nabla V_\varepsilon) \quad (6)$$

Three-dimensional harmonic and transient electromagnetic problems with zero value Dirichlet boundary condition for free space boundary according FEM formulation are utilized for the field analysis in human tissues.

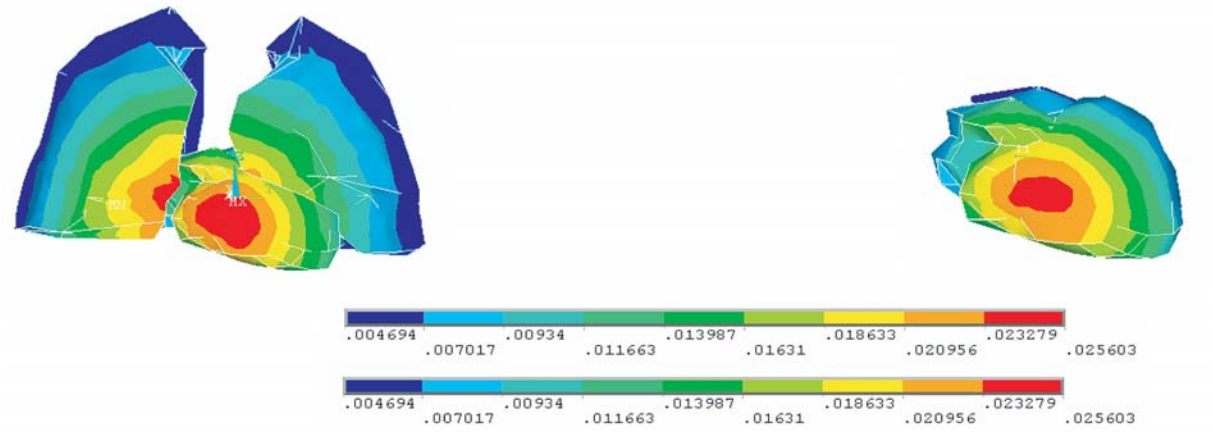
The finite element implementation of the A-V, A formulation has been carried out by using four-nodded, first-order, tetrahedral elements. The problem is solved by ANSYS software [19].

Heating effect on the tissue is calculated with Joule heating Q

$$Q=\rho J^2 \quad (7)$$

where $\rho=1/\sigma$, is specific electrical resistance.

Constructed realistic 3D anatomy models are imported in ANSYS software, the electromagnetic properties of human tissues are imbedded and the electromagnetic models are built according to the described FEM formulation. Computer



(a) Inner organs (b) Heart

Fig. 23. Magnetic flux density distribution

modeling of electromagnetic field distributions in the organs and parts of the human body during magnetic stimulation is realized.

Thorax

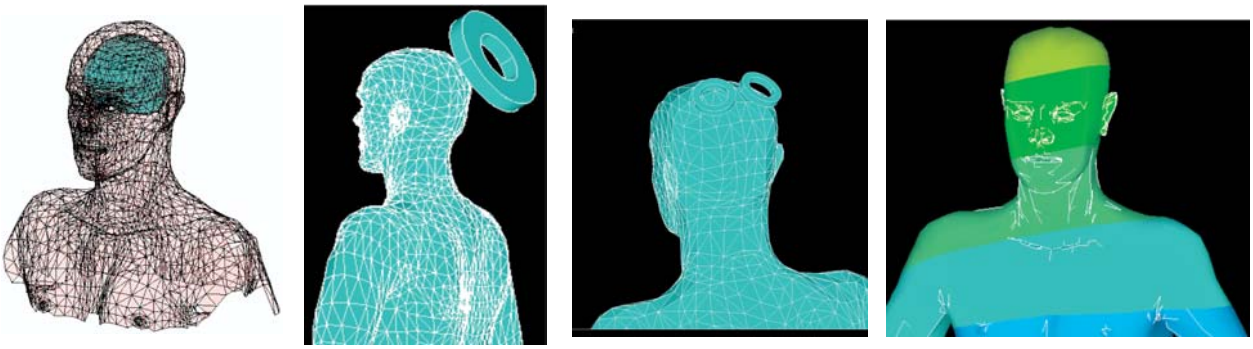
Computer modeling of electromagnetic field distributions in human the thorax under magnetic stimulation field is obtained. Field source is a cylindrical coil. Coil dimensions are: outer radius – 100mm, inner radius – 60mm and height – 20mm. Total current is 2000A. Magnetic flux density distribution on the surface of inner organs and on the heart surface is shown in Fig. 23. Flux density maximum values are below the inner diameter of the coil.

Head

A 3D model of the human head with magnetic stimulation coil is shown in Fig. 24. The model consists of scalp, skull, cerebrospinal fluid and brain. Field source is a cylindrical coil and its position is shown in Fig. 24(b). Coil dimensions are: outer radius – 60mm, inner radius – 50mm and height – 8mm. Total current is 400A.

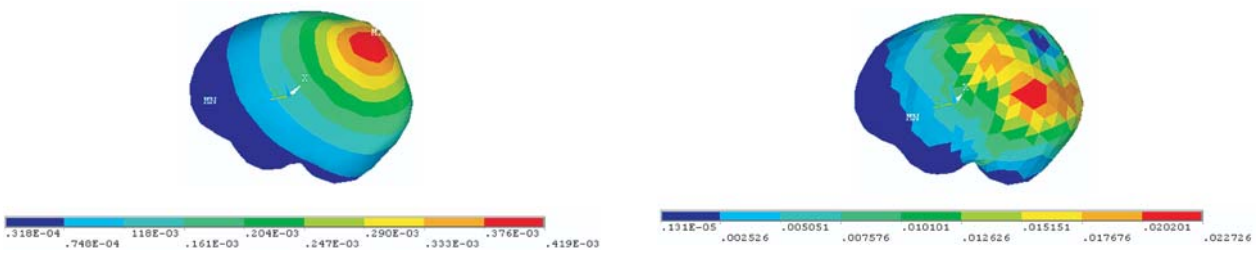
Analyzed magnetic vector potential distribution is shown in Fig. 24(d). Magnetic flux density over the brain volume is demonstrated in Fig. 25(a). Joule heating calculated for each element of brain region using (7) is shown in Fig. 25(b).

Computer modeling of the human head during magnetic stimulation with a "8-sahaped" or butterfly TMS coil is realized. Both coils have same dimensions as follows: outer radius – 40mm, inner radius – 35mm and height – 6mm. The angle between the coils axes is 15 deg., total current for each is 180A. Both currents have equal directions. Magnetic flux density over the brain volume enclosure surface with butterfly



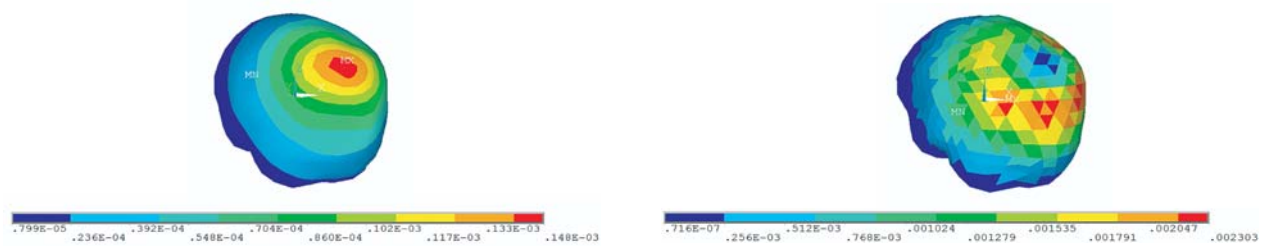
(a) 3D human head with brain volume (b) TMS coil (c) TMS butterfly coil (d) Magnetic vector potential distribution in model skin surface

Fig. 24. 3D model of human head for Transcranial Magnetic Stimulation(TMS)



(a) Magnetic flux density distribution (b) Joule heating in each element of the treated organ

Fig. 25. Magnetic stimulation of the human head with single coil



(a) Magnetic flux density distribution (b) Joule heating in each element of the treated organ

Fig. 26. Magnetic stimulation of the human head with butterfly coil

TMS coil is demonstrated in Fig. 26. Joule heating for that case is shown in Fig. 26(b).

CONCLUSION

In this paper we developed a methodology for computer modeling of electromagnetic fields in biological structures. The proposed methodology combined a method for automatic 3D geometry model building of anatomical objects, developed computerized system and method for measurement of electromagnetic properties of human tissue, (A-V, A) formulation of electromagnetic field problem and computer script for ANSYS software.

The method for 3D geometry model building of anatomical objects uses image data acquired by modern medical diagnostic equipment. The computerized measurement system gives possibility for precise determination of electromagnetic properties of human tissue. The magnetic vector potential and scalar electric potential (A-V, A) formulation of the electromagnetic problems, finite element method and computer software ANSYS are capable to analyze the electromagnetic field distribution in human tissues at different modes and conditions.

The presented implementations show that the developed approach is suitable for electromagnetic field investigations and treatment by outer field source, such as magnetic stimulation, defibrillation, impedance tomography, etc.

The developed methodology and realized numerical and physical experiments could be applied for solving many forward and inverse electromagnetic problems in order to realize the optimal energy interaction between biological structures and electromagnetic devices.

Acknowledgment

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КОМПЮТЪРНО МОДЕЛИРАНЕ НА ЕЛЕКТРОМАГНИТНОТО ПОЛЕ В БИОЛОГИЧНИ СТРУКТУРИ

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Резюме

Компютърното моделиране на електромагнитни полета в биологичните структури е от изключителна важност при изследване на процесите и явленията при медицинска диагностика и терапия. В тази статия е представена разработена методология на процеса на компютърното моделиране. Методиката съдържа метод за автоматично изграждане на 3D модел на анатомичен обект, измервателна система и метод за определяне на електромагнитни свойства и изчислителен алгоритъм за определяне на разпределението на електромагнитното поле в биологични обекти, основаван на метода с крайни елементи (МКЕ). Последните постижения на МКЕ дават възможности за моделиране на триизмерен обект с нелинейни, нехомогенни или анизотропни свойства на материалите. С приложението на предложената методика при изследване на разпределението на електомагнитни полета в различни части на човешкото тяло при магнитна стимулация се демонстрират възможностите за решаване на такива сложни задачи.

THE ARCITEC–PROJECT AND THE DOCUMENTATION AND INTERPRETATION OF ARCHAEOLOGICAL SITES AS A CONTRIBUTION TO PROTECTION OF CULTURAL HERITAGE IN BULGARIA

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Abstract

In the following article an outline of activities carried out during the first half of the project on documentation and interpretation of cultural-historical heritage funded by the Bulgarian Scientific Foundation is given. An archaeological survey in the valley of the river Struma has been carried out as a case study, where new methods of finding, description and evaluation of archaeological sites have been tested. A team of archaeologists, geographers, geologists and geophysicists from various institutions has been looking for regularities in the location of archaeological sites, which have been approached as important sources for economic and social processes in the past. A digital, easily accessible documentation of the archaeological sites and surface finds will allow a better understanding of settlement patterns in the past and will help authorities to prevent more efficiently the destruction of cultural heritage by looters, construction of buildings and roads. One of the accents during the first period of the project was preparation of an archaeological exhibition for children and their parents, which aims to present cultural heritage in an unconventional way and therefore to grow public intolerance against treasure-hunting and looting of monuments.

INTRODUCTION

Cultural heritage is a non-restorable resource. Once destroyed it is lost forever. Neither reforestation, nor artificial breeding or re-cultivation can compensate for its demolition. That is why an interdisciplinary research team consisting of archaeologists from the Department of Archaeology at the New Bulgarian University, the Archaeological Institute in Sofia, the Museums in Blagoevgrad and Sandanski, as well as geographers from the Sofia University St. Kliment Ohridski and geologists from the Earth and Man Museum in Sofia participated in a competition of the Bulgarian Research Foundation with a project which aims to contribute to decreasing the loss of monuments and information about them [1]. The most common reason for the destruction of cultural heritage in Bulgaria is looting by treasure hunters. Ploughing of agricultural fields, construction of buildings and motorways as well as natural processes like erosion are other factors contributing to this destruction. Looting by treasure hunters has been so devastating for Bulgarian cultural heritage during the last two decades [2], that in some parts of the country nearly all (!) archaeological sites from periods which are rich in gold and silver finds (Antiquity and Medieval times) have been partially or totally destroyed [3]. How could members of the scientific community act in this situation and fulfill their duty to the protection of cultural heritage, without taking on the role of police or the court? The initiatives could be divided into two groups. The first relates to the development and application of new methods of discovering, evaluation and documentation of archaeological sites. The second concerns the wider public and aims to provoke higher intolerance toward illegal excavations and looting of settlements and burial grounds in Bulgaria. We believe that the scientific community should involve more actively in public work and educational activities, which demonstrate to the Bulgarian society that **our archaeological heritage does not consist simply of precious objects**

(coins, golden masks, jewelry and other treasures)**, but of the contextual information about remains from the past** [4].

DISCOVERING, DESCRIPTION AND EVALUATION OF ARCHAEOLOGICAL SITES

Full and easily accessible information about location, character and value of archaeological sites is needed not only by the police and municipalities, but also by the tourist industry [5]. The most effective way to collect this information is the archaeological field survey. Observation and description of antiquities in Southeastern Europe was initiated by western travelers in the late centuries of the Ottoman period. After creation of national states in the 19-th century archaeological services emerged as a result of growing interest on the historical and archaeological past, which played constituting role in the construction of the new national identities in modern Balkan states [6]. The wave of industrialization in Bulgaria in the decades after the Second World War, which brought a boom in the building of factories, dams and roads, was accompanied by extensive archaeological projects during which numerous data about spatial aspects of human behavior in the past was collected. One of the consequences of the isolation of the country during the Cold War was ignorance about the major methodological and theoretical advances of the scientific metropolises. This resulted in a rather conservative way of carrying out field surveys. A methodological change came with the Bulgarian-Polish regional archaeological projects in the valleys of the rivers Mesta (1970s) and Struma (1980s) [7], when the tenets of the German *Siedlungsarchäologie* were adopted [8]. One of the consequences of the experience gathered during these projects was creation of the *Archaeological Map of Bulgaria* [9]. A database, where detailed archaeological as well as geographic and geological information can be stored, was set up. Fed with data from surveys carried out since the early 1990s, the *Archaeological Map of Bulgaria,* hosted in the National Archaeological Institute in Sofia, was a watershed in the way of documentation of surveys but did not lead to substantial changes in fieldwork methods or theoretical evaluations.

CASE STUDY: THE STROUMA ARCHAEOLOGICAL SURVEY

In order to apply and test new methods of

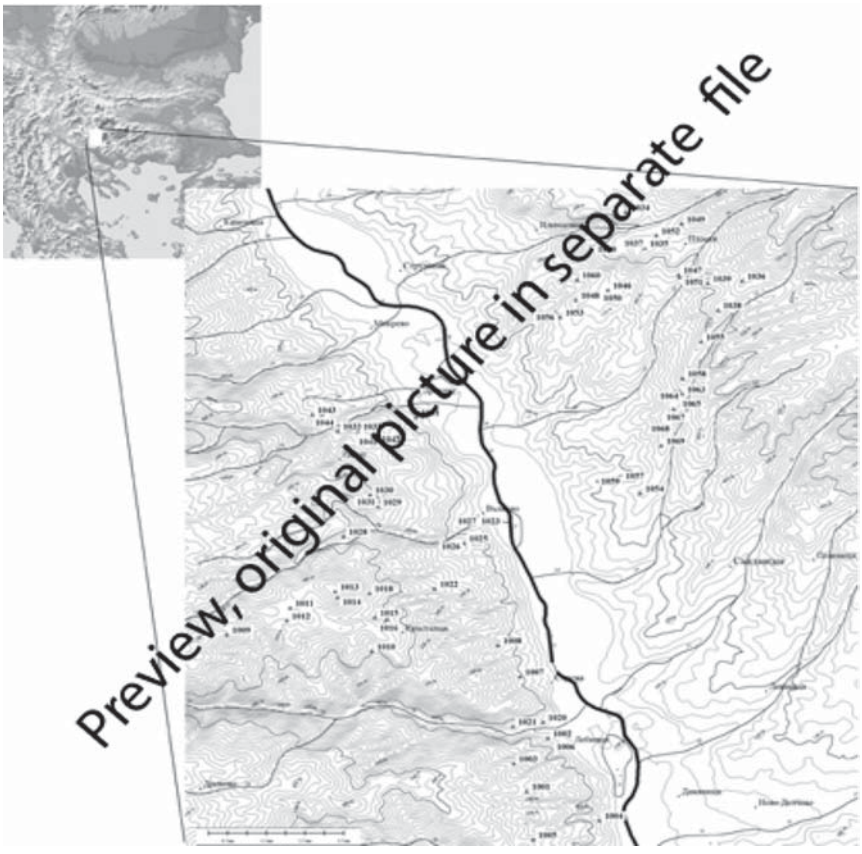

Fig. 1. Map of the researched area in the Middle Struma valley with archaeological sites surveyed

discovering and evaluating archaeological sites, a fieldwork in the middle valley of the river Struma (i.e. between Kresna and Koulata/Promahon, Fig. 1) was designed as part of the ARCITEC-project. Financial support of the Bulgarian Scientific Foundation enabled the acquisition and use of GPS-devices (simple and referential), mobile computers, GIS-software and photographic equipment. The technical panoply would make little sense without testing the methodological and theoretical advances of surveying in Southeastern Europe, which have led to new understanding of the past settlement patterns [10]. In the spirit of the pioneering archaeological projects in Boeotia, Melos and Kithira, attention has been focused on the catchment of sites or economic and other potential of site territories, as well as on a better understanding of settlement hierarchies, and transformation of landscapes in the past. The experience in Thessaly, which appears to be one of the most densely populated regions in the Neolithic period, demonstrated that consideration of environmental criteria alone can not explain the distribution of the sites [11]. Therefore elaboration of a theoretical component as an important factor for the part in the generation of some predictive models about the location of archaeological sites is among the aims of the ARCITEC-project.

Realization of the goals outlined above imposed on the one hand the choice of a restricted geographic area, where methodological and theoretical approaches could be tested, and on the other hand the involvement of a big interdisciplinary team. The middle part of the valley of the river Struma was chosen as a pilot research area also because of the anticipated destruction of archaeological sites due to intensive building of roads, industrial structures and living edifices as well as by local and organized treasure hunters. Another reason for the choice of the region of Struma was that the valley of the river is supposed to be an important communication axis between the Aegean and the continental part of the Balkan Peninsula [12]. In what follows, some results from the first campaign (2009) will be presented [13]. The fieldwork started from the line reached by the Bulgarian-Polish extensive surveying project in the 1980s [14]. First objective of the new campaign was to cover extensively the remaining unsurveyed part of the valley of Sandanski, that is, a territory of some 25 x 10 km, situated between the mountains of Ograzhden from the West and Pirin from the East, reaching the pass of Kresna to the north and the delta of the right tributary of the Strouma Lebnishka river to the south. At a second stage intensive surveys will be carried out at important parts of the landscape. During the first campaign 69 archaeological sites present in an area of 45 m^2 were localized and described. The average of 1,5 sites per square kilometer is by far smaller than the results of the old campaign from 1978-1982, when an average of 2,14 sites per sq km (1800 sites on 240 km^2) were discovered. Probable reason for this difference is the plowing conditions of the surface soil: plowed soil allows a considerably better degree of observation. A comparison with the 2,75 sites per square kilometer located in the plain of Blagoevgrad [15] is indicative, since here much more agricultural land and less pastures and forests exist currently.

A regularity concerning the distribution of archaeological sites in the landscape refers to the distance between sites and the river Struma. A zone of yearly floods of the river, which can reach a height of 2-3 meters above the average level of the water, did not provide any traces of human activities from the past (i.e. from Early Prehistory till the mid of the 20th cent.). This type of land (Fig. 2) seems to be avoided for building activities because of the danger of floods and was probably kept free for agricultural plots. The river alluvium makes it the most fertile terrain in the landscape, preferred till nowadays for cultivation. This landscape was considerably changed in the 20th cent. AD, when the river bed was straightened and some meanders filled up and turned into agricultural areas. A first belt or zone with archaeological sites was located in the foothills of Maleshevska Mountain on the right riverbank and the lowest slopes on the left bank, several meters higher or at the same level as the lowest modern villages (Lebnitsa, Struma-village, Valkovo and Drakata) [16]. It is probably not a coincidence that the lowest located archaeological sites are predominantly from the Late Antiquity (4th-6th cent.

A.D., 1006-Ugrinovi spot, 1002-Ganska Chuka, 1007-Ornicheto, 1024-Valogo, 1027-Diado Vassiliovata niva, Fig. 1). The distance of some 4,5 km between 1007-Ornicheto and 1024-Valogo is not indicative of the real distribution of settlements in Late Antiquity, and possibly another settlement between them is buried by a modern dumping ground. The existence of isolated necropolises from the Late Antiquity like 1004-Vesselinitsa and 1008-Beliovitsa demonstrates that we did not succeed to find some settlements, obviously totally destructed by erosion. In the lower zone with archaeological sites, no settlements from the Neolithic or Copper age have been found. This is surprising given the low location of the Late Neolithic settlement Damianitsa.

Fig. 2. The valley of Struma

The second zone with sites lies in the hills of Ograzhden and Pirin, some 2 to 5 km away from the river Struma. It is not a homogenous category of the landscape and the only common feature is the lack of fertile agricultural land. The earliest site (probably a settlement) discovered in this zone is 1051-Podo Yug, dated in the Early Neolithic, whereas the settlement with the highest location is 1013-Krastilski vrah/Lokvata, where pottery from the Late Copper Age (5^{th} mill. BC) and the Late Antiquity was collected. Noteworthy are remains of intensive human activity during Late Antiquity in the area of 1014-Krastilski peak (810 m a.s.l.), whereas finds on the neighboring 1030-Palatski (586 m a.s.l.) are much more scarce. The distribution of sites in different periods does not surprise, because similar picture (esp. Late Antiquity and Early Medieval period) has been reported from other surveys in the Eastern Balkans [17]. Noteworthy is the number of settlements from the Late Bronze Age in Middle Struma valley, which is considerably smaller than in the plain of Blagoevgrad, situated some 30-40 km to the north. A possible explanation might be the lack of fortified buildings of the type of Kamenska chukka, in the plain of Sandanski and Petrich (i.e. Middle Struma) in the Late Bronze Age, which are easy to be found because of their substantial stone architecture, existed in the Late Bronze Age. The rich necropolis from this period in Sandanski [18] proves that the plain of Sandanski and Petrich was populated in the second half of the second millennium BC. Settlements from this period were not discovered probably because of the fact that they were thin-layered or destructed by erosion and agricultural activities.

The second stage of the fieldwork will continue with intensive investigation of selected parts of the Landscape.

PUBLIC ARCHAEOLOGY. EXHIBITION FOR CHILDREN AND THEIR PARENTS "ARCHAEOLOGY BEHIND THE SCENE"

The fieldwork in Struma confirmed the general tendency of destruction of archaeological sites by treasure hunting. We realized that over 90% of the sites from Late Antiquity have been looted by treasure hunters, while some necropolises are totally destroyed (Fig. 3).

The collected documentation, which is on average about 20-25 gigabytes of pictures, maps and other files from a single campaign (Fig. 4), will partially compensate for the loss, since future generations will have information about archaeological sites, which are expected to disappear in the years after our field campaigns. This is of course not enough and prevention of the destruction must be strived for.

Fig. 3. Prospection of a looted grave from the Late Antiquity during fieldwork in 2009

The experience of scientific metropolises has shown that educational activities and particularly initiatives targeting children and youth are effective mechanisms for public engagement in tackling sensitive issues such as treasure hunting, illegal excavations and export of cultural-historical heritage [19]. Particularly efficient is the hands-on approach, which involves exhibition visitors in the process of discovering the story behind the objects. This approach has an exceptional comparative advantage as it engages the visitor emotionally and makes sure that important messages are received and remembered [20]. It is expected that most people will not tolerate the misuse of archaeological finds and sites once they associate them with a particular archaeological site and story of the past. We envisage the hands-on approach as particularly appropri-

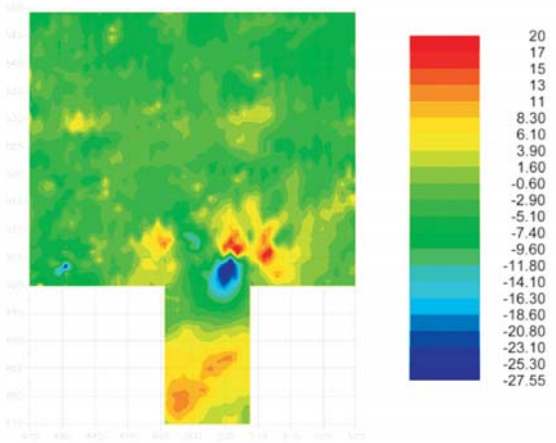

Fig. 4. Results from geomagnetic scanning of a surveyed site 1058. With little effort and no excavation a picture of the probable character of structures under the surface is given (P. Zidarov, NBU)

ate for presenting archaeological heritage to a broad public in Bulgaria. This is the reason for our choice to present the results from the field-work in the valley of Struma not in a traditional exhibition, with show-cases full of objects with small labels, but in an exhibition adapted for children and young people based on the hands-on principle. Interactive exhibition integrates major trends in current museum pedagogy [21] and allows visitors to participate in the process of scientific interpretation and in the construction of the past. This represents an innovation in the museum practice in Bulgaria and most of Eastern Europe, where visitors are usually given the role of passive receivers of the scientific knowledge, which could only be offered by expert scientists.

The exhibition developed under the ARCITEC project aims to communicate the importance of preserving cultural heritage in Bulgaria and to demonstrate the damages of treasure hunting and illegal excavations. The ARCITEC interactive exhibition is based on the presumption that each visitor could have a solution and hence important role in preventing the damage of cultural heritage in Bulgaria. Involving visitors in an active role is a recognized mechanism in modern museology, employed particularly in the case of exhibitions presenting endangered heritage [22]. It is foreseen that the ARCITEC exhibition will be displayed during the second phase of the project (2011-beginning of 2012) in popular public locations in Sofia and Blagoevgrad and will be thus more easily accessible to families, school groups and broader public as compared to the ones attracted to traditional museum. The exhibition consists of seven separate modules, each presenting important stage of the archaeological investigation: desk research and processing of preliminary information, interviewing local people and collecting oral histories; terrain surveys; study and interdisciplinary research of collected materials; interpretation and publication. Hands-on activities for visitors are based both on traditional techniques of archaeological survey and on modern means such as GIS and contemporary geophysical methods in registering archaeological sites.

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ПРОЕКТЪТ ARCITEC ЗА ДОКУМЕНТИРАНЕ И ИНТЕРПРЕТИРАНЕ НА АРХЕОЛОГИЧЕСКИ ОБЕКТИ КАТО ЧАСТ ОТ СТРАТЕГИЯ ЗА ОПАЗВАНЕТО НА КУЛТУРНО-ИСТОРИЧЕСКОТО НАСЛЕДСТВО В БЪЛГАРИЯ

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Резюме

ARCITEC е проект на Департамент "Археология" в Нов български университет и партньори от български и чуждестранни музеи, университети и научни центрове, ръководен от проф. дин Ив. Гацов и финансиран от Фонд „Научни изследвания" към Министерството на образованието, младежта и науката на Република България. Сред целите на участниците в проекта е по-качественото документиране и интерпретиране на паметници на културно-историческото наследство в България с помощта на съвременни технологии; тяхното опазване, публикуване в престижни международни медии и популяризирането им сред българската и световна общественост. В статията е направен кратък преглед на по-важните дейности от първия период на дейността по проекта. Представени са резултатите от експедиция за документиране на археологически паметници в долината на река Струма, както и концепцията за изложба, представяща опазването на културно-историческото наследство, насочена към деца и младежи. Интердисциплинарността на проекта е резултат от съчетанието на следните методи: археологически обходи, геофизика, геология, географски информационни системи, фотография, трасеология, технологичен анализ, информатика, културна антропология, музеология.

BULGARIAN ADDED VALUE TO ERA

DEVELOPMENT OF A SPECIALIZED CENTER FOR SCIENTIFIC, TRAINING AND DIAGNOSTIC WORK FOR THE NEEDS OF APIDOLOGY AND SERICOLOGY IN BULGARIA

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STATE OF SCIENTIFIC RESEARCH IN BULGARIA

In the field of apidology

Since 1999 it has been worked on the implementation of the National Programme for breeding and improving work with bees and organization of queen-bee breeding in Bulgaria. Its purpose is conservation of the gene fund (search, reproduction and movement) of the Bulgarian honey bee. With this regard, a morpho-ethological analysis by specific characteristics has been carried out in our country in order to determine the race belonging (Petrov, 1997). On the other hand, biochemical-genetic researches of polymorphism in some protein and isoenzymic systems have been carried out ever since 1992 (Ivanova et al, 1991; Ivanova, 1991; Ivanova et al, 1994; Ivanova and Popov, 1996-1997; Ivanova et al, 1996; Ivanova et al, 2004; Ivanova et al, 2007). In the recent years by reuniting of different approaches (classical and geometric morphometry, isoenzyme and DNA analysis), different local populations have been studied on the territory of our country aiming at clarifying the race belonging of the local honey bees

In the field of sericology

Scientific research work on sericology in Bulgaria to the present moment has been mainly directed to studies of biological and technological features important for the selection of the mulberry silkworm with a view to a mathematical prognosis and a complex evaluation of the breeds raised in our country (Nacheva et al., 2004; Vasileva et al., 2004; Petkov et al., 2006); creation of new breeds, lines and hybrids (Nacheva et al., 2000; Kipriotis and Grekov, 2000; Grekov, 2002); creation of genetically sex-marked lines (Petkov et al., 2004, 2006); selection, agro-equipment and use of mulberry (Petkov and Petkov, 2004). Increasing the productive and adaptive potential of the newly created breeds, lines and hybrids of silkworm requires the use of a greater selection of initial populations with different origin and introduction of reliable genetic markers (isoenzymic, DNA) in order to determine the level of genetic mutability in the initial populations. Studies in this trend in our country are very poor and are narrowed only in the research of polymorphism in some isoenzymes (esterases, phosphoglucomutases, superoxiddismutases) from the haemolymph and some tissues in some strains raised in Bulgaria (Shabalina, 1990; Stoykova et al., 1998, 1999, 2003; Popov et al., 1999; Staykova, 2006; Staykova, 2008). Corre-

lation between the isoenzymic spectra of the parental forms and the selective-significant features in the generations has not been studied yet. Suitable DNA markers for analyzing the genetic heterogeneity of the initial populations included in breeding programmes and their use for determining the degree of genetic similarity and distance have not been searched yet. The performance of complex scientific research uniting specialists specialists from different trends and different methods (both genetic and selective) would be important with a view to preserve the national genetic resource from the breeds *Bombyx mori* L. and the varieties *Morus spp.*, and its enrichment. They will also help us make a database serving the selection programmes.

AIM OF THE PROJECT

The aim of the current project is **development of a specialized center for scientific, training and diagnostic work for the needs of apidology and sericology in Bulgaria.**

The specialized center:

• **Will function as a scientific and research center** – for carrying out interdisciplinary scientific research in the field of apidology and sericology, including complex evaluation of the studied populations through phenotype and genotype characterization of different levels, uniting the scientific research units of Paisii Hilendarski University of Plovdiv, Agricultural University - Plovdiv and Sericulture Experiment Station at the Agricultural Academy, their production facilities and the production facility of the National Breeding Association of Apiculture;

• **Will function as a training center**, on the one hand, for work with outstanding students, students, masters, graduates and PhD students, and on the other hand, for training and improving the qualification of farmer-beginners and active farmers through organization of courses and workshops;

• **Will be specialized for diagnostic activity** in the field of apidology and sericology, selective-genetic based assessment through established reliable markers and mathematical prognosis.

TYPES OF ACTIVITIES

In compliance with the project objective, we envisage the following activities: scientific-research; training and diagnostic.

Scientific and Research Work

The scientific and research work within the consortium will be allocated according to sections, as follows:

Laboratory of Genetics at the Department of "Developmental Biology" at "Paisii Hildendarski" University of Plovdiv

• Electrophoretic analysis in polyacrylamide and starch gels for specifying suitable isoenzymic markers of selective value upon assessing the gene fund and the genotype structure of the local populations of *A. mellifera* and *B. mori*.

• DNA analysis for specifying genetic markers suitable for both species, which may be successfully used in the selection programmes for the purposes of the gene fund characterization, preservation and enrichment.

• Population and genetic analysis for assessing the level of genetic heterogeneity in local populations, determining phylogenetic dependencies and selection of options suitable for hybridization.

• Complex of classic statistical methods and specialized software for analyzing the results obtained.

Agricultural University – Plovdiv

• Classical and geometric morphometry and ethological analysis of honey bees.

• Complex of methods for reporting the main biological indicators of the silkworms and technological indicators of the cocoons and the silk filament: hatch level of the silkworm eggs, vitality of the silkworms, raw cocoon weight, silken sheath weight, raw cocoon silkness, dry cocoon silkness, silk filament length, yield, raw cocoons yield per a small box of silkworm eggs, yield per a small box of silkworm eggs.

• Complex of statistical methods for analyzing the results obtained.

Sericulture Experiment Station – Vratza

• Analysis of the morphological indicators of the silkworm at the phase of egg, larva and pupa.

• Phenotype characteristics of the silkworm populations by determining the average values of the main biological and reproductive indicators of butterflies. Analysis of the mutability.

• Genotype characterization of the silkworm

populations by determining the correlation and regression dependencies between the main reproduction indicators. Analysis of the inheritance and prognosis on the efficiency of selection according to the main reproduction indicators.

• Improvement of methods and schemes for maintaining and improving the silkworm breeds, lines and hybrids raised in the production, for spring and summer-autumn industrial silkworm rearing.

• Improvement of the technology of production of tribal (super-elite and elite) and industrial (F1 hybrid) silkworm eggs for spring and summer-autumn industrial upbringings.

• Research, preservation and management of the gene fund of the populations for the mulberry *Morus spp*.

National Breeding Association of Apiculture

• Maintenance of the gene-fund of the local honey bee.

• Artificial insemination and ensuring the queen-bee producing farms with highly productive queen bees of the race that is local for Bulgaria.

• Providing material for isoenzymic and DNA analysis.

Training Activity

• In the Laboratory of Genetics at the University of Plovdiv training of students, masters, graduates and PhD students will be carried out in the field of genetics of both species of insects and in the field of genetics of both species of insects of economic significance, namely: *Apis mellifa* and *Bombyx mori*. The apparatuses supplied will give the opportunity of implementing new quality training, of adopting modern methods of genetic analysis and carrying out competitive researches. All this will lead to formation of a new generation of scientists prepared to accept challenges of free work and high quality of work within the European Union.

• At the Agricultural University – Plovdiv, training of students, masters, graduates and PhD students will be carried out in the field of apiculture and sericulture.

• At the Sericulture Experiment Station quality training of farmers-beginners and active farmers will be carried out by organizing courses.

• The National Breeding Association of Apiculture will carry out training for beekeepers by organizing seminars on the territory of the whole country.

Diagnostic Activity

Through the mutual work at all levels included into the consortium, and the complex approach of research in the field of apidology and sericology, the established Center will carry out diagnostic activity connected with:

• genetic assessment of initial material by using isoenzymes and DNA markers with regard to preservation, enrichment and management of the generic resource (analysis of the gene fund and the genotype structure of initial populations, determining the level of polymorphism and genetic heterogeneity, determining the level of genetic similarity and genetic distance for analyzing phylogenetic dependencies);

• assessment of the general and specific combinative ability of the initial material for the selection of options stable to non-favourable conditions and diseases;

• formation of a database for management of the genetic resource of both species in Bulgaria in view of determining suitable donors and recipients upon creating new highly productive and selective populations;

• mathematical prognosis upon the selection of initial populations in view of creating new highly productive breeds, lines and hybrids.

EFFECT AND OUTCOMES EXPECTED

By establishing and developing a specialized center for scientific, training and diagnostic work for the needs of apidology and sericology in Bulgaria we expect:

1. Improvement and modernization of the necessary equipment and conditions for research and training activity.

2. Specific outcomes, analyses, summaries and conclusions of specific types of activities stipulated under the project and with clear underlined opportunities for achieving long-term visible effect within the field of genetics and selection of both species of insects of economic significance in Bulgaria.

In view of the great economic significance of the sites under research we stipulate that analysis of the results obtained by us will be of both fundamental and applied importance. Within the

process of the research planned we expect the following in particular:

Regarding the genetic researches:

• Selection of isoenzymic markers, which are most suitable for both species of insects in view of characterization of the genetically determined polymorphism.

• Analysis of the contents of the gene fund and the genotype structure of the populations being researched according to the selected isoenzymic markers.

• Characteristics of the genetic heterogeneity by determining the level of polymorphism and heterozygote level according to isoenzymic markers.

• Selection of suitable DNA-markers for analyzing the genetic mutability within the initial populations.

Regarding the morphological and ethological indicators for *Apis mellifera*:

• Establishing an optimum complex of morphological and morphometrical indicators, on the basis of which a comparative analysis will be made on the populations of honey bees under research.

• Selection and analysis of suitable behavioral indicators for the populations of honey bees under research.

Regarding the biological and technological indicators for the species analyzed of *Bombyx mori*:

• Complex assessment of the species used for the research in view of the main biological indicators of the silkworms – hatch level of the silkworm eggs and the silkworm vitality.

• Complex assessment of the species used for the research in view of the main technological indicators of the cocoons and the silk filament - raw cocoon weight, silken sheath weight, raw cocoon silkness, dry cocoon silkness, silk filament length, yield, raw cocoons yield per a small box of silkworm eggs, yield per a small box of silkworm eggs.

3. Creating new opportunities for integration between different institutions and establishment of networks for scientific cooperation:

• Establishing opportunities for permanent cooperation in the field of scientific and research activity and information activity between the Laboratory of Genetics at the Faculty of Biology at "Paisii Hilendarski" University of Plovdiv, Scientific and Research Apiculture Facility at the Faculty of Plant Protection and Agroecology, and Scientific and Research Sericulture Facility at the Faculty of Agriculture at Agricultural University – Plovdiv, and Sericulture Experiment Station – Vratza at the National Center of Agricultural Sciences.

• Creating opportunities for integration of science and production through cooperation between scientific fields and production structures.

4. Creating new opportunities for international cooperation:

• New opportunities for work within the European university and scientific space by establishing an optimum environment for international cooperation upon analysis and interpretation of the results obtained according to generally adopted international criteria.

• Long-term cooperation within the field of apidology and sericology between the center established and other European universities, institutes and infrastructure complexes of similar subject of activity.

5. Creating potential for intensive development of apiculture and sericulture within specific regions in the country.

6. Establishment of a favourable environment for young scientists.

PROJECT MANAGEMENT

This project has been financed by the National Science Fund at the Ministry of Education, Youth and Science with contract DO 02-63/2008 in the competition "Development of Scientific Infrastructure" - 2008. The project management is implemented in accordance with the regulation stipulated by the National Science Fund at the Ministry of Education, Youth and Science.

RISK ASSESSMENT FOR NON-PERFORMANCE OF THE PROJECT

The establishment and functioning of the specialized center for scientific, training and diagnostic work for the needs of apidology and sericology will reveal great opportunities for research and cooperation in the field of biochemi-

cal, molecular and selective genetics of *Apis mellifera* and *Bombyx mori*. After the center being equipped, and in case that the research team does not find new ways and means for financing (rather than financing the activities under the project), due to the high price of the reagents for DNA analyses, the research in this direction at a subsequent stage could be delayed or temporarily suspended.

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REINFORCEMENT OF THE RESEARCH CAPACITY OF THE BULGARIAN INSTITUTE OF BIOLOGY AND IMMUNOLOGY OF REPRODUCTION

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In 2009 the Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences (IBIR-BAS) prepared a project **ReProForce** to be submitted to the *SEVENTH FRAMEWORK PROGRAMME OF EUROPEAN COMMISSION, ACTIVITY 4, CAPACITIES - REGPOT-2009-1.*

The project was highly evaluated and received 14 points out of 15 maximal points, gaining the competition among more than 1300 submitted projects, as 312 were nominated and just 16 of them received finances from the Program REGPOT. Coordinator and beneficiary is the Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences. Project Coordinator is an associate professor Margarita Mollova, PhD and scientific expert is associate professor Elena Kistanova, PhD. Project duration is 42 months with the starting date December 2009. Total sum of money for this project awarded by the European Commission is 3 000 000 leva.

The project **ReProForce** includes active international collaboration with foreign research laboratories, and the foreign partners are:

• French National Institute for Health and Medical Research (INSERM) - **Prof. G. Chaouat**.

• University of Jena, Germany, Department of Obstetrics – **Prof. U. Markert.**

• University of Essex, UK, Department of Biological Sciences- **Prof. N. Fernandez**.

• Private Hospital for Gynaecology, Magdeburg, Germany - **Prof. H. Donat**.

• University Clinic for Endocrinology and Reproduction, Germany, Magdeburg **– Prof. J. Kleinstein**

• Laboratory of Biomedical Embryology - Center for Stem Cell Research, University of Milan, Italy - **Prof. F. Gandolfi**

• University of Veterinary Medicine, Biotechnology in Reproduction, Clinical Department of Reproduction and Animal Breeding, Vienna, Austria- **Dr. U. Besenfelder.**

• Department of Animal Sciences, Wageningen University, Netherlands - **Prof. K. Teerds**.

The main objectives and strategic vision of ReProForce is an increase of the human and technological capacities of IBIR-BAS; reinforcement of its local networks and establishment of partnerships in order to reach European scientific level.

The main ReProForce goal is the creation of favourable conditions for the reinforcement of IBIR research potential in order to restore its leading positions in the field of biology and immunology of reproduction. The strategic objective of ReProForce would be to provide opportunities to create a regional European Centre on Biology and Immunology of Reproduction within IBIR with significant regional influence, attracting young and talented researchers from Bulgaria and abroad by its research excellence and modernized infrastructure.

The ReProForce Project is an important means for IBIR to *enhance, to further develop and to disseminate better* its scientific results on the elucidation of the cellular and molecular mechanisms of reproductive processes and to elaborate new biotechnologies applicable in human medicine and agricultural practice for sustainable reproduction of animal resources.

The Specific Objectives of the ReProForce project include strengthening of IBIR research capacity by enhancing the human resources capabilities of IBIR and its research collaboration with excellent European support institutions; stimulation of employment through hiring new researchers and attracting young and talented researchers; improving the research environment of IBIR through purchase of equipment;

strengthening of IBIR cooperation with both human and veterinary medicine practice and industry; building of a strong long-term collaboration with outstanding organizations and enterprises from the European Union and South-East Europe; obtaining an independent evaluation strategy for long-term IBIR development.

The ReProForce project itself is structured in different units called Work Packages according to the requirements and the practice of the European Commission. Each Work Package (WP) is planned to conduct a certain volume of specific activities as the summed up activities will build up the project as a whole entity.

Work Package 1 (WP1) consists of efforts to strengthen the research capabilities of IBIR. The leaders of WP1 (Dimitrina Kacheva, Stanimir Kyurkchiev) will organize the 2-way mobility visits with 8 support partner research organizations; organization of *scientific seminars*; recruitment of *new researchers.*

Work Package 2 (WP2) activities are directed to strengthening the technical capacity of IBIR - the leaders Maria Stamenova, Rossitza Konakchieva. The main objective of WP 2 is to upgrade the technological capacity of IBIR-BAS. The equipment with new generation research apparatus is an important prerequisite for the achievement of high-quality scientific results comparable to those obtained by leading European and world research centers, and imposes the necessity of substantial investments. An additional element aimed at enhancement of the research capacity of IBIR-BAS will be a subscription to some large specialized online scientific databases providing access to research quotations and publications.

Work Package 3 (WP3) with leader Rayna Georgieva is going to organize activity for the reinforcement of IBIR strategic partnerships; dissemination of scientific results by organizing scientific international events (*13th International Symposium on Immunology of Reproduction* - June 2012, *2-nd Conference of Balkan Network for Animal reproduction biotechnology "European Achievements in Biotechnology in Animal Reproduction"* with Supporting Workshop - March 2011) and regional events (*Training Course for Farmers, Breeding Associations Representatives "Biotechnology of Embryo Transfer in Farm Animals,"* September 2010, *Round Table "Assisted Reproduction Techniques"(ART)* -October 2011, *Workshop "Mitochondria and Reproduction"* - June 2010).

Work Package 4 (WP4) consists of activities for promotion and visibility of ReProForce results. The leaders of WP4 Maria Ivanova, Maria Kicheva will take care of preparation of *Information web-Portal of IBIR* including research and training database; organization of Open Days at IBIR (October 2010); *presentation* of the new lab of IBIR and opening of its doors *to business partners* from medicine and agriculture; joint actions with the *Bulgarian Scientists' Associations*; issue of *communication and publication materials*. The communication strategy of the project will be implemented during the whole project cycle and will ensure visibility of IBIR activities as well as cooperation with ReProForce 8 support partners.

Work Package 5 (WP5) activities are devoted to the Management and Coordination of ReProForce. Its leader is Margarita Mollova who will be responsible for the general management of all activities, coordination of all exchanges of visits, mobility activities for training of young researchers, for the financial discipline with strict observing of the regulations of Bulgaria and the European Commission.

Work Package 6 (WP6) with leader Margarita Mollova will provide the Institute with an external Research and Technological Development and Information strategy review by independent experts selected by the European Commission. The evaluation facility will further support the *recognition of IBIR as a visible research European center* and its integration into the European Research Area.

Program of the ReProForce Advisory Board Meeting:

- Kick-off meeting: first discussion over an overview of the research institutions in Europe that are involved in the research related to Biology and Immunology of Reproduction, definition of existing problems, mapping of common interests, determination of *narrower research themes for cooperation*.
- M12 - second meeting to developing an ac-

tion plan in order to deepen a further cooperation between IBIR and its 8 SP, to set time schedule for activities for the coming two years, to define a collaboration agenda and to agree on the general principles for establishing a collaborative research network.

• M24 - third meeting - launch of the QUALITY OF REPRODUCTION NETWORK. The network will essentially consist in exchange of knowledge and research results sharing. Rules to expand the network will be developed, roadmap and deadlines issued, and people in charge of planned activities appointed.

• M36 - final project meeting - discussion of new topics/projects of research cooperation for submission to EU programs, possible new members, and a continuing expansion of the network, dissemination of information.

„CAD/CAM/CAE IN INDUSTRY" – LABORATORY PROFILE

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Laboratory "CAD/CAM/CAE in INDUSTRY", Machine Technology Faculty, R&D division of Technical University - Sofia was established in 1990. Today it is continuously developing and improving as capabilities, using contemporary CAD/CAM/CAE technologies and the potential of its team of highly qualified engineers.

TECHNICAL INFRASTRUCTURE

The Technical Infrastructure of the laboratory is presented in Fig. 1. Laboratory facilities include internal network with connected power workstations for CAD and CAE activities, servers, power computational resources, a small documentation centre and full possibilities for rapid prototyping and tooling of developed parts, including micro sized products.

Laboratory partners are provided with engineering and consulting services at development and improvement of new products, their prototyping and industrialization for fast and effective realization of new ideas and minimizing the time to market.

Available activities are conceptual design, design, modelling and optimization, prototyping and interactive documentation creation as well as increasing of the CAD/CAM/CAE technology professional qualification.

The laboratory has successful continuous collaboration with companies from France, Italy, Germany and USA.

WORK TECHNOLOGY

The work technology is a multipurpose process - from 3D modelling and assembling through re-engineering of separate parts to product development (Fig. 2). Basic stages covered by the laboratory are: conceptional design; 3D design and assembly; FEM and Reliability analysis; Prototyping and Equipment Test.

There are sets of CAD/CAM software applicable to each stage.

The following CAD/CAM/CAE software is available in the laboratory: 3D modellers (AutoCAD (Autodesk), SolidWorks & CATIA (Dassault Systemes), Pro/ENGINEER (PTC)); analysis software (ANSYS family of products); NC/CAM programs (SurfCAM, Power Mill (Delcam)); reliability analysis software (RELEX); product data management software (Windchil, Audros).

This set of products entirely covers CAD/CAM/CAE technology. Additional possibilities are available through the existing experience and infrastructure of other laboratories in the university.

THE BASE LABORATORY ACTIVITIES

Conceptual design

• Product conception;
• Functional models;
• Virtual prototypes;
• Product prototyping by Rapid Prototyping / RP/ and NC technology.

3D Modelling (using Pro/ENGINEER , Solid

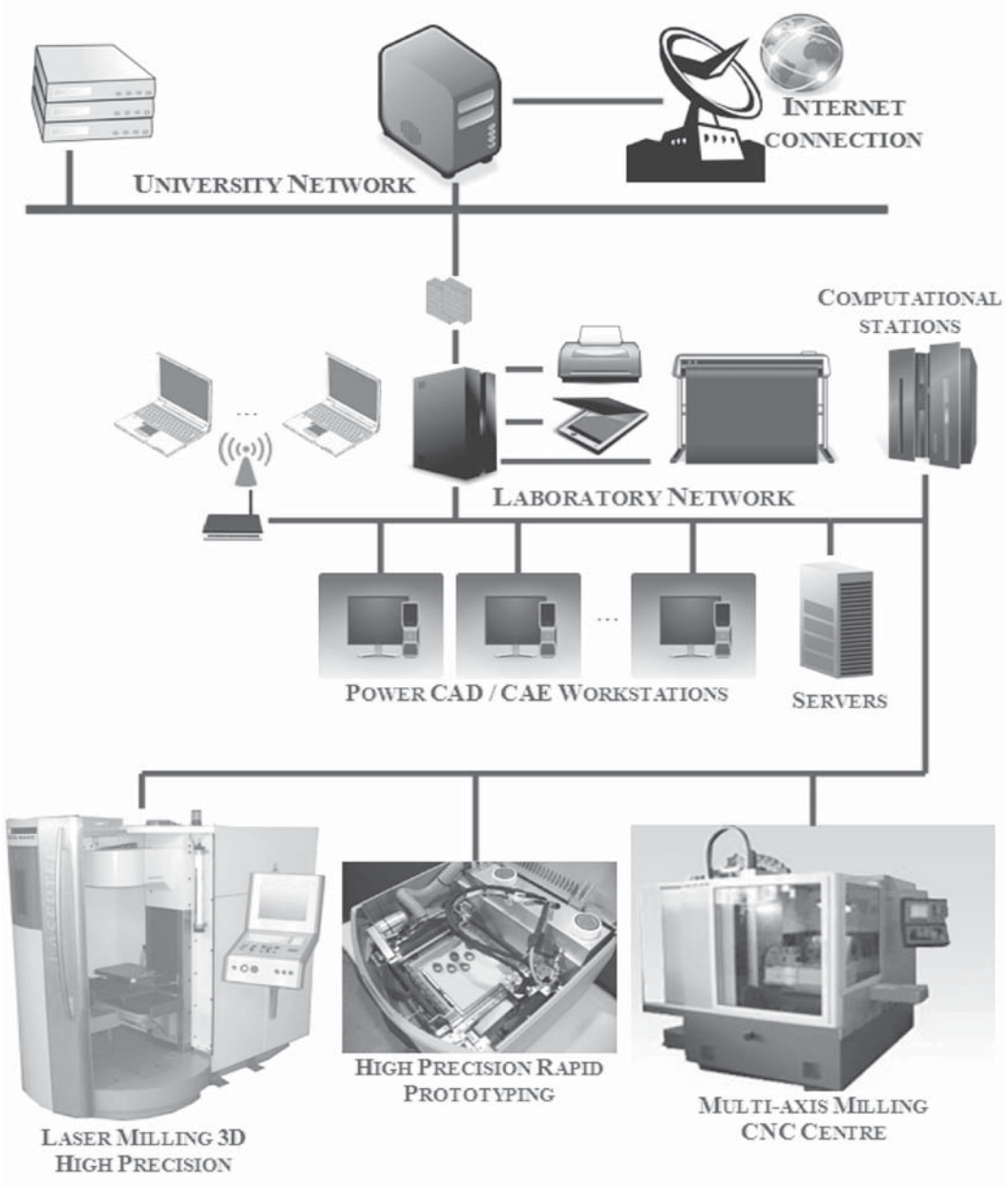

Fig. 1.

Works and CATIA V5)

- Complex 3D models;
- Assembly model;
- Product structuring and specifying;
- 2D documentation, based on 3D models, BOM Lists;
- 3D kinematics test

Structure Analysis and Optimisation of parts and assemblies by the finite element method (FEM) using available software:

- static - deflections and stresses in 3D models under complex load conditions;
- modal - natural frequencies and eigen vectors;
- dynamic - determination of the structures dynamic response under complex dynamic loads;
- contact - analysis of complicated 3D areas contact presures and deflections;
- thermal - including phase changes;
- plastic - modeling of the elasto-plastic deformations and processes;
- electromagnetic and electrostatic analysis;
- complex shape optimization of parts and assemblies using parametric optimization module.

These analyses allow the quality of the products to be increased and the period of development and prototypes and tests resources to be reduced. At the same time increased and predictable reliability of the products is assured.

Reliability Analysis

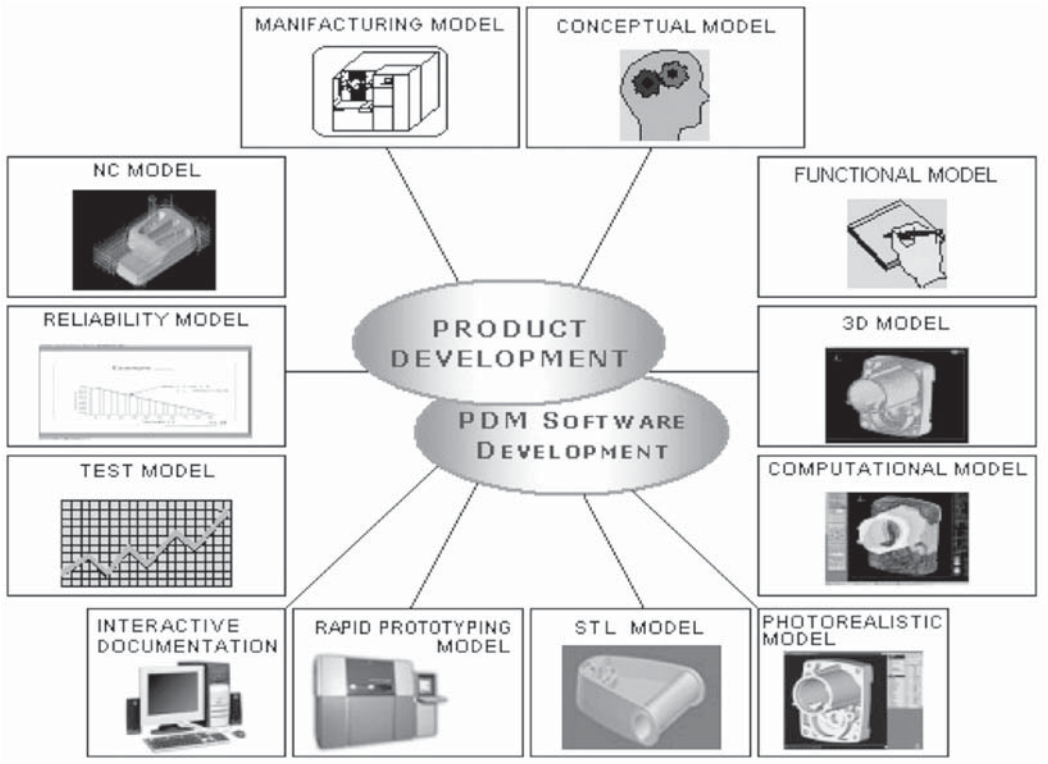

Fig. 2.

Reliability models are built by the RELEX software. This means that at the design stage it is possible to predict and balance the reliability parameters (as mean time between failures (MTBF); criticality, etc) of the product's separate parts. Through this product failure modes, effects and criticality analyses (FMECA) and maintainability analysis are also available.

The possibility of life cycle cost (LCC) analysis, i.e. design, conformed to the life cycle of the products (design, manufacture preparation, manufacture management, marketing, service, recyclability) is of general importance for the customers. The basic goal is to determine and to minimize the cost of ownership.

Interactive Technical Documentation

Interactive technical documentation creation - user manuals, catalogs, etc. on CD or HTML format for web publishing, including 3D models, graphics, animations, presentations.

PDM software development

• Conceptual Design and Data modeling of Industrial Software;

• Software Development: In DBMS and WEB design the software development team has the following experiences in Oracle 8,MS SQL Server 7.0, JAVA, JNI, SERVLET and JAVA BEANS.

Rapid Prototyping & Rapid Tooling

• Verification of Product conception;

• Product prototyping using RP using SLS (DTM SintherStation 2500 Plus);

• 5 axis NC technology;

• Functional models.

Pro/ENGINEER Demo Center

Laboratory "CAD/CAM/CAE in INDUSTRY" is a unique authorized Pro/Engineer's demo center by Bulgarian PTC's dealer. Present and future users may become familiar with software by presenting real projects manufactured by it. The laboratory performs consulting activities and technical support of users, based on its experience in work with Pro/Engineer.

The laboratory high motivated team provides effective and successful collaboration with industrial partners (SPARKY M&T, Berlin, GERMANY, tel: +49/30/20 39 16-0, Full product development of Power Tools; ASSETIUM - Villeurbanne, FRANCE, phone: +33 (0)478 684641; DBMS Development: PDM/Product Data Management/, PLM /Product Life-Cycle Management/ systems; SPARNEX N.V. Antwerp, BELGIUM, tel: +323/ 237 3330, Thermal and Reliability Analysis and research&design laboratories) **and research&design laboratories.**

MADE IN BULGARIA WITH EUROPEAN SUPPORT

DEVELOPMENT OF INNOVATION MODULES AND MECHATRONIC SYSTEMS FOR FUNCTIONAL REHABILITATION OF PARAPLEGICS AND PATIENTS IN A PERIOD OF CONTINUOUS IMMOBILITY

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The Institute of Mechanics at the Bulgarian Academy of Sciences since its foundation combines both sides of scientific investigations – fundamental investigations in mechanics and biomechanics and applicable investigation with innovative aspect.

Innovation modules for massage and acupuncture are presented further down, which have been build up in the process of development of biomechatronic systems for functional rehabilitation of paraplegics and patients in a period of continuous immovability.

ACTUALITY OF THE PROBLEM

After trauma or a disease of the spinal cord brain the neuro-muscular regulation is disturbed and as a consequence serious functional deviations of the support and locomotion human apparatus appear. Patients lose the ability for independent mobility and this is one of the most dramatic situations. The spinal-brain trauma divides the human living and the human himself into two parts. This is one of the most dreadful diseases, which medicine is still helpless to fight with. The nerves are saved, but the spinal cord brain does not respond, it is not stuck with. These patients are classified in the group of patients with the smallest perspective for health regeneration for life and social rehabilitation.

One important reason for appearance of this problem is the increasing transport traumatism. As a result of so many car crashes on a world scale, also unsuccessful jumps into water, diving and diseases of the spinal cord brain with different ethiology, every year the number of patients in heavy impediment (paraplegics) increases immensely.

This is the reason for seeking of effective methods and relevant devices for rehabilitation of patients in heavy locomotion impediment.

Investigations on the building up of biomechatronic systems in the Institute of Mechanics in BAS are performed within the frame of an international project between BAS and the Russian Academy of Sciences. On their part two world-known scientific institutes participate – the Pavlov Institute of Physiology in Saint-Petersburg and the Keldysh Institute of Applied Mathematics in Moscow.

United efforts of the three institutes are scheduled towards creation of principally new **automation systems for functional rehabilitation of paraplegics and patients in the period of continuous immobility.** The investigations are based on the influence on the neurons of the spinal cord brain by means of natural canals for connection – the receptor system of the limbs in conditions similar to those in a normal mobile activity. The systems developed within the frame of the project are scheduled preferably to rehabilitation of patients in **the**

early stage after trauma of the spinal cord brain with an objective: diminishing of the stage of atrophy of the bone-muscular system, linked with the absence of motion, and diminishing of the neuron conductivity (and its full termination 28 days after trauma); stimulation of the central locomotion generator of the spinal cord brain by means of the information current from the foot receptors, activated in conditions similar to those of natural walk (but without a real walk process); stimulation of the internal organs and systems of the patient in the period of continuous immovability; restoration of activity of the central locomotion generator of the spinal cord brain with the help of simultaneous application of epidural (electrical) and biomechanical simulations of the spinal cord brain.

The approach, the structure and functions of the newly created technical devices are a result of the mutual work of the scientific groups from the three institutes. The building up of the technical devices according to the mutual topic is performed on the basis of the technical project, according to the investigation on the functional specification, technical project, mechanical modules, controlling collectors and test programs of the new technical devices. The developing of the technical devices on the mutual topic is fulfilled on the basis of the technical project, prepared by the executive scientific groups from the three institutes.

The group of specialists from the Institute of Mechanics, BAS, Sofia, participating in the mutual project is responsible for development of the functional specifications, technical project, mechanical models, controlling collectors and test programs of the new technical devices.

The participants in the mutual project from the Keldysh Institute of Applied Mathematics, RAS, Moscow are responsible executors for creation of specialized software of the new technical devices and performing of technical experiments.

The scientists, participants in the mutual project from the Pavlov Institute of Physiology, RAS, Saint Petersburg, are responsible executors for creation of the healing methodology, laboratory and clinical experiments of the new technical devices.

Two innovation systems are developed:

1. Biomechanical system "GAITSIM" for rehabilitation of paraplegics by means of a reception activation of the foot, type "Walking".

2. Biomechatronic system "FOOTREACT" for a functional rehabilitation of paraplegics and patients in a period of continuous immobility by means of activation of the receptors in the zone of pedopuncture.

Both innovation mechanical modules are developed as a result of the work of Bulgarian scientists in the joint project (Device for a massage and a device for acupressure), executive mechanisms of the biomechatronic systems **"GAITSIM"** and **"FOOTREACT",** protected by a patent (2009).

CONFIGURATION OF THE SYSTEM "GAITSIM"

The system comprises: 1 – module for dynamic loading of the foot; 2 – module controller, 3 – computer for the system control /compatible with IBM PC/ (Fig.1). Two mutually equal modules 1 are included in the configuration for the left and right leg respectively. The devices perform feet loading according to a defined computer programme and according to the walk rhythm. A walk with a definite speed is simulated [1, 2, 4].

The periods with a singular and double support are realised according to the time history with the loading of each limb respectively.

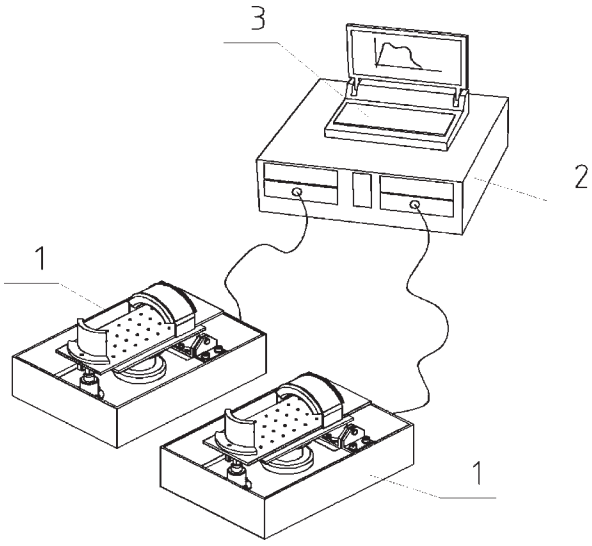


Fig. 1. Basic scheme of biomechatronic system **"GAITSIM"**

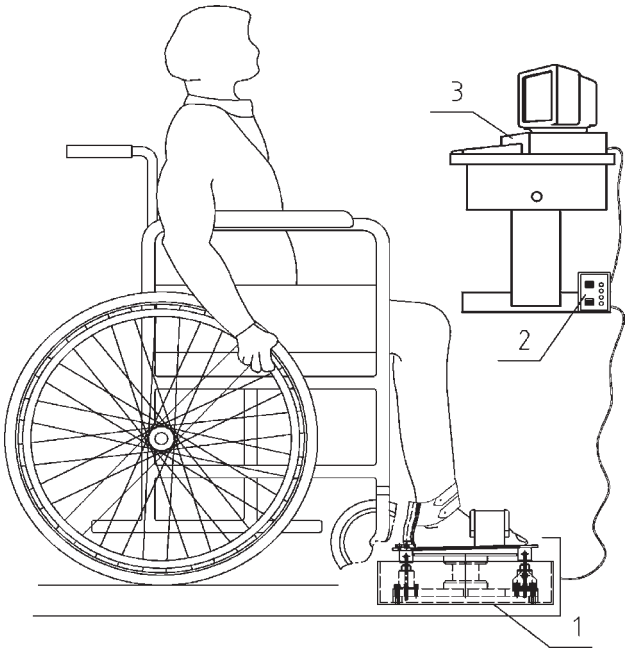


Fig. 2. View of system **"GAITSIM" with a patient**

Module for dynamic foot loading (patent №65911 – Device for massage)

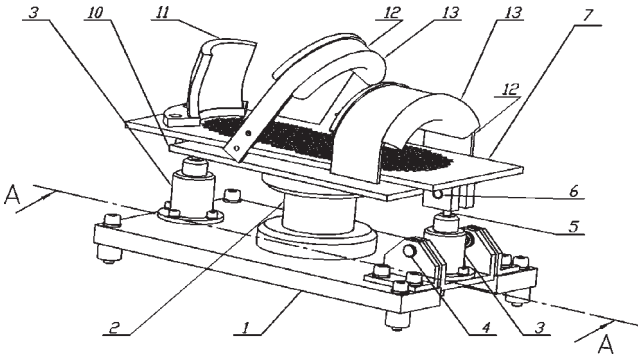
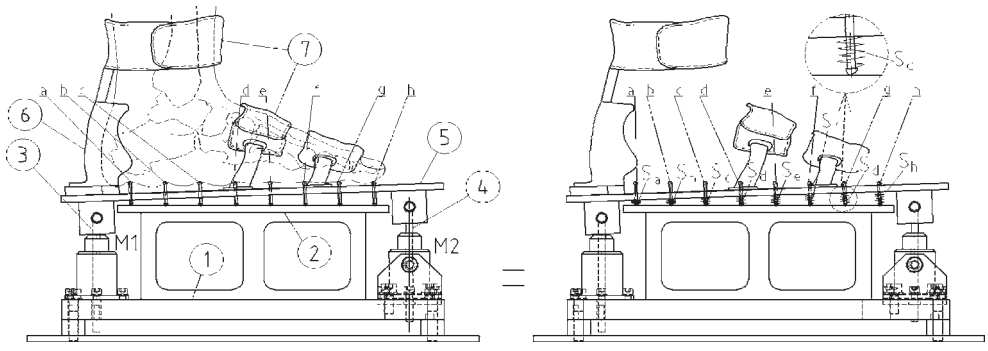


Fig. 3. Axonometric view for dynamic foot loading module

Model of the module for foot loading and reception activation in the walk rhythm

A virtual prototype of the mechanical module of a biomechatronic system is developed for locomotion rehabilitation of patients – paraplegics.



a) **b)**

Fig. 4. Model of the module for feet loading and reception activation in the walk rhythm

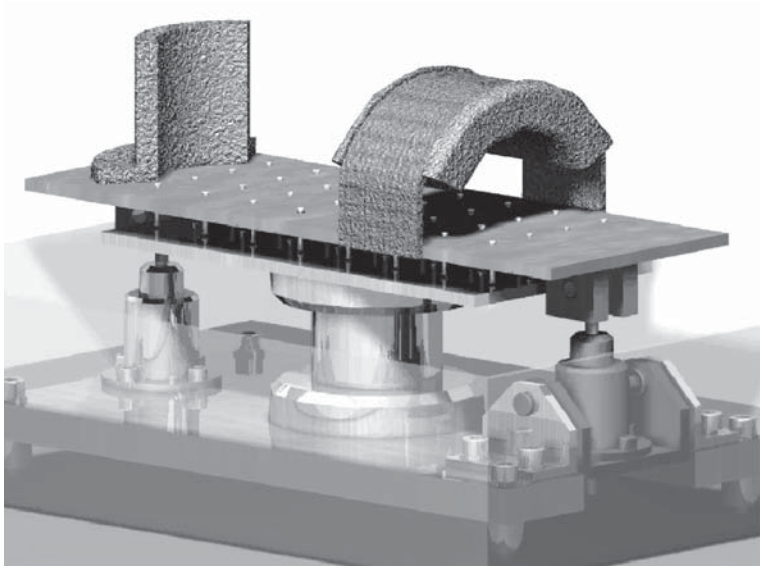


Fig. 5. 3D model for the mechanical module of the biomechatronic system

Motion simulation of the linear drives is performed on the basis of the input parameters and a special program product is implemented. The derived 3D virtual model allows the testing of different laws for the device control, the choice of the drive and selection of materials for manufacturing of separate elements.

Controllable parameters of the simulated walk are velocity and maximal speed of the foot loading. The conclusions derived after modelling and simulation help the successful realisation of the prototype of the innovation mechanical module and the specialised system.

TECHNICAL SOLUTION OF THE BIOMECHATRONIC SYSTEM "FOOTREACT"

The general scheme of the mechatronic system for the foot receptor stimulation comprises a device for the foot receptor activation (two in number), servo controller for the electric drives (two in number), a computer for the system control /compatible with IBM PC/ [3].

Module for foot basing, measurement and acupressure (patent №65876 – Device for acupressure)

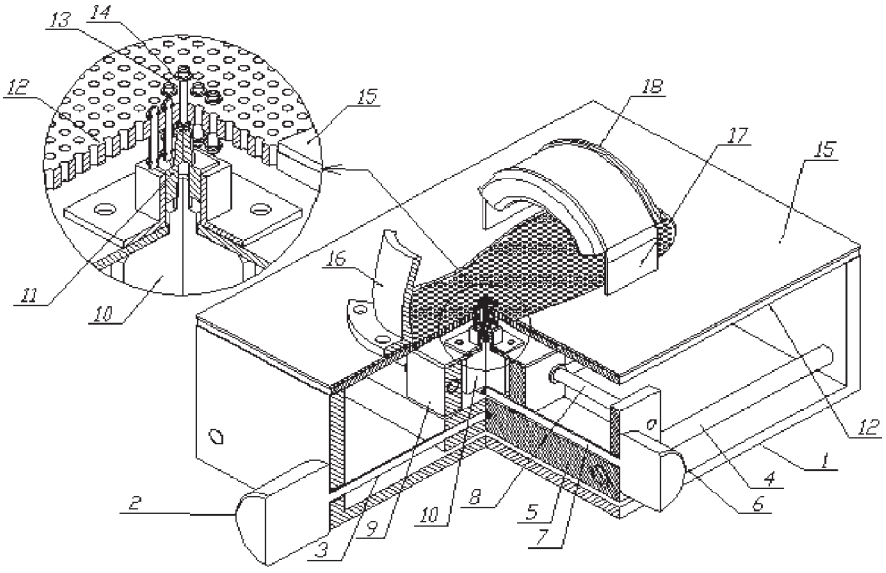


Fig. 6. Axonometric view of the module

Model of the module for the foot basing, measurement and acupressure

The derived model (Fig. 7) allows module function simulation: planned supporting of the measuring sensor, motion definition of the driven mechanism and performing acupressure on the foot in three ways – in the acupuncture point, along a given trajectory and on the pitch of projection of a definite human organ according to the method of the surface multi-needle acupuncture.

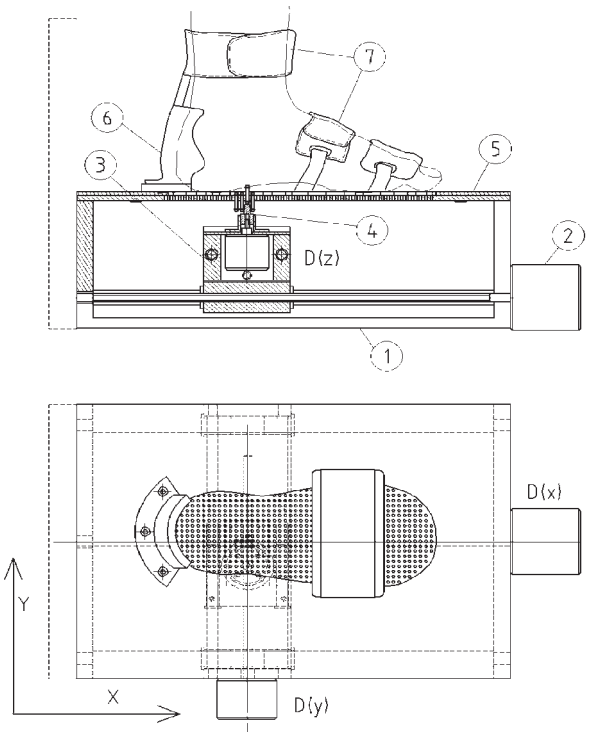


Fig. 7. Model of the module for foot basing, measurement and acupressure

Final notes

Forthcoming is the manufacturing of prototypes of the innovation modules and biomechatronic systems as a whole, as well as developing of their technical, laboratory and clinical investigation.

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AUTOMATED SYSTEM FOR COMPLEX NON-DESTRUCTIVE TESTING OF THE STRUCTURE AND MECHANICAL PROPERTIES OF METALLURGIC METERIALS

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INTRODUCTION

The requirements to the quality and reliability of machine-building products and equipment become increasingly strict, which necessitates investigation, development and industrial application of automated systems for non-destructive testing and diagnostics of the physical-mechanical properties and the structure of industrially used materials. Complex investigation and estimation of the characteristics of materials and devices, which uses simultaneously several non-destructive methods, combines and complements their possibilities. For this purpose we have developed a modular automated system "MULTITEST" for non-destructive control and testing of the structure, composition and physical-mechanical properties of machine building materials in laboratory and industrial conditions. It is based on the complex use of several physical methods (magnetic, acoustic, electrical, etc.), where each method can be used in combination with the others or independently.

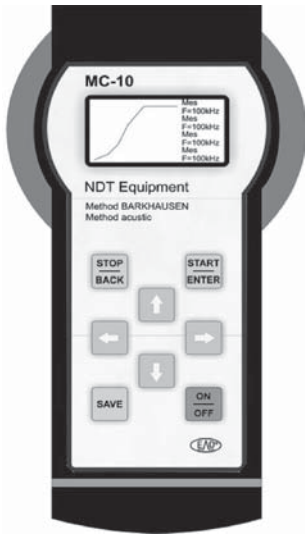
AIM OF THE WORK

The aim of the present work is to present main elements of the system and to describe the complete modular device. We present a complete independent device "MULTITEST MC 010" – a modular device for complex magnetic-noise and magneto-acoustic control of ferromagnetic materials. It can operate as a stand-alone device for non-destructive testing of machine-building samples or diagnostics of industrial equipment, and it can also be incorporated into a system for automated multi-parameter control [3,4]. We have performed measurements of the informative parameters of the chosen non-destructive methods – Barkhausen magnetic noise (BN) and magneto-acoustic emission (MAE), and we have tested their applicability for complex control of the properties of samples and details from structural steel and cast iron.

DEVICES, METHODS AND TESTED MATERIALS

The automated system "MULTITEST" consists of three parts – PC for automatic data processing, visualization and estimation of the testing results, as well as control of the appliances; modular devices for measurement of the non-destructive informative parameters; appliances (testing pliers, robots, manipulators) for holding, moving and classification of the tested samples, details and materials. The modular devices can operate with autonomous power supply and they can work independently or in a common automated system, depending on the tasks set [3]. The module "MULTITEST MC 010" uses methodology based on the effect of Barkhausen – magnetic noise method and magneto-acoustic emission method [2,3,4]. The informative parameters measured are the magnetic noise voltage E_{NB}, the magneto-acoustic voltage E_{MAE} and their dependences on the frequency f_B of the magnetic noise and the magnetizing current I_B. The outlook of the device is shown in Fig. 1. The buttons on the front panel of the device "MULTITEST MC 010" are as follows (see Fig. 1):

ON – turn on the device; ***OFF*** – turn off the device; ▶ – go to setting of the next parameter; ⩔ – choice of adjustable digit; ▼ – parameter set down; ▲ – parameter set up; **M** – manual mode of operation, adjustment of current and frequency, measures the magnetic noise of a chosen point; **I** – current scan mode, adjusted current scan limits, scan step and frequency; **F** – frequency scan mode, adjusted frequency scan



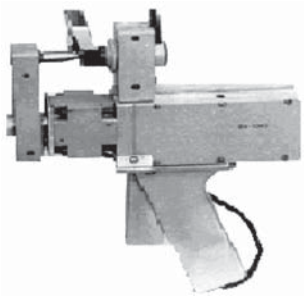


Fig. 1. Outlook of the modular device "MULTITEST MC 010" and the testing pliers CD 100

limits and current, measures the noise for the adjusted current and frequency from **F1** to **F2**; **Set** – adjustment of basic parameters; **Mem** – memory operation, assigns measurement number and step number, the results for the adjusted step are shown; **Sel** – rejection mode, setting of the voltage limits for certain current and frequency, the respective LED is on; **B** – battery status, shows the battery voltage; **L** – illumination of the screen; **START/STOP** – measurement beginning and end.

The new buttons of the "MULTITEST MC 010" device (left) are as follows:

Buttons ▲,▼,► and ⩊ increase and decrease the selected value, move between the adjustable parameters (cursor movement) and change the variable digit (change the variation speed). Continuous pressing of buttons up and down leads to increased speed of variation of the active digit. Values 9.99, 99.99 and 9999 correspond to values outside the range. The device operates in the following modes:

Setting of reference speed – ***Ce***; setting of reference size –***De***; Adjustment of the ultrasound tester (adjustment of the speed in the material) – ***SET***; Working mode – ***W***; Memory operation – button ***Mem***; button ***R*** is reserved for future modes; ***ENTER*** – serves for memorizing the adjusted values in modes ***Ce, De*** and ***SET,*** storing in the memory of the measured values in working mode and confirmation in Memory mode.

Fig. 2 shows a schematic view of appliance – testing pliers for complex non-destructive control with two informative parameters. The appliance for complex measurement of informative characteristics magnetic noise voltage and magneto-acoustic voltage, together with the modular device "MULTITEST MC 010" operates as follows: When releasing the trigger 1, the spring 6 and the manipulator arm 2 press with constant force the tested material 4 to the magnetic noise transducer 5 and the piezo transducer 3.

In order to determine the correlation between the non-destructive informative characteristics, the structure and mechanical properties of the tested materials, it is necessary to investigate comparative (reference) samples. For calibration of the system we have chosen reference sam-

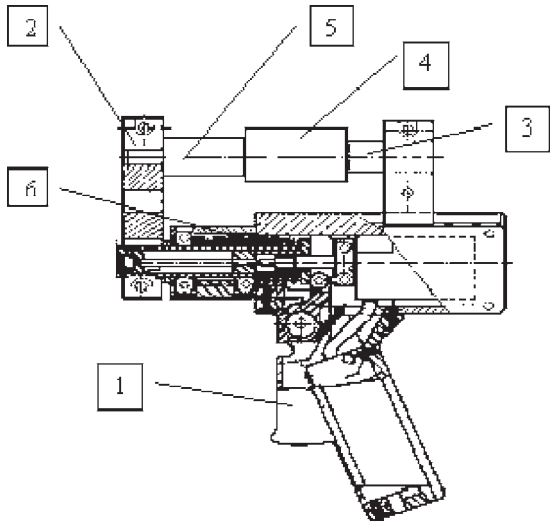


Fig. 2. Scheme of the appliance CD 100 for complex measurement of two non-destructive informative parameters

Table 1. Thermal processing of reference samples made of steel 40X

Groups of reference samples	Thermal processing				Hardness
	Annealing	Duration	Normalization	Duration	Mean value
	$T_z, ^oC$	t_z'	$T_o, ^oC$	t_z'	HB
1	890	20	-	-	395
2	890	15	400	30	365
3	890	15	480	30	300
4	890	15	610	30	245

ples made of structural steel 40X. The 40X structural steel samples are plane-parallel with Φ30x20 *mm* and different thermal processing, performed in inert medium of CO_2 (5 samples in each group). The type of thermal processing and the respective mean value of the Brinnel hardness for the reference samples are given in Table 1. The reference samples are classified into groups with similar structure and mechanical characteristics.

ALGORITHMS AND SOFTWARE PRODUCTS FOR AUTOMATIC COMPLEX NON–DESTRUCTIVE TESTING

The informative weight of the measured non-destructive parameters of the magnetic noise and magneto-acoustic emission – magnetic noise voltage E_{NB} and magneto-acoustic voltage E_{MAE} is based on their dependence on the frequency f_B and the magnetizing current I_B. Therefore, the dependences $E_{NB}(f_B)$ are analyzed for several groups of standard (reference) samples from the tested material at arbitrary values of the magnetizing current I_B. The obtained dependences are approximated with functions of the type [1]:

$$E_{NB} = (a_k f_B^2 + b_k f_B + c_k)^{-1} \quad (1)$$

Dependences (1) allow determining of the optical frequency $f_{B,\ opt}$ and thus – the dependences of the magnetic noise voltage E_{NB} on the magnetizing current I_B - $E_{NB}(I_B)$ [7]. The obtained dependences are approximated with functions of the type:

$$E_{NB} = a_k \exp\{ b_k [\exp(c_k I_B)]\} \quad (2)$$

Optimization of the control technology with respect to the frequency f_B and the magnetizing current I_B, is obtained through analysis of the dependences by using a procedure for finding the maximal differences between the values of the approximated data. Based on this methodology, we have developed an algorithm based on magnetic noise method for optimization of non-destructive informative parameters during complex testing of the structure and mechanical properties of ferromagnetic materials. Mathematical and software programs allow automatic adjustment of the frequency and magnetizing current values for different structural states of the tested ferromagnetic materials. Following a similar procedure according to Eq.(1) and (2), it is possible to optimize the measurements of the dependences of the second informative parameter – the magneto-acoustic voltage E_{MAE} – $E_{MAE}(I_B)$ and $E_{MAE}(f_B)$ and to determine the optimal magnetizing current for the testing.

In order to determine the correlation between the structure and characteristics of the tested material and the non-destructive informative parameters, as well as to formulate criteria for classification, we have developed algorithms and software programs to be used in the following sequence:

1. Device calibration

Each modular device has its own calibration algorithm. For the modular device "MULTITEST – MC 010", which measures the informative parameters of the magnetic noise and Barkhausen magneto-acoustic emission (magnetic noise voltage E_{NB} and magneto-acoustic voltage E_{MAE}), the dependences $E_{NB}(f_B)$ for several groups of reference samples of the tested material are obtained, for arbitrarily chosen value of the mag-

netizing current I_B. These dependences are approximated with functions according to Eq.(1) and (2) according to the algorithm described in [7] and thus, the optical regimes for measurement of E_{NB} and E_{MAE} are found.

2. Defining of characteristic areas

The so calibrated devices are used to measure the groups of reference samples. For each of the groups a trust region is determined, which allows to identify a real detail based on the testing results. The trust region can be automatically calculated by using statistical methods for experimental data processing or it can be chosen interactively with the computer mouse from the graphic window. The measurements of the next nondestructive informative parameters are repeated and characteristic areas for multi-parameter non-destructive control are defined. Each group of standard (reference) samples has its own characteristic area. When characteristic areas overlap, another more appropriate criterion or informative parameter is chosen to form the trust regions. The characteristic areas are visualized in a common graphic window.

3. Processing of raw data. Classification of real samples

After each measurement of the tested material it is classified into its corresponding trust region or characteristic area. The details or materials, measurement results of which do not fall within the required characteristic area, do not possess the required characteristics. For a modification of the automated system with a different modular device (for example for measurement of non-destructive informative parameters velocity and damping of ultrasound waves in the tested material), the program is changed in the part described in 1. – Processing of calibration data, while it is the same in part 2.

ANALYSIS OF THE EXPERIMENTAL RESULTS

In order to obtain reliable results from the non-destructive control, it is necessary to check the usefulness of the reference samples and their proper selection for definition of the classification criteria. This task can be performed manually after visual analysis of the obtained results, or automatically by means of an automatic system for analysis and evaluation of the experimental data, which is followed by the procedure for adjustment and criterion definition, according to the program described in part 2. As an example, Fig. 3 shows the magnetic noise dependences $E_{NB}(I_B)$ of the reference samples from Table 1, measured for "MULTITEST MC 010" in the frequency scanning region (10-100) *kHz*, in accordance with part 1. for device calibration.

It can be seen that for some samples it is not possible to perform the scanning up to the predefined limits of the magnetizing current, therefore such values of the current are too high for the specific task. A predefined value of the magnetizing current of $I_B = 80$ *mA* is automatically chosen, for which all samples are frequency scanned in order to determine the optimal frequency of the magnetic noise. Fig. 3b. shows the frequency dependences $E_{NB}(f_B)$ of the samples. They are approximated according to Eq.(1) and the optimal frequency f_B *(opt)* is determined by use of a special algorithm. A frequency f_B *(opt)* $= 55$ *kHz* is chosen, where the dispersion between the curves is maximal. Again the samples are scanned, this time at a frequency of 55 k*Hz*, in order to find the optimal current. The magnetizing current is scanned *n* by *m* times and in a similar way the optimal current *I(opt)* = 75 *mA* is is determined (Fig. 3c). Simultaneously, the magnetizing current for the second informative parameter (the magneto-acoustic voltage E_{MAE}) is scanned *n* by *m* times (Fig. 3d). It can be seen that the magnetizing current value of *I(opt)* = 75 *mA* is enough for the simultaneous measurement of the two informative parameters by means of the appliance of Fig. 2a. The optimized parameters *I(opt)* and f_B *(opt*) are stored in the device memory and are used for the *k* time measurements of each of the *n* by *m* samples. The results are stored in the memory and now the classification criteria can be defined. Fig. 4 shows a graphic representation of the characteristic areas of the groups of reference samples of Table 1. The areas are determined on the basis of the complex measurement of the informative parameters E_{NB} and and E_{MAE}.

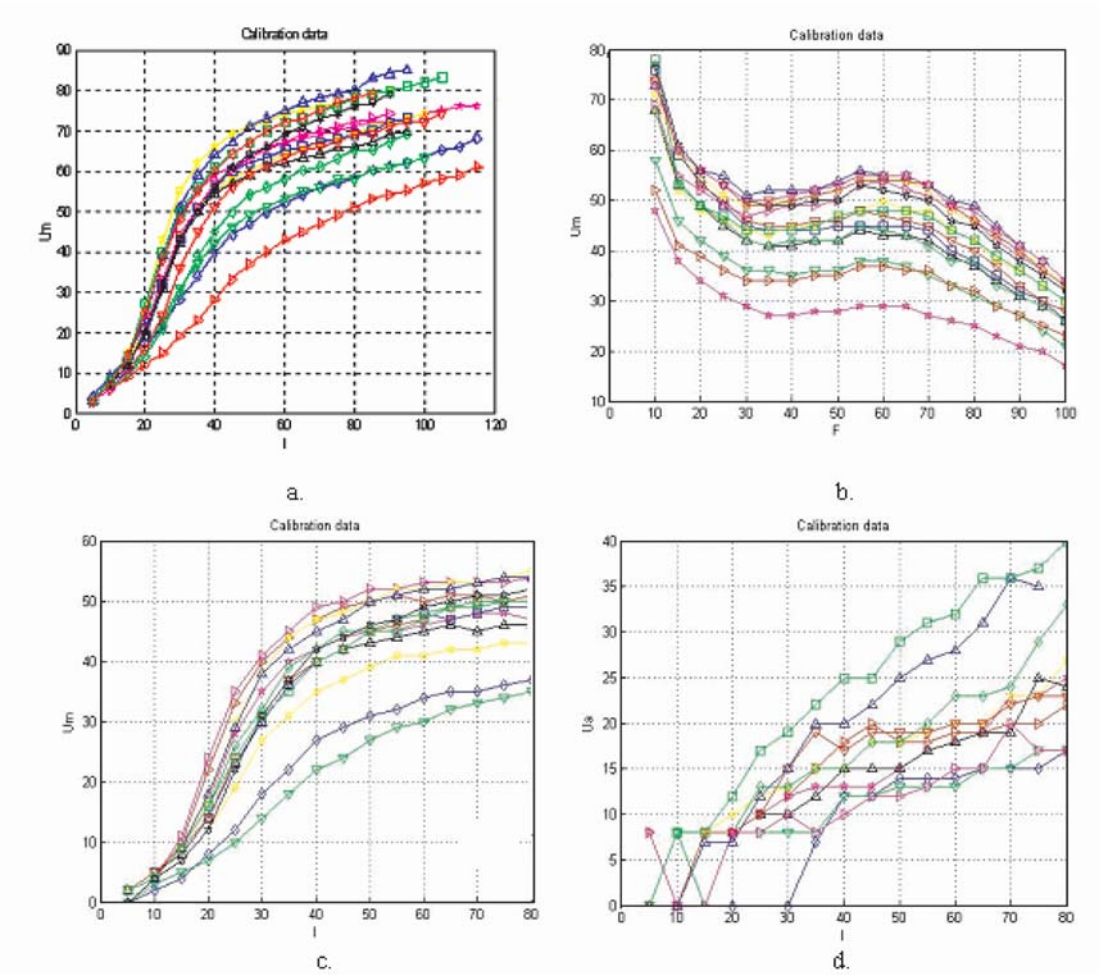


Fig. 3. Visualization of the calibration of the device "MULTITEST MC 010" by means of the reference samples

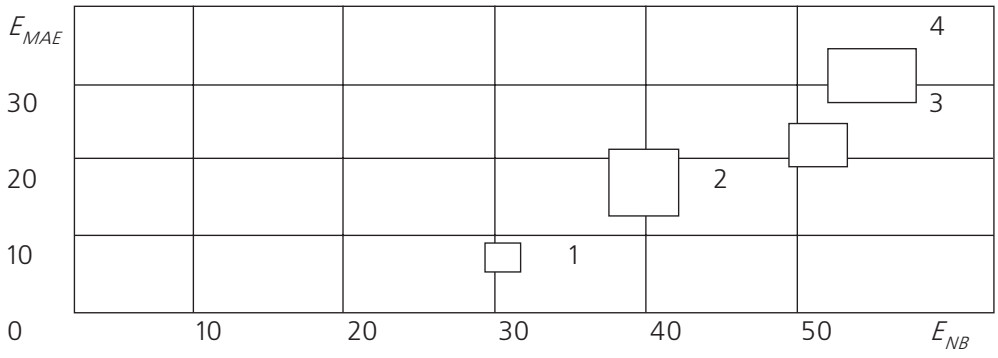


Fig. 4. Characteristic areas of the samples from steel 40X. The informative non-destructive parameters are the magnetic noise voltage E_{NB} and the magneto-acoustic voltage E_{MAE}

CONCLUSIONS

We have shown that the automated system for complex non-destructive control based on the modular device "MULTITEST - MC010" (which measure non-destructive informative parameters magnetic noise voltage E_{NB} and magneto-acoustic voltage E_{MAE}) can be used for automated classification of samples and details made of structural steel and separated into groups according to the thermal processing.

The use of computer control, software and statistical methods for data processing and evaluation for the system calibration and preparation of reference samples are a necessary prerequisite for reliable complex non-destructive control of the mechanical properties of machine-building materials and devices.

Acknowledgments

This work was carried out under a project funded by the European Regional Development Fund through the Operational Program "Development of competitiveness of Bulgarian economy", Contract № 02-22/09.

References

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HIGH PRECISION LASER CUTTING MODULAR SYSTEM

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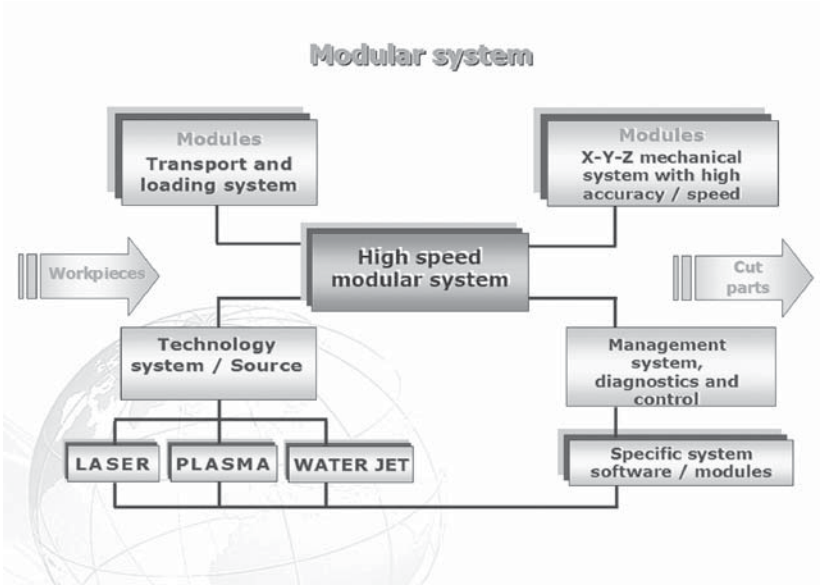
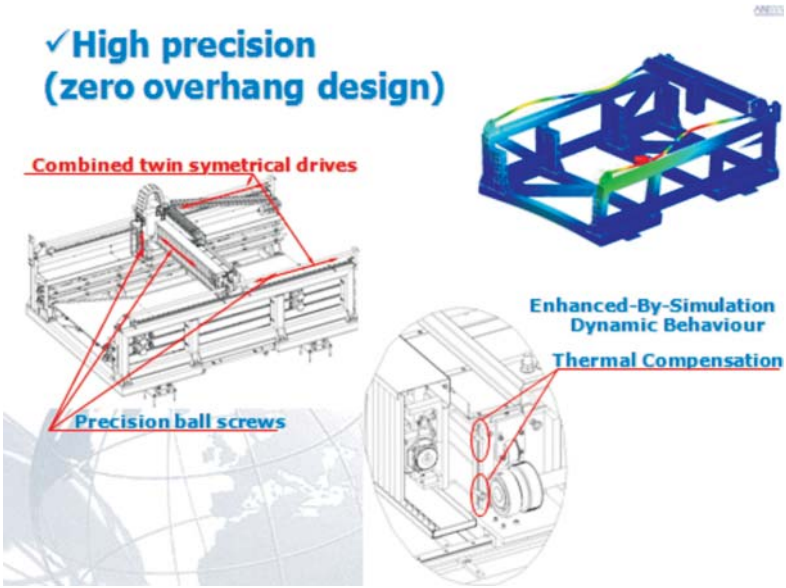
The project for development of a "MODULAR SYSTEM FOR HIGH SPEED PRECISE CUTTING OF SHEET MATERIAL WITH HIGH TECHNOLOGY SOURCES – LASER, PLASMA, WATER JET", with work title MasterCut was built by Laboratory "CAD/CAM/CAE in Industry" in MTF, Technical University – Sofia. Project's main target is to generate an innovative high speed precise cutting system for sheet metal and non-metal parts. This modular system allows to create cutting systems of different type, based on a set of base modules and additional assemblies and components, depending on the specifics of different technology processes and/or the specifics of customers needs – application, machining precision, workpiece dimensions, level of automation and other specific requirements. These systems are developed for mass, small serial or even single production. They are applicable for cutting different sheet materials up to 100mm thickness and maximal work area of 2000mm x 6000mm, using the most effective technology (laser, water jet or plasma) for each material, thickness or needed accuracy.

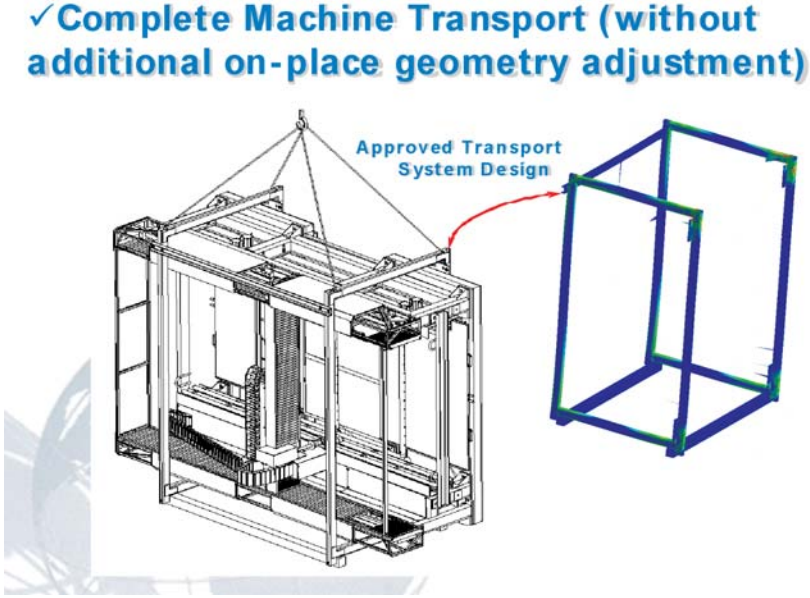
Direct result of performed industrial researches and marketing during the project development was an effective and competitive on the market product – cutting system using high technology sources, having main characteristics as:

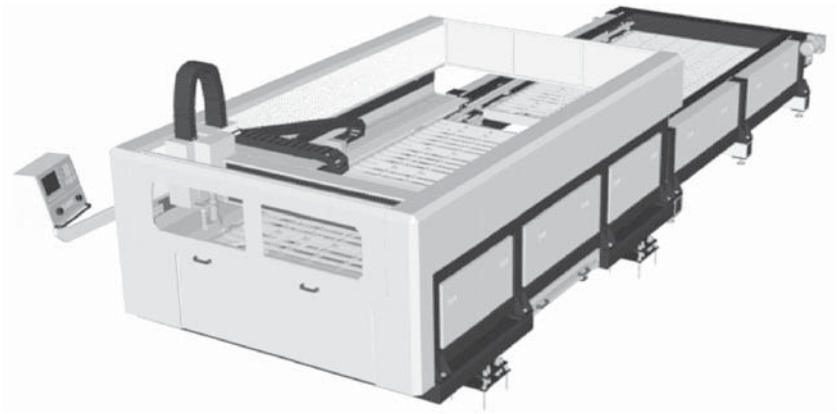
- Modularity – allows flexibility and maximal correspondence with customer needs;
- High performance and accuracy;
- Correspondent to the environmental/ecological requirements;
- Economic in resources and energy.

This project gives also possibilities for contemporary cutting technology transfer (espe-


Modular system
Modules
Transport and loading system
Modules
X-Y-Z mechanical system with high accuracy / speed
Workpieces
High speed modular system
Cut parts
Technology system / Source
Management system, diagnostics and control
LASER
PLASMA
WATER JET
Specific system software / modules


✓High precision
(zero overhang design)
Combined twin symetrical drives
Enhanced-By-Simulation
Dynamic Behaviour
Thermal Compensation
Precision ball screws


✓Complete Machine Transport (without additional on-place geometry adjustment)
Approved Transport
System Design



cially in the field of optics technology) to Bulgarian market, as well as to some traditional and new markets for Bulgarian manufacturers.

This project originates from the development strategies of the industrial companies that are participants in it, as well as from connected to them companies – existing and future customers of the product. This collaboration sets contemporary technical and exploitation characteristics at attractive for the cutomers end prices of the product. This allows the company owner of the product to go for expansion of its market share, bringing a competitive product to its existing and future partners.

Examined additional market potential is about 20 million year production, shared equally between the internal market and export. Bulgarian companies, customers of the developed cutting systems will gain additional indirect effect as they will reach decrease of about 40% of their initial investments and over 60% decrease of the expenses for post guarantee period.

High cutting Performance by Different Sources

Laser

+

- cutting, burning, engraving:
- for all types of metals (without high reflecting ones), some plastics, glass and wood;
- high cutting precision 0.05mm;

–

- heating, deformations and structural material changes;
- thin plate cutting – 3 to 10 mm.

Plasma

+

- thick workpieces – 8 to 40 mm.

–

- limited implementation – only for metal cutting;
- low cutting precision.

Water Jet Cutting

+

- for all types of materials;
- material thickness – 10 to 50 mm;
- without thermal material loads.

–

- low cutting precision 0.2 mm;
- high level of process noise;
- great quantity of wasted material.

System Innovations

• **High Efficiency and Precision** – high speed parameters, dynamics and precision – close to the leading world manufacturers at BETTER PRICE PERFORMANCE VALUE;

• **Modular Design** – short delivery terms and low product price of a machine with different cutting sources (laser, plasma, water jet);

• **Ecology** – corresponds to the actual European and world standards for laser safety and ecological requirements;

• **Low process cost** – decreased by 30-50% because of its high efficiency and precision;

• **High process automation** of loading/unloading from the work area leads to better work conditions.

EQUAL IN EUROPEAN RESEARCH AREA

BULGARIAN VIPs

Prof. ILIANA IONKOVA, DSc

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Professor on Pharmacognosy and Phytochemistry, Head of Section of Pharmaceutical Biotechnology, Department of Pharmacognosy at the Faculty of Pharmacy, Medical University of Sofia.

Prof. Iliana Ionkova was born in Veliko Tarnovo, Bulgaria. In 1979 she graduated in MSc degree Pharmacy, Medical University of Sofia. In 1983 she presented her PhD Thesis on "Phytochemical investigation of Astragalus genus". In the period 1983-1998 she works as an assistant professor, from 1999 she is an Associate Professor on Pharmacognosy and Phytochemistry. In 2008 she presented her DSc Thesis and since 2009 she is Professor on Pharmacognosy and Phytochemistry at the Faculty of Pharmacy, Medical University of Sofia. Prof. Ionkova is a member of 4 international professional societies. She delivered lecture courses and seminars on Pharmacognosy and Pharmaceutical biotechnology for bachelor, master degree and PhD students.

During 1987-1989 and many years later she worked at the University of Dusseldorf, Germany; with the group of Prof. Alfermann, Prof. Proksh, at the University of Graz, Austria; with Prof. Kartnig, etc. She is an Invited lector at University of Dusseldorf Germany, Vrije Universiteit Brussel, Belgium, Karl-Franzes-University of Graz, Austria. She is a board editor of many international scientific journals - Pharmacognosy Magazine, Pharmacognosy Review, Pharmacognosy Research, Pharmacy and invited reviewer in international scientific journals. She is a member of the Expert commission within the Sixth and Seventh Framework Program of the European Union - SEE-ERA.NET. In 2008 she was elected Expert at European Directorate for the Quality of Medicines & Health Care on Phytochemistry (European Pharmacopoeia) – EDQM, Strasbourg, France.

Prof. Ionkova is a coordinator of 5 international scientific projects financed by NATO, UNESCO, Conference of German Academies of Sciences, DFG, BMFT, Germany and of 19 projects financed by National Science Fund and Scientific Fund of Medical University of Sofia. In 2006 she received Scientific Awards - Badge of Honour "Signum Laudis pro scientiae meritis" for the best research work in the field of Pharmacy.

Prof. Ionkova is an author of more than 100 papers in international scientific journals, 11 International Books, 2 Patents and more than 60 Reports and published contributions to international congresses. Her scientific work and achievements are cited in more than 200 international journals, patents, and monographs.

Prof. IVAN GATSOV, DSc

Department of Archaeology, New Bulgarian University
Phone: +359 2 8110 289, E-mail: igatsov@nbu.bg

Head of the Department of Archaeology at New Bulgarian University; Corresponding member of the German Archaeological Institute (DAI).

Prof. Ivan Gatsov received his MSc degree in the Department of History at Sofia University in 1972 and a PhD degree at the Archaeological Institute of Jagiellonian University, Krakow, Poland. He defended his doctoral thesis on the topic of "The Archaeological Cultures of the Late Pleistocene and Early Holocene in the Western Black Sea region and their Significance for the formation of the Neolithic Industries" in 1978.

Prof. Gatsov defended his DSc thesis "Prehistorical Chipped Assemblages from Eastern Thrace and Marmara region – VII-VI mill. BC" in 2007.

He is currently leading the Department of Archaeology at New Bulgarian University in Sofia and acts as an Associated Professor at the National Institute of Archaeology with Museum at the Bulgarian Academy of Sciences. His professional field is lithich technology – Neolithic, Chalcolithic and Bronge Age periods in Eastern Balkan and NW Turkey.

Prof. Ivan Gatsov has participated in archaeological excavations and processing the material of many sites in Bulgaria ("Temnata" cave, "Bacho Kiro" cave, Karanovo, Drama), Vietnam (Dong Khan and Rock Shelter Dieu), Turkey (**Hoça Çeşme, Aşağı Pinar, Troia, Kanlıgeçit, Ilıpinar, Menteşe, Yenibademli Höyük, Küllüoba, Barcin settlements**), Greece (Micro Vouni, Samothrace), Romania (Pietrele). These projects are held in partnership with international organizations such as Istanbul University, Tubingen University, German Archaeological Institute (DAI) – Berlin, Netherlands Institute in Turkey.

Prof. Ivan Gatsov is a former Mellon Research Fellow in Turkey with a grant from the American Research Institute in Turkey for the project: "Prehistoric Chipped Stone Assemblages and the Problem of Connections between Anatolia and Thrace" and in Greece; British Academy of Sciences Lecturer in 2003.

He has 3 monographic publications on materials researched in Turkey and Bulgaria, and over 50 co-authored publications and articles. He has published 2 students' guide books.

Prof. Gatsov is a Corresponding member of the German Archaeological Institute (DAI) since 2009.

Member of Society for American Archaeology; representative for Bulgaria in ARCANE; Member of Zentrums fur Archäologie und Kulturgeschichte des Schwarzmeerraumes, Hale, Germany; Member of SAA PQEM Group: Prehistoric Quarries and Early Mines Interest Group, USA.

Assoc. Prof. DIMITAR MITOV, PhD

Institute of Neurobiology, Bulgarian Academy of Sciences
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Coordinator of the Department Sensory Neurobiology and Head of the Vision Information Processing Laboratory, Institute of Neurobiology at Bulgarian Academy of Sciences.

Mr. Dimitar Mitov graduated from St. Petersburg Polytechnic University, Russia. He defended his MSc degree in 1971 and his PhD thesis "Investigations of some temporal characteristics of grating perception" in 1983 at the Institute of Physiology (now Institute of Neurobiology), Sofia. He specialized at Laboratory of Physiology of vision, Pavlov's Institute of Physiology, St. Petersburg, Russian Academy of Sciences, as well as at the Department of Psychology, University of California in Los Angeles, USA.

Dimitar Mitov is an Associate Professor at the Institute of Neurobiology, Bulgarian Academy of Sciences. He is the coordinator of the department "Sensory Neurobiology" and the head of the "Vision information processing" laboratory. More specifically he has been working on spatio-temporal interactions in the visual system, motion perception, reaction time, visual evoked potentials as well as on development of PC equipment for visual investigations.

Assoc. Prof. Dimitar Mitov has about 30 publications, about 220 citations and over 20 presentations at international conferences. He is a member of the Bulgarian Society of Physiological Sciences.

Participation in research projects:

• 1994-1997: "Luminance and chromatic signals in structure and motion perception". Sponsored by National Science Research Fund, Ministry of Education and Science. Principal investigator.

• 1998–2002: "Mechanisms extracting image contours in human visual system. Spatial and temporal properties". Sponsored by National Science Research Fund, Ministry of Education and Science. Principal investigator.

• 1998-1999: "Visual perception and motor control" - with the University Louis Pasteur - EP 618 du CNRS, Strasbourg, France. Sponsored by the University Louis Pasteur. Participant.

• 2002–2004: "Human vision: a psychophysical search for the S-cone OFF channel" - with the Vision Science Research Group, University of Ulster, Coleraine, U. K. Sponsored by the Welcome Trust. Participant.

• 2009–2012: "Sensory and cognitive processes in perception of brightness and contrast defined visual stimuli. Investigations in healthy volunteers and patients with diabetes type 2". Sponsored by National Science Research Fund, Ministry of Education, Youth and Science. Principal investigator.

Assoc. Prof. GEORGI TODOROV, PhD

Department of Machinetools and Machinebuilding, Technical University of Sofia
Phone: +359 2 965–2574, +359 2 965–3323; E-mail: gdt@tu-sofia.bg

Associate Professor in "Computer-Aided Engineering and CAD systems", Author and co-author of more than 70 papers in scientific journals and conference proceedings, Dean of Machine Technology Faculty.

Education and Career

Professor Dr. Georgi Todorov was born in 1961 in Haskovo. He graduated from Technical University of Sofia, Faculty of Machine Technology in 1986. In 1987 he gained a Master degree in Robotics and Applied Mathematics, Technical University of Sofia. Specializes in Staffordshire University, UK in 1993 and 1994. In 2002 he specializes in computer design in Yokohama, Japan. In 1998 he defended his PhD thesis "Structural Design and Parametric definition of the Frontal Rotary Milling systems" supervised by Prof. Alexander Lubenov. His professional activity began in Sofia Technical University in 1987 as Assistant in the Machinetools and Machinebuilding Department, MTF. He is an Assistant Professor of "Computer Aided Engineering and CAD systems" (2002). Since 1992 he heads the Laboratory "CAD / CAM / CAE in industry", MTF; from 2009 he was head of the "Center for Virtual Engineering" of the Technical University of Sofia, in the same year he founded "Center of Excellence" at Technical University of Sofia. Since 2008 he has been the deputy dean of the MTF on "Scientific research" and since 2010 he is dean of the MTF.

Major areas of research and teaching

Theory of mechanisms, machines and automatic lines, mechanics, synthesis and analysis of mechanisms, special and specialized robotics

Publications

Author of over 70 scientific publications, leader of more than 80 R&D projects.

Inventions

11 inventions related to various technical solutions of industrial projects. Member of the National Scientific and Technological Society of Automation and Informatics.

Awards

'Icarus' Award of the Bulgarian Industrial Association for 2008 for excellence in the field of pneumatic hammer mechanisms. "Pythagoras" Award of the 2009 Fund for Scientific Research in Education of Applied Sciences, First prize for "Innovative research organization" as head of the scientific team, achieved significant results in developing research projects in 2009.

Expertise and advisory capacity

Development and dimensional design (synthesis and analysis) of machines and systems, appliances and devices for different purposes, mechatronic devices, MEMS, materials handling equipment, machine tools and tools for flexible automation. Engineering analysis and simulation, optimization systems, CAD / CAM / CAE technologies, PLM, PDM.

AWARDS

PRESIDENT GEORGI PARVANOV HANDED JOHN ATANASOFF AWARD FOR THE YEAR 2010

At an official ceremony on October 4, 2010 President Georgi Parvanov bestowed **John Atanasoff Award**. The carrier of the award is Dr. **Peter Popov**. The award is bestowed for his contribution to the development of computer and information technologies.

In 1999 Peter Popov graduates in applied mathematics from Sofia University St. Kliment Ohridski with specialization in mechanics. He defends his doctoral degree and works as a researcher at Texas A&M University. Since 2009 he is a researcher at the Institute of Parallel Processing of Information at Bulgarian Academy of Sciences.

Peter Popov is a co-author of the book on mathematical modeling published by Springer (2008). He is the author of 19 scientific works published in journals and proceedings of prestigious international conferences.

He works in the field of numerical methods for solving problems connected with interaction between fluid and elastic bodies. The results of the offered by him methods find application in medicine in development of cardiologic stents, as well as in geology. At present he works on an original international project with Zurich Polytechnic connected with creation of methods for individual diagnostics of osteoporosis.

A diploma for contribution connected with the development of computer and information technologies was handed to **Kuzman Ganchev**. He graduates from a college in Pennsylvania and works in the sphere of machine self-education and computer linguistics. Since autumn 2010 he works for Google.

For the first time this year the head of state handed the newly-established **Diploma for applied projects in the field of e-government and information society development in Bulgaria** to **Peter Georgiev**, who works in the sphere of new software technologies. He has over 22 research projects for the government, and BULSTAT system is his deed.

Anton Atanasov became carrier of the **John Atanasoff Award for students**. He graduated from National High School of Mathematics and Natural Sciences in the town of Haskovo and took part in over 34 national and international mathematical Olympiads. He is silver medal winner at the 16-th Balkan Olympiad (2008), bronze medal winner at the 21-st International Olympiad in Plovdiv, gold medal at the 17-th Balkan Olympiad (2009), gold medal at the Central European Olympiad, silver medal at the 22-nd International Olympiad (2010) in Canada.

The award for achievements in the development of information society in the name of the famous scientist with Bulgarian origin and creator of the first in the world computer John Atanasoff was established by President Parvanov in 2003. It is adjudged every year to a young Bulgarian who has considerable contribution to the development of computer and information technologies and information society in Bulgaria.

SCIENTISTS DISTINGUISHED IN THE CONTEST OF THE UNION OF SCIENTISTS IN BULGARIA FOR HIGH SCIENTIFIC ACHIEVEMENTS IN 2010

YOUNG SCIENTISTS

Diploma and prize money

Res. Assoc. TODOR IVANOV ARABADZHIEV, PhD from Laboratory of biomedical engineering Prof. Iv. Daskalov at BAS for 11 publications in the field of biophysics of extracellular potential fields generated from transversely furrowed muscle fibers. Seven of his publications are in specialized international journals with impact factor and have 18 citations in international scientific works

Honorary Diploma for research achievements

Res. Assoc. MILENA GEORGIEVA KIRILOVA, PhD from the Institute of Molecular Biology at BAS for 8 scientific works and developed new method for chromatin investigations at single cells level.

Res. Assoc. YANA IVAILOVA SIMOVA, PhD from Specialized hospital for treatment of cardiovascular disorders for 9 research publications in prestigious journals, three of which have high impact factor and 43 citations.

SCIENTISTS OVER 35 YEARS OLD

Honorary Diploma for research achievements

Monograph "Geology of Bulgaria", vol. II, part "Mesozoic Geology", Prof. M. Drinov Publishing House, S., 2009, 766 pp. It is created by a team of authors with participation and under the editorship of **Corresp. Members Ivan Zagorchev** and **Hristo Dabovski** and **Acad. Todor Nikolov**.

Diploma and prize money

Prof. RUMEN GEORGIEV PANKOV from Cytology, Histology and Embryology Chair at Biological faculty of Sofia University St. Kl. Ohridski for 12 publications, 7 of which in prestigious international journals with impact factor, connected with organization and functions of biological membranes.

Diploma for research achievements

Corresp. Member ATANAS DIMITROV KOVACHEV from Forestry University for the monograph "Town and Country Planning" (second revised and enlarged edition). Sofia – Moscow. PENSOFT Publishing House, 2009, 403 pp.

Prof. GARO HUGASOV MARDIROSYAN from the Space Research Institute at BAS for the monograph "Natural Disasters and Ecological Calamities (study, prevention, protection)". Prof. M. Drinov Acad. Publishing House, Sofia, 2009.

Diploma for socially important work

Assoc. Prof. PETKO STEFANOV PETKOV from St. Cyril and St. Methodius University of Veliko Turnovo for the monograph "One Hundred and More Years Ago – Investigations and Essays on the New History of Bulgaria". St. Cyril and St. Methodius University Publishing House, V. Turnovo, 2009, 292 pp.

Chief Assist. Prof. KALINA VELCHEVA GALUNOVA from St. Cyril and St. Methodius University of Veliko Turnovo for the monograph "Uncle Ganyo the Bulgarian: an Attempt over Bulgarian National Psychology and Bulgarian Modernity". Book One. Faber Publishing House, V. Turnovo, 2009, 384 pp.

TZANKA STOYANOVA KONSTANTINOVA, PhD from Sofia for the monograph "Toponymy of Kazanluk Region", St. Cyril and St. Methodius University Publishing House, V. Turnovo, 2008, 940 pp.

Diploma for scientific applied contribution

Chief Assist. Prof. ANTON KIRILOV GROZDANOV from the Law faculty of Varna Free University "Chernorizets Hrabar" for the monograph "Contract for Sea Transportation of Cargoes". VFU Ch Hrabar Publishing House, Varna, 2008, 271 pp.

NIKOLAY PEYCHEV POPPETROV from the Institute for Historical Studies at BAS for two books: monographic study "Fascism in Bulgaria: Development and Activities", Kama Publishing House, Sofia, 2008, 126 pp., and collection of works "Social – to the Left, Nationalism – For-

ward (program and organizational documents of Bulgarian authoritarian nationalistic formations for the period before 1944)". Guttenberg Publishing House, Sofia, 2009, 900 pp.

AWARDS FROM THE CONTEST OF UNION OF SCIENTISTS IN BULGARIA AND THE SUPREME ATTESTATION COMMISSION SAC FOR RESEARCH ACHIEVEMENTS OF DOCTORAL STUDENTS IN 2010

Young researches under the age of 35 who successfully defended their dissertations in 2009 and claiming scientific achievements in their works also take part in the contest for 2010.

Diploma and prize money

Research Assoc. PhD IRENA EMILOVA ANDONOVA from the Institute of Experimental Pathology and Parasitology at BAS for research achievements in the dissertation on "Investigation of Genetic Polymorphisms of Glutathione – S – Transferases in European Populations and Association with Multifactor Disorders".

Chief Assist. Prof. PhD DIANA VALENTINOVA CHESHMEDZHIEVA from Applied Organic Chemistry Chair at Faculty of Chemistry at Sofia University St. Kliment Ohridski for scientific achievements in the dissertation "Mechanism and Reactivability at Alkaline Hydrolysis of Amides".

Diploma for research achievements

Senior Assist. Prof. PhD EMILIA DIMITROVA RAEVA from Mineralogy and Petrography Chair at St. Ivan Rilski University of Mining and Geology for scientific achievements in the dissertation "Geochemistry of Granites and Metagranites from the Upper Course of the Arda River, Central Rhodope Mountains, Bulgaria".

ARTICLES

RECENT PUBLICATIONS OF BULGARIAN SCIENTISTS

Title: **Management of students' participation in e-learning collaborative activities**

Authors: Daniela Tuparova[1], Georgi Tuparov[1,2]

Source: Procedia - Social and Behavioral Sciences, Vol. 2, 2, (2010), 4757-4762, Innovation and Creativity in Education

Author Affiliations: [1]South West University "Neofit Rilski", 66, Ivan Mihailov Str., 2700 Blagoevgrad, Bulgaria;
[2]Institute of Mathematics and Informatics, Bulgarian Academy of Sciences, 8, Acad. G. Bonchev Str., 1113 Sofia, Bulgaria.

ISSN: 1877-0428

Title: **The application of the derivative IR-spectroscopy and HPLC–ESI-MS/MS in the analysis of archaeology resin**

Authors: S. Zareva, I. Kuleff

Source: Spectrochimica Acta, Part A: Molecular and Biomolecular Spectroscopy, Vol. 76, 2, (Jul. 2010), 283-286

Author Affiliations: Faculty of Chemistry, Sofia University "St. Kliment Ohridski", 1, James Boucher Blvd., 1164 Sofia, Bulgaria

ISSN: 1386-1425

Title: **Optical characterization of thin chalcogenide films by multiple-angle-of-incidence ellipsometry**

Authors: R. Todorov[1], A. Paneva[2], K. Petkov[1]

Source: Thin Solid Films, Vol. 518, 12, (2 Apr. 2010), 3280-3288

Author Affiliations: [1]Central Laboratory of Photoprocesses, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 109, 1113 Sofia, Bulgaria;
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ISSN: 0040-6090

Title: **Radiation measurements inside a human phantom aboard the International Space Station using Liulin-5 charged particle telescope**

Authors: J. Semkova[1], R. Koleva[1], St. Maltchev[1], N. Kanchev[1], V. Benghin[2], I. Chernykh[2], V. Shurshakov[2], V. Petrov[2], E. Yarmanova[2], N. Bankov[3], V. Lyagushin[4], M. Goranova[5]

Source: Advances in Space Research, Vol. 45, 7, (2010), 858-865

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ISSN: 0273-1177

Title: **Ozone decomposition on Ag/SiO_2 and Ag/clinoptilolite catalysts at ambient temperature**

Authors: Penko Nikolov[1], Krassimir Genov[2], Petya Konova[2], Katya Milenova[1], Todor Batakliev[1],Vladimir Georgiev[1],Narendra Kumar[3],Dipak K. Sarker[4],Dimitar Pishev[5], Slavcho Rakovsky[1]

Source: Journal of Hazardous Materials, Vol. 184, 1-3, (15 Dec. 2010), 16-19

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ISSN: 0304-3894

Title: **Functionalized electrospun mats from styrene–maleic anhydride co-polymers for immobilization of acetylcholinesterase**

Authors: O. Stoilova[1], M. Ignatova[1], N. Manolova[1], T. Godjevargova[2], D. G. Mita[3,4], I. Rashkov[1]

Source: European Polymer Journal, Vol. 46, 10, (Oct. 2010), 1966-1974

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ISSN: 0014-3057

Title: **A novel parallel-field double-Hall microsensor with self-reduced off set and temperature drift**

Authors: Siya Lozanova, Chavdar Roumenin

Source: Procedia Engineering, Vol. 5, (2010), 617-620, Eurosensor XXIV Conference

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ISSN: 1877-7058

Title: **The combination of neuronavigation with transcranial magnetic stimu lation for treatment of opercular gliomas of the dominant brain hemi-sphere**

Authors: T. Shamov[1], T. Spiriev[2], P. Tzvetanov[3], A. Petkov[1]

Source: Clinical Neurology and Neurosurgery, Vol. 112, 8, (Oct. 2010), 672-677

Author Affiliations: [1]Department of Neurosurgery, Medical Military Academy, 3, St. Georgi Sofiiski Str., 1606 Sofia, Bulgaria;

[2]Department of Neurosurgery, Tokuda Hospital-Sofia, 51b, "Nikola Vaptsarov" Blvd., 1407 Sofia, Bulgaria;
[3]Department of Neurology, Medical Center "Medica 2005", 3700 Vidin, Bulgaria.
ISSN: 0303-8467

Title: **Energy demand for selected bread making processes: Conventional versus part baked frozen technologies**
Authors: Alain Le-bail[1], Tzvetelin Dessev[2], Vanessa Jury[1], Ruben Zuniga[1], Thomas Park[3], Martin Pitroff[4]
Source: Journal of Food Engineering, Vol. 96, 4, (Feb. 2010), 510-519
Author Affiliations: [1]UNAM – ENITIAA – GEPEA – UMR CNRS 6144, BP 82225, F-44322 Nantes Cedex 3, France;
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ISSN: 0260-8774

Title: **A new asymptotic bound of the minimum possible odd cardinality of spherical (2k–1)–designs**
Authors: Peter Boyvalenkov[1], Maya Stoyanova[2]
Source: Discrete Mathematics, Vol. 310, 15-16, (28 Aug. 2010), 2170-2175
Author Affiliations: [1]Institute of Mathematics and Informatics, Bulgarian Academy of Sciences, 8, G. Bonchev Str., 1113 Sofia, Bulgaria;
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ISSN: 0012-365X

Title: **Lanthanoid ions solvent extraction with 4–benzoyl–3–phenyl–5–isoxazolone: Synergistic effects in the presence of long chain alkylammonium salts**
Authors: Maria Atanassova, Ivan Dukov
Source: Separation and Purification Technology, Vol. 74, 3, (6 Sep. 2010), 300-304
Author Affiliations: Department of General and Inorganic Chemistry, University of Chemical Technology and Metallurgy, 8, St. Kliment Ohridski Blvd., 1756 Sofia, Bulgaria
ISSN: 1383-5866

Title: **Microstructure and stability of medieval glass bracelets from Drastar Castle, Bulgaria (11th–13th cent. AD): Four case studies**
Authors: Ralitsa Georgieva[1], Albena Detcheva[1], Yanko Dimitriev[2], Elena Kashchieva[2]
Source: Journal of Non-Crystalline Solids, Vol. 356, 28-30, (15 Jun. 2010), 1526-1529
Author Affiliations: [1]Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, Acad. Georgi Bonchev Str., Bl. 11, 1113 Sofia, Bulgaria;
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ISSN: 0022-3093

Title: **Agrobiodiversity, farm profits and land fragmentation: Evidence from Bulgaria**
Authors: Salvatore Di Falco[1], Ivan Penov[2], Aleksi Aleksiev[2], Tom M. van Rensburg[3]
Source: Land Use Policy, Vol. 27, 3, (Jul. 2010), 763-771
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ISSN: 0264-8377

Title: **Lateral displacement MEMS sensor**
Authors: V. Stavrov[1], E. Tomerov[1], G. Stavreva[1], C. Hardalov[2], A. Shulev[3]
Source: Procedia Engineering, Vol. 5, (2010), 649-652, Eurosensor XXIV Conference
Author Affiliations: [1]AMG Technology Ltd., Microelectronica Industrial Zone, 2140 Botevgrad, Bulgaria;
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ISSN: 1877-7058

Title: **Optimal sizing of a grid-connected PV system for various PV module technologies and inclinations, inverter efficiency characteristics and locations**
Authors: G. Notton[1], V. Lazarov[2], L. Stoyanov[1,2]
Source: Renewable Energy, Vol. 35, 2, (Feb. 2010), 541-554
Author Affiliations: [1]Laboratory "Systиmes Physiques de l'Environnement", University of Corsica Pascal Paoli, UMR CNRS 6134, Route des Sanguinaires, F-20000 Ajaccio, France;
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ISSN: 0960-1481

Title: **On some fractional generalizations of the Laguerre polynomials and the Kummer function**
Authors: S. P. Mirevski[1], L. Boyadjiev[2]
Source: Computers & Mathematics with Applications, Vol. 59, 3, (Feb. 2010), 1271-1277, Advance in Fractional Differential Equations
Author Affiliations: [1]Hewlett-Packard Global Delivery Center Bulgaria, 55, Vaptzarov Blvd., EXPO 2000, 1407 Sofia, Bulgaria;
[2]Department of Applied Mathematics and Informatics, Technical University of Sofia, 8, St. Kl. Ohridski Blvd., 1156 Sofia, Bulgaria.
ISSN: 0898-1221

Title: **Systematic palaeontology (Vertebrate palaeontology)**
Latest Cretaceous hadrosauroid (Dinosauria: Ornithopoda) remains from Bulgaria
Authors: Pascal Godefroit[1], Neda Motchurova-Dekova[2]
Source: Comptes Rendus Palevol, Vol. 9, 4, (Jun. 2010), 163-169
Author Affiliations: [1]Institut royal des sciences naturelles de Belgique, 29, rue Vautier, 1000 Bruxelles,

Belgium;
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ISSN: 1631-0683

Title: **Using virtual communities in tourism research**
Authors: Steven F. Illum[1], Stanislav H. Ivanov[2], Yating Liang[1]
Source: Tourism Management, Vol. 31, 3, (Jun. 2010), 335-340
Author Affiliations: [1]Missouri State University, 901 South National Ave., Springfield, MO 65897, USA;
[2]International University College, 3, Bulgaria Str., 9300 Dobrich, Bulgaria.
ISSN: 0261-5177

Title: **Probabilistic safety analyses developments resurgence in Bulgaria–Kozloduy nuclear power plant example**
Authors: Bozhana Marinova[1], Pavlin Groudev[2], Valentin Papazov[3]
Source: Nuclear Engineering and Design, Vol. 240, 4, (Apr. 2010), 880-885
Author Affiliations: [1]Risk Engineering Ltd., 10, Vihren Str., 1618 Sofia, Bulgaria;
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[3]Kozloduy NPP Plc, 3321 Kozloduy, Bulgaria.
ISSN: 0029-5493

Title: **Potential application of Candida melibiosica in biofuel cells**
Authors: Hubenova, Y.[1], Mitov, M.[2]
Source: Bioelectrochemistry, Vol. 78, 1, (Apr. 2010), 57-61
Author Affiliations: [1]Department of Biochemistry and Microbiology, Faculty of Biology, Plovdiv University, 24, Tsar Asen Str., 4000 Plovdiv, Bulgaria;
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ISSN: 1567-5394

Title: **Improvement of Yeast–biofuel cell output by electrode modifications**
Authors: Hubenova, Y.[1], Rashkov, R.[2], Buchvarov, V.[2], Arnaudova, M.[2], Babanova, S.[3], Mitov, M.[3]
Source: Industrial & Engineering Chemistry Research, (2010) DOI: 10.1021/ie1000949, Publication Date (Web): June 15, 2010
Author Affiliations: [1]Department of Biochemistry and Microbiology, Faculty of Biology, Plovdiv University, 24, Tsar Asen Str., 4000 Plovdiv, Bulgaria;
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EVENTS

EUROPEAN RESEARCHERS' NIGHT 2010

European Researchers' Night is held for the sixth consecutive year in the whole Europe with support from the European Commission within the framework of the biggest program for funding research and technological development – the 7-th Framework Programme. It is dedicated to all those who are professionally engaged in science, as well as to young people for whom it is not only magic, but also future career.

In Bulgaria the project dedicated to Researchers' Night 2010 - REACT (REsearchers in Industry and ACademy for Technology development) is realized by a consortium of participants: Young Talents Club (coordinator), Technical University – Sofia, DIPKU of Thracian University – Stara Zagora, Ruse University "Angel Kanchev", Plovdiv University "Paisii Hilendarski" with partnership on the national and local levels of Bulgarian Academy of Sciences, Union of Scientists in Bulgaria, the National Museum of Polytechnic, Oborishte district of the Capital Municipality, GIS Transfer Center Foundation and many other organizations. Media partners of the project are: Bulgarian national radio, "Obekti" and "Osem" periodicals, "AzBuki" newspaper, "ITI – Inventions-Transfer-Innovations" journal, "I read – the site for your book".

On September 24, 2010 different creative and entertaining events took place in Sofia, Plovdiv, Stara Zagora, Ruse, Burgas, and Shumen. Major part of them was connected with the topic of this year **"Researchers in industry and the Academy for higher technological development of the country"** with the aim to better inform citizens for popularization of benefits from cooperation between industry and the Academy with relation to training, career development, exchange of skills and technologies for the society, and for young people in particular, and progress and results of the research in Bulgarian industry.

The multiform program of the project includes: Fair of innovations with participation of Bulgarian companies and researchers; exhibitions dedicated to Bulgarian scientists, inventors and discoverers; attractive demonstrations in Curiosity Rooms; scientific discussions and shows; musical and poetic performances by scientists; awarding the winners in competitions for young people, including schoolchildren and students with innovative ideas and skills in different research areas; awarding the winners in the contest for journalists for publication on the topic "Summital achievements of Bulgarian scientists", etc.

EUROPEAN DAY OF THE ENTREPRENEUR 2010

On October 28, 2010 the eighth edition of the European Day of the Entrepreneur took place in Sofia.

Every year the forum brings together researchers working in humanitarian and applied spheres and entrepreneurs from Sofia. The goal of the joint initiative of the Capital Municipality and Sofia University St. Kliment Ohridski is to encourage research teams working in the public sphere in applying European experience in innovations and their application in public services management.

This year the forum passed under the motto: **"Innovations – meeting of science and business in the capital city"**, which is in synchroniza-

tion with the European Commission policy for competitiveness of European cities and regions through innovations and transfer of technologies.

The participants were greeted by Mrs. Zhulieta Hubenova – counsellor in the political cabinet of the Minister of Economy, Energy and Tourism, Mr. Paul Bevan – Secretary General of EUROCITIES, vice-rector of Sofia University Prof. Anastas Gerdzhikov, and vice-minister of Education, Youth and Science Mrs. Petya Evtimova.

The event is a part of the project financed by "Programme Europe – civil projects and European practices" of the Capital Municipality, as well as a part of the CITY Network Initiative 2010 and one of the leading events in the "Open Doors" week of the Capital Municipality.

The topics of the European Day of the Entrepreneur this year which caused interest of over 180 participants were as under:

- The link science – business for application of the innovative policy of the EC;
- Cultural heritage – research developments and investigations of interest to business;
- Innovative approaches and methods for carrying out training;
- Commercialization of research results in the public sector;
- Intellectual property – a resource and chance for economic development;
- Innovations in rendering public services by SMEs;
- Implementation of new technologies and innovations in the public and economic sectors of the municipality;
- Cultural heritage as a resource for municipal entrepreneurship;
- Announcement of the results of the students' contest for innovative entrepreneurial idea, etc.

SEVENTH NATIONAL CONTEST FOR INNOVATIVE ENTERPRISE OF THE YEAR 2010

Applied Research and Communications Fund, Enterprise Europe Network – Bulgaria, jointly with the Ministry of Economy, Energy and Tourism and the World Bank office in Bulgaria organize for the seventh time a National Contest for Innovative Enterprise of the Year 2010.

The aim of the contest is to encourage innovation activities of Bulgarian enterprises, and promote Bulgarian achievements in the field of innovation. The contest is open to innovative enterprises from all sectors of the economy.

The annual Innovative Enterprise of the Year Award acknowledges Bulgarian enterprises that have successfully introduced innovations or scientific accomplishments, thus transforming their operation mode and achieving sustainable economic effect.

A company is considered to be innovative provided that it has developed and marketed new or advanced products (goods or services) and/or processes over the last three years.

The **first stage** of the contest is devoted to collecting nominations for innovative companies. Nominations can be made by companies, organisations or individuals. In the second stage the nominees are invited to submit an application form which gives further information on their innovative product/service/process.

The innovation performance of participant companies will be evaluated by an expert panel. The companies with highest ranking in the two categories – "micro and small enterprises" and "medium-sized and big enterprises" will be visited by ARC Fund's experts. The **final step** is evaluation by a jury comprising representatives from ministries, research and non-government organisations, the World Bank's Mission to Sofia, and others.

The award ceremony will be presented at the Seventh National Innovation Forum that will be held on May 17, 2011.