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## NANOBIOCATALYSTS FOR BIOFUEL CELLS AND BIOSENSOR SYSTEMS

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FIELD: Chemical Technology

### Abstract:

*This overview summarizes the application of enzymes in the manufacture and design of biofuel cells and biosensors. The emphasis will be put on the protein engineering techniques used for improving the properties of enzymes such as nanobiocatalysts, e.g. immobilization orientation, stability, activity and efficiency of electron transfer between immobilized enzymes and electrodes. Some possible applications in the military and some future designs of these electric devices will be discussed as well.*

Key words: *nanobiotechnology, enzyme logical gates, directed evolution, high throughput screening, microfluidics, glucose oxidase, army, cryptography.*

## Introduction

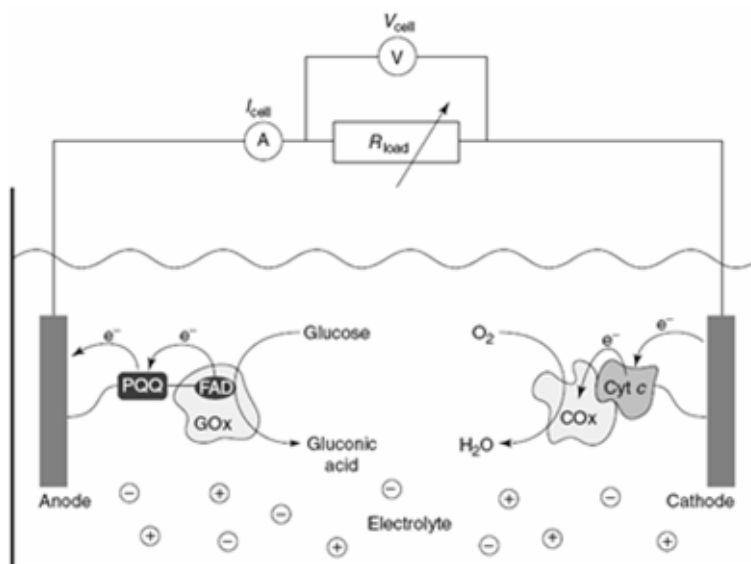
A nanobiocatalyst is a term referring to a biocatalyst in the form of enzyme or cell immobilized or modified with nanostructured materials, such as nanoporous materials, nanoparticles, nanofibers and nanotubes [1].

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Recent advances in the nanoscale science and technology have brought a new wave in the field of biocatalysis. Nanoscale engineering of biocatalysts is greatly promising for the development of high performance biofuel cells and novel biosensor systems [2, 3]. In addition, advancements in protein engineering techniques and bioinformatics are opening new possibilities for further improvements of biocatalyst performances in these devices [4].

## Nanobiocatalysis in Biofuel Cells

Biofuel cells use biocatalysts to convert chemical energy into electrical. A biofuel cell requires an anode and a cathode with immobilized enzymes or cells, a supporting electrolyte medium to connect the two electrodes and an external circuit to use extractable power [5]. On the anode, organic compounds oxidise, helped by an enzyme/cell, giving electrons to the electrode and on the cathode another enzyme/cell receives these electrons from the electrode and transmits them to the oxygen or another oxidizer like hydrogen peroxide. One of the first enzymatic biofuel cells reported in the year 1963 was made using glucose oxidase on the anode [6]. Today most of enzymatic cells are obtained using glucose oxidase on the anode and cytochrome c oxidase on the cathode (Scheme 1).



*Scheme 1* – An enzymatic biofuel cell. GOx- glucose oxidase; Cyt c- cytochrome c; COx- cytochrome c oxidase; PQQ- pyrroloquinoline, FAD- flavine adenine dinucleotide

*Šema 1* – Enzimski biogorivna ćelija. GOx- glukoza oksidaza; Cyt c- citohrom c; COx- citohrom c oksidaza; PQQ- pirololinoline, FAD- flavin adenin dinukleotid

### *Biocatalysis on the anode*

Enzymes used on the anode are glucose oxidase [7, 8], formaldehyde & formate dehydrogenase [9, 10], alcohol dehydrogenases [10-12] and sugar dehydrogenases (fructose, glucose, cellobiose) [13-15]. Glucose oxidases (GOx) have been isolated from many organisms, but most often used forms in biofuel cells and biosensors are GOx from *Aspergillus niger* and *Penicillium amagasakiense*. Three different classes of dehydrogenases are used at the anodic compartment: quinoprotein-dehydrogenase, flavin dependent dehydrogenases and NAD<sup>+</sup> dependent dehydrogenase.

### *Biocatalysis on the cathode*

On the cathode, most often used enzymes are multicopper oxidases such as laccases and bilirubin oxidase [16, 17], peroxidases such as horseradish, microperoxidase [18] and cytochrome c oxidase [19]. Laccases and bilirubin oxidase contain four Cu centers in one protein molecule and in a four-electron reduction process, the final electron acceptor oxygen is reduced to water. Fungal laccases have broad substrate specificities and are able to oxidize a wide range of organic compounds. Peroxidases from plants like horseradish contain heme and they can transfer electrons to water and generate hydrogen peroxide [20].

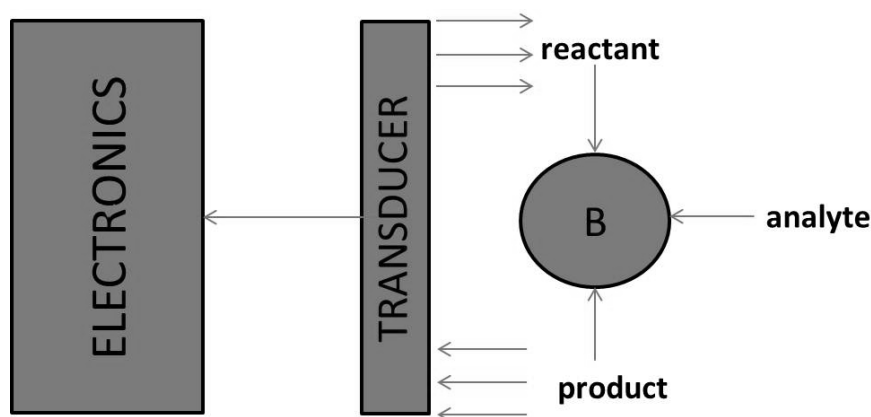
### *Miniature biofuel cells*

Due to recent developments in nanotechnology, biofuel cells can be miniaturized and used as power sources for electric medical devices such as implantable sensors, pacemakers and insulin pumps [21, 22]. They use glucose and oxygen from blood as a source of electric energy. The choice of enzymes for the nanobiocatalyst preparation in implantable miniature biofuel cells is limited by the blood composition and physiological conditions (e.g. pH, ions, glucose concentrations). Mainly cytochrome c oxidase, laccase or bilirubin oxidase have been employed on the cathode, while glucose oxidase from *A.niger* and glucose dehydrogenase from *A.calcoaceticus* are preferentially used on the anode.

## Nanobiocatalysis in Biosensor Systems

### *Biosensors*

A nanobiocatalyst can also be used for manufacturing biosensors, devices that convert a chemical signal into an electrical one. The main components of biosensors are a biological component, a transducer and electronics, Scheme 2.



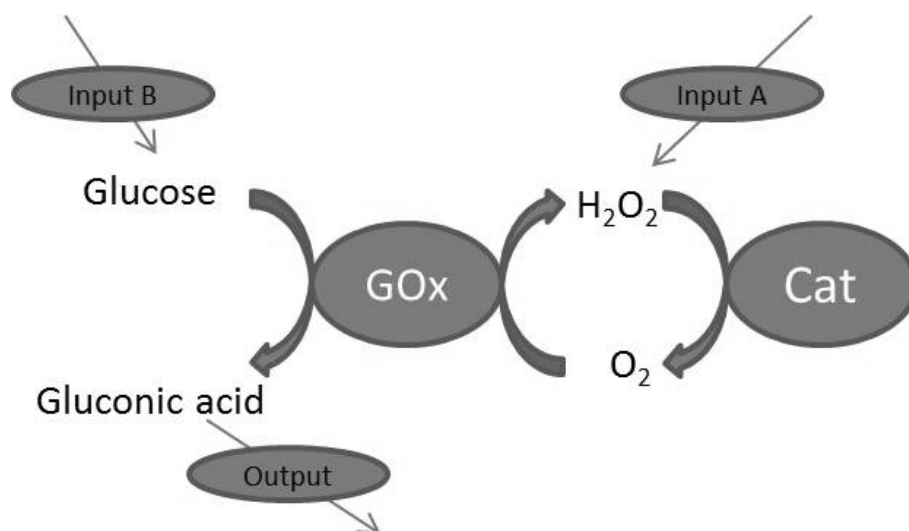
Scheme 2 – Biosensor with a bioactive element (B)  
 Šema 2 – Biosenzor sa bioaktivnim elementom (B)

A transducer senses a biochemical event and converts it to a potential change, electron transfer, light emitted or adsorbed by a product or reactant, heat or mass change. Based on transducers, biosensors can be: electrochemical, optical, piezoelectrical or thermal. The most often used electrochemical biosensors can be based on amperometric, potentiometric and impedance detection.

Bioactive components are cells or biological macromolecules such as: enzymes, monoclonal antibodies, nucleic acids, and lipids. The most often used biological components for biosensor manufacturing are enzymes (glucose oxidase), usually in an immobilized form [23].

### *Enzyme Logical Gates*

For injured civilians or soldiers at an accident spot, a rapid and reliable diagnosis of physiological conditions would allow immediate medical intervention. Since the majority of battlefield deaths occur within the first half an hour after injury, a rapid diagnosis and treatment are crucial for a survival rate. In order to determine the type and the extent of an injury, it is usually necessary to monitor several physiological parameters. A multiple biosensor device (enzymatic reactions) connected similarly to electronic circuits in computer logical gates can perform biocomputing and process various biochemical information received from body fluids to determine the injury type [24, 25]. Various Boolean logic gates such as AND, OR, XOR, NOR, NAND, INHIB and XNOR were made using biomolecular switchable systems (proteins/enzymes, DNA, RNA, whole cells) [26-29]. An example of the AND logical gate is shown in Scheme 3.

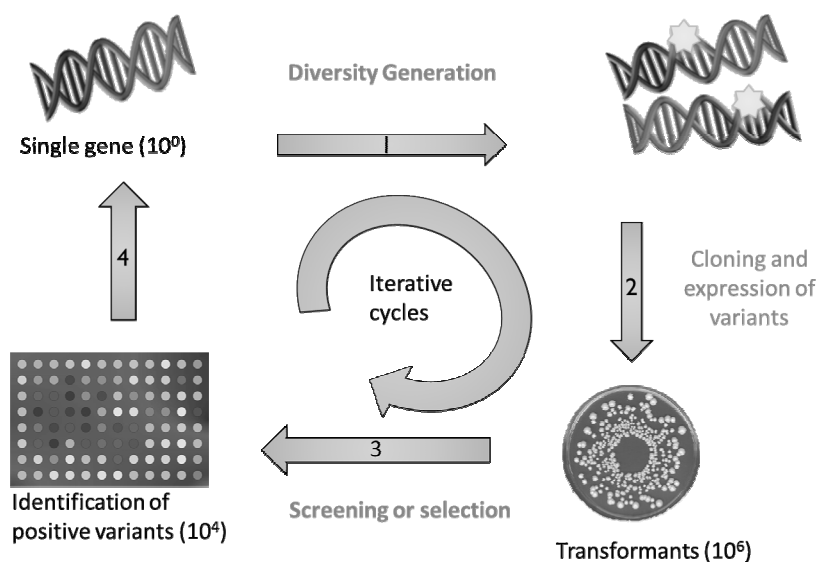


*Scheme 3* – AND logical gate made from glucose oxidase (GOx) and catalase (CAT)  
*Šema 3* – AND logičko kolo napravljeno od glukoza oksidaze (GOx) i katalaze (CAT)

## Protein Engineering for Nanobiocatalysis

Biocatalysts are not optimized for the application in bioelectrocatalysis. Nonoptimal operating conditions, radical formation during electron transfer and a poor electric contact with the electrode diminish the power output and the operational life of these devices. Immobilization of biocatalysts on nanostructured materials, rational design and directed evolution have successfully been used to improve the nanobiocatalyst properties such as activity towards artificial cofactors or electron mediators, electrical contact between the enzyme and the electrode, stability in the presence of an organic solvent, thermostability and stability in the presence of oxidizing reagents like hydrogen peroxide [4].

The rational protein design requires the knowledge of the protein structure and the understanding of structure-function relationships. Due to the lack of deeper understanding of these relations, directed enzyme evolution is becoming increasingly important in protein design. Directed evolution does not require a detailed knowledge of protein structures and uses the principles of the Darwinian evolution from the nature and applies them in the lab. In the iterative process of mutation and selection, biocatalysts are „forced“ to evolve in the direction needed for a better performance on the electrode, Scheme 4.



Scheme 4 – Basic steps of directed evolution.

Šema 4 – Osnovni koraci dirigovane evolucije.

The most limiting step in directed evolution is a screening process and there is an enormous effort to develop high throughput screening systems for gene libraries generated in directed evolution experiments. These screening systems are usually performed in aqueous microdroplets of water in oil emulsions where in 1 mL of an emulsion it is possible to perform  $10^{10}$  different reactions and screen libraries with sizes of up to  $10^8$ . For screening and sorting these microcompartments, scientists use flow cytometry [30] or microfluidic devices [31, 32].

In the literature there is a growing number of articles with examples of successful applications of protein engineering and directed evolution in developing more efficient biocatalysts like glucose oxidase [30, 33], glucose dehydrogenase [34], formaldehyde dehydrogenase [35], lactate dehydrogenase [36], horseradish peroxidase [37] and laccase [38, 39] for applications in biofuel cells and biosensor systems.

### Glucose Oxidase

Glucose oxidase (GOx) from *A.niger* is the most studied enzyme in electrochemistry for applications in biofuel cells and biosensors. It has a molecular mass of around 155-160 kDa in its glycosylated form and consists of two identical subunits. The  $K_m$  value for  $\beta$ -D-glucose has been reported between 11.0 and 41.8 mM. GOx catalyzes oxidation of glucose

by molecular oxygen to gluconic acid and hydrogen peroxide. In order to be better suitable for the application in miniature biofuel cells, glucose oxidase has been evolved for higher activity and stability at physiological conditions (pH 7.4, 4 mM glucose) and better activity with artificial electron mediators like ferrocene. A ferrocene-based assay for glucose oxidase was used for screening approximately 2000 GOx mutants. A double mutant of GOx (T30S, I94V) has increased  $k_{\text{cat}}$  compared to wt (69.5 1/s WT; 137.7 1/s T30S I94V) and increased pH and thermal resistance [33]. In another directed evolution experiment, an ultrahigh throughput screening system based on emulsion technology and FACS (fluorescent activated cell sorter) was used for screening a high error prone PCR GOx gene library containing  $10^5$  different mutants (30). Mutant M12 contained five mutations (N2Y, K13E, T30V, I94V, K152R) and 3.3 times increased specificity constant compared to wt (2.49 mM/s WT; 8.26 mM/s M12).

### *Glucose Dehydrogenase*

Using directed evolution techniques (gene shuffling approach), glucose dehydrogenase from *Bacillus megaterium* was improved in its thermal stability. The improved mutant contained two amino acid substitutions, Glu170Lys and Gln252Leu [34]. To select active variants high-throughput screening was performed in two steps: a filter-based prescreen of clones grown on agar plates using as a redox system 5-ethylphenazinium ethylsulfate and tetrazolium salt and an NADH based quantification assay in microtiter plates. A crystal structure of the double mutant showed that these two residues strengthen the subunit-subunit interactions by stabilizing a hydrophobic cavity.

### *Formaldehyde Dehydrogenase*

By substituting only one residue Ser 318 to Gly, the formaldehyde dehydrogenase from *Pseudomonas putida* was improved in activity by 1.7 times [35]. The enzymatic assay was done by placing nylon membranes soaked with detergent and p-nitroblue tetrazolium on diformazan agar plates. The positive colonies that developed blue colour were further analysed by the NADH-based assay in microtiter plates. The obtained mutant unfortunately decreased its thermal residence.

### *Lactate Dehydrogenase*

The lactate dehydrogenase from *Bacillus stearmophilus* is a thermostable L-2-hydroxyacid dehydrogenase used in biofuel cells. It is allosterically activated by fructose 1,6-bisphosphate (FBP) resulting in a 100-fold drop in  $K_m$  and 2.5-fold drop in  $k_{\text{cat}}$  due to the tetramerization of the dime-



ric form. After three rounds of family shuffling and screening 3000 clones variants of lactate dehydrogenase, a mutant was found that could form a tetrameric oligomer in the absence of FBP (36). The obtained mutant had three substitutions (Arg118Cys, Gln203Leu, Asn307Ser) and  $K_m$  for pyruvate was reduced to 0.07 mM.

### *Horseradish Peroxidase*

A plant horseradish contains several peroxidase (HRP) isoforms and C isoenzyme is the most abundant. Thermal stability and resistance to peroxide inactivation was improved for this enzyme using the directed evolution [37]. Increased activity and changed specificity and enantioselectivity were also obtained by directed evolution [37, 40, 41]. Using the emulsion technology and microfluidic devices, HRP also evolved for 7-fold increase in activity [31]. Such high increase in activity was achieved due to the screening of big gene libraries  $10^7$  in multiple rounds of directed evolution. This is one of the best examples that can be done by directed evolution if there is a good high throughput screening system.

### *Laccase*

Laccases belong to the group of copper containing enzymes that can oxidize various phenols by oxygen. It has been successfully expressed in yeasts for directed evolution [42]. Using *in vivo* the recombination approach, the thermostability of versatile (VL) and high redox potential laccases (HRPL) was improved [39]. The activity at 65°C for VP was improved 3-fold while at 75°C the improvement was over 10-fold. The HRPL with evolved thermostability was subjected to further rounds of directed evolution and the activity of the best mutant OB-1 had 34000-fold enhanced activity [39].

## **Military Application of Nanobiocatalysts**

### *Enzyme Logic Systems for Battlefield Injuries*

There is a growing interest in the armies worldwide for developing a field hospital-on-a-chip that could monitor a soldier's injuries and administer medications. The Office of Naval Research is funding a program entitled „Integrated Sense and Treat Enzyme Logic Systems for Battlefield Injuries“ that if successful would provide U.S. soldiers with a wearable device to constantly monitor vital signs and help treat wounds. These microfluidic laboratories on-a-chip would allow unskilled personnel to perform specialized tests in the field. The chip would fluids like blood and sweat for the „biomarkers“ of common battlefield

injuries like shock or fatigue and then automatically inject the appropriate drugs. Preliminary results in these field with enzymatic logical gates are making this approach quite realistic [24, 43].

### *Information Security: Biomolecular keypad lock systems, steganography and encrypting*

The computing networks composed of enzyme logical gates can be also used for mimicking a biomolecular keypad lock [44, 45]. A designed biochemical reaction chain was composed of several enzymatic reactions: hydrolysis of sucrose to glucose, oxidation of glucose to oxygen and then oxidation of ABTS dye to green product [46]. These reaction steps were catalysed by invertase, glucose oxidase and microperoxidase. The enzymes were immobilized on glass beads. The experiment was performed when the order of the enzyme-encoded input signals varied in 6 different combinations. Only one correct order of the input signals resulted in output 1 (generation of green product). A similar enzyme-based keypad lock was integrated with the biofuel cell where only a correct „password“ (specific order of adding enzymes) resulted in the activation of the biofuel cell, while all other „wrong“ permutations of the enzyme inputs preserved the „OFF“ state of the biofuel cell [45].

### *Keypad Lock Security, Steganography and Encrypting Based on Immunochemical Systems*

A keypad lock device can also be made using an immuno-based biorecognition system. Such a device was integrated with a switchable biofuel cell that was giving power output only after the correct input of the „password“ encoded in the antibody-sequence.

Steganography and encrypting was also demonstrated by using immunochemistry systems. IgG antibodies were used as invisible ink developed with complementary antibodies labelled with enzymes producing color spots [47]. This approach in the future could provide information protection and watermark-technology and scaling down the encoded text to a micro size is also feasible with the use of nanotechnology.

## Conclusion

The development of nanotechnology, protein engineering and novel concepts in molecular biocomputing have opened new possibilities in the manufacture and design of biofuel cells and biosensor systems. These

technologies will help in continuous monitoring of human health conditions and increase survival rate under shock conditions. Novel concepts in molecular biocomputing could also provide new ways of information storage and protection.

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## NANOBIOKATALIZATORI ZA BIOGORIVNE ĆELIJE I BIOSENZORNE SISTEME

OBLAST: hemijske tehnologije

### Rezime:

*U ovom preglednom članku je sumirana primena enzima u proizvodnji i dizajnu biogorivnih ćelija i biosenzora. Naglasak u pregledu literature je stavljen na tehnike proteinskog inženjeringa, koje se koriste za poboljšanje osobina enzima u nanobiokatalizatorima kao što su orijentacija kod imobilizacije, stabilnost, aktivnost i efikasnost transfera elektrona između imobilizovanog enzima i elektrode. Na kraju pregleda je dato nekoliko primera moguće primene u vojsci.*

*Nanobiokatalizatori su biokatalizatori u obliku enzima ili ćelija imobilizovani na nanomaterijalima.*

*Koriste se kao sastavni elementi gorivnih ćelija u vidu imobilizovanih oksidoreduktaza na elektrodama. Na anodi se uz pomoć enzima oksiduju hemijska jedinjenja i elektroni predaju elektrodi, dok se na katodi elektroni uz pomoć druge oksidoreduktaze prebacuju sa elektrode na vodu ili kiseonik. Enzimi koji se koriste na anodi su glukoza oksidaza, formaldehid dehidrogenaza, alkohol dehidrogenaza i druge oksidaze šećera. Na katodi se uglavnom koriste lakaze, bilirubin oksidaza, peroksidaze i citohrom c oksidaza. Zahvaljujući razvoju nanotehnologije razvijaju se i minijaturne biogorivne ćelije koje proizvode električnu energiju za implantirane medicinske uređaje (insulinske pumpe, pejsmejkere, biosenzore) koristeći glukozu i kiseonik iz ljudske krvi.*

*Biosenzori predstavljaju uređaje koji se sastoje iz biološke komponente, transducera i električne komponente. Oni pretvaraju koncen-*

*traciju hemijske supstance u električni signal i koriste se za analitiku. Kao biološka komponenta se mogu koristiti enzimi, monoklonska antitela, nukleinske kiseline i lipidi.*

*Enzimski logička kola predstavljaju kombinaciju različitih biosenzora (enzimskih reakcija) koji mere nekoliko ulaznih parametara i na osnovu njih daju odgovarajući izlazni signal. Koristeći znanja kompjuterske tehnologije enzimskim logičkim kolima mogu se simulirati AND, OR, XOR, NOR, NAND, INHIB i XNOR logička kola.*

*Za poboljšanje osobina biokatalizatora u cilju efikasnije primene u bioelektrokatalizi koriste se tehnike proteinskog inženjeringa kao što su racionalni dizajn i dirigovana evolucija. Dirigovana evolucija koristi iterativne korake mutiranja i selekcije, kako bi biokatalizator evoluirao u pravcu koji nam je potreban. Najsporiji stupanj u ovoj tehnologiji predstavlja „skrining“, te se u novije vreme pomoću protočne citometrije i mikrofluidike pokušavaju razviti nove metode visoko propusnog skrininga. U literaturi opisani primeri dirigovane evolucije glukoza oksidaze, glukoza dehidrogenaze, formaldehid dehidrogenaze, laktat dehidrogenaze, peroksidaze i lakaze.*

*Kombinacijom enzimskih logičkih kola i mikrofluidne tehnologije se pokušavaju napraviti laboratorije na čipu koje bi omogućile kontinuirano praćenje zdravstvenog stanja vojnika na bojnopolju i u slučaju šoka (ranjavanja) primenu odgovarajuće terapije u toku prvih 30 minuta od povrede. To bi obezbedilo veći stepen preživljavanja vojnika u ratu. Takođe upotrebom enzimskih logičkih kola i antitela moguće je postići uskladištenje i šifrovanje informacija, kao i zaštitu lozinkom, odgovarajućih elektronskih uređaja kao što su biogorivne ćelije*

*Razvoj nanotehnologije, proteinskog inženjeringa i molekularnog računarstva otvara vrata novim mogućnostima u proizvodnji i dizajnu biogorivnih ćelija i bisenzorskih sistema, kao i u skladištenju i zaštiti informacija.*

*Ključne reči: nanobiotehnologija, enzimski logička kola, dirigovana evolucija, visoko propusni skrining, mikrofluidika, glukoza oksidaza, vojska, kriptografija.*

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