Hungary-Serbia
IPA Cross-border Co-operation Programme

Jó szomszédok a közös jövőért
Good neighbours creating common future
Dobri susedi zajedno stvaraju budućnost

The project is co-financed by the European Union through the Hungary-Serbia IPA Cross-border Co-operation Programme

Project title:
Development of innovative technology's for prevention and treatment of female genital infections

Project abbreviation: DEVTEGEN

Duration: 01/2012- 12/ 2013

Total project budget: 271.100 Euro

Grant rate of the project: 95,55 %

Member organizations of Consortium:

University of Szeged

University of Novi Sad - Faculty of Medicine of severe liability

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Project description

Problem areas occuring at partners on both sides of the border:

(1) Through gynecological examination feasible, immediate, objective diagnostic method for identification of microbial vaginitis

(2) Lack of therapeutic remedy considering the discrete appaerance of microbial vaginitis

The overall and long-term goals of the project are to decrease and mitigation the effects of microbial vaginal inflammation (decrease of fertility and the incidence of premature birth) in favor of

- identification of predisposing genetic factors, selection of patients with increased risk factor;

- development of nanotechnology based therapy considering the personal aptitudes and without side and teratogenic effects;

- operation of crossborder innovative researchers' cooperation and exploration of new lines for infertility's research

Result outcomes of the project:

(1) Identification of genetic polymorphisms of microbial vaginosis

(2) Development of pH sensitive nanocomposite hydro gel,

that in the future can be upgraded according to different genetically determined marker specificities

(3) Organizational framework of international, innovative researchers' team based on multidisciplinary knowledge base;

(4) Research protocol for supporting the implementation of standardised activities regarding genetic examinations and sampling at vaginal infections

(5) Publications and presentations published in international science and at international conferences

(6) Contracted relation between Hungarian and Serbian research institutions and companies

Project management and administration

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Members of the consortium:

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Objectives of the research program:

1. Department of Obstetric and Gynecology, Faculty of Medicine, University of Szeged

1 Sampling for identification of genetic factors

1.1 Protocol definition for clinical examinations and sampling, with detailed description of selection methodology for staff involved, sampling method and documentations requirements, evaluation, data management principles.

1.2 Protocol submission for the permission of clinical examinations

1.3 Protocol based collection of blood samples

1.4 Evaluation of samples, data base creation and data processing

2. Department of Dermatology and Allergology, Albert Szent-Györgyi Medical Center, University of Szeged

2 Identification and analysis of genetic factors playing a role in the genetic predisposition to recurrent vulvovaginal candidiasis (RVVC) in the Hungarian population

2.1 Preparation of the project: selection of the studied genes

The investigated disease within this current research grant is **vulvovaginal candidiasis** (VVC), a recurrent and opportunistic mucosal infection caused by *Candida albicans* infection of the vagina, associated with the dermatitis of the vulva.

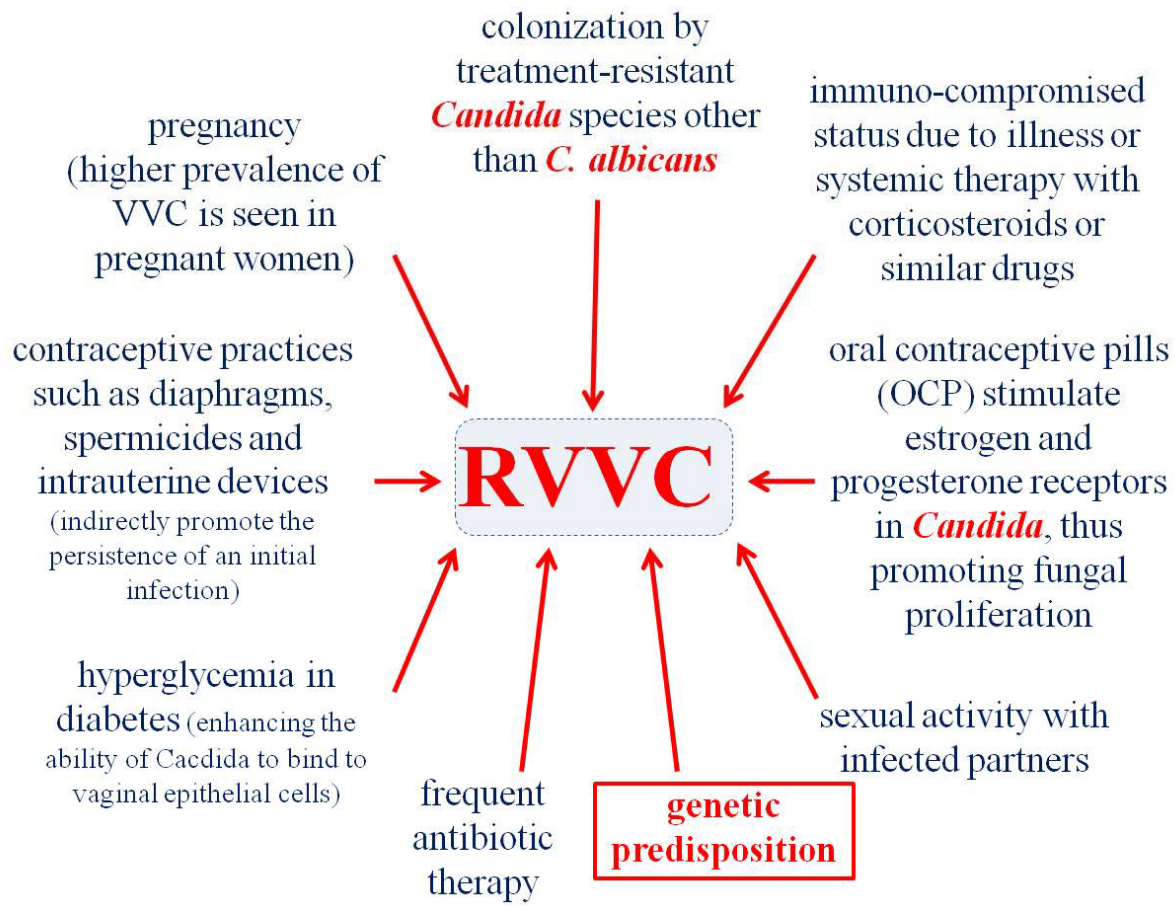

Figure 1. RVVC is a multifactorial disease, several different- self and environmental factors contribute to the pathogenesis of the disease. Among them, inherited genetic factors play an important role in determining the affected persons' individual susceptibility.

The fungus *Candida albicans* accounts for approximately 80-90% of all vulvovaginitis cases. It is estimated that 75% of women have at least one episode of VVC within their lifetime, with 40% to 45% having two or more episodes (van Vranken, 2007). Recurrent VVC (RVVC) is diagnosed in females who suffer from three or more VVC episodes per year. It is estimated that approximately 5–8% of menarchal women are affected (Sobel, 1992).
RVVC can be idiopathic or caused by several different mechanisms (Adib, 2011) (Figure 1.).

Immunity in higher animals is broadly divided into two systems, one acting as the front-line defence (innate immunity) and the other providing pathogen-specific immunity and memory (adaptive immunity). The innate immune system, also known as non-specific immune system acts as a first line of defense. The cells of this system recognize and respond to pathogens in a generic way, which is rapidly initiated in a few hours after the pathogenic attack. Later on, as a result of these events a pathogen-specific adaptive immune activation is also initiated (Janeway, 2002).

Important players of the innate immune activation are the so-called pathogen recognition receptors (PRR) capable of the recognition of pathogen-associated molecular patterns (PAMPs) connected to microbial pathogens or cellular stress, as well as damage-associated molecular patterns (DAMPs) that are released during cell damage.

Fungal recognition is mediated via interaction of PAMPs on fungi cells, with pattern recognition receptors on host cells; some of which include Toll-like receptors and C-type lectin receptors (CLR) (Salek-Ardakani, 2012). Dectin-1, Dectin-2 and Mincle belong to the CLR family of proteins. These receptors play an important role in the modulation of the release of inflammatory mediators playing a key role in the shaping the hosts' protective immune response in order to eradicate the fungal burden (Willment, 2008; Drummond, 2011; Salek-Ardakani, 2012) (Figure 2).

Dectin-1 (DECT1) and -2 (DECT2) recognition of fungi induces activation of innate intracellular pathways through Syk kinase and the adaptor molecule, **CARD9** that leads to transcription of innate specific genes, production of reactive oxygen species and phagocytosis of yeast cells (Salek-Ardakani, 2012). Dectin-1 is a transmembrane protein that contains a single CTLD in the extracellular region and an immunoreceptor tyrosine-based activation (ITAM)-like motif within its intracellular tail (Drummond, 2011). The PAMP identified for Dectin-1 is β-glucan, a carbohydrate found in fungal cell walls and in some bacteria and plants (Brown, 2001; Brown, 2006). Upon receptor activation as a result of the initiated signaling cascade the production of lipid mediators (Suram, 2006; Alvarez, 2010) and

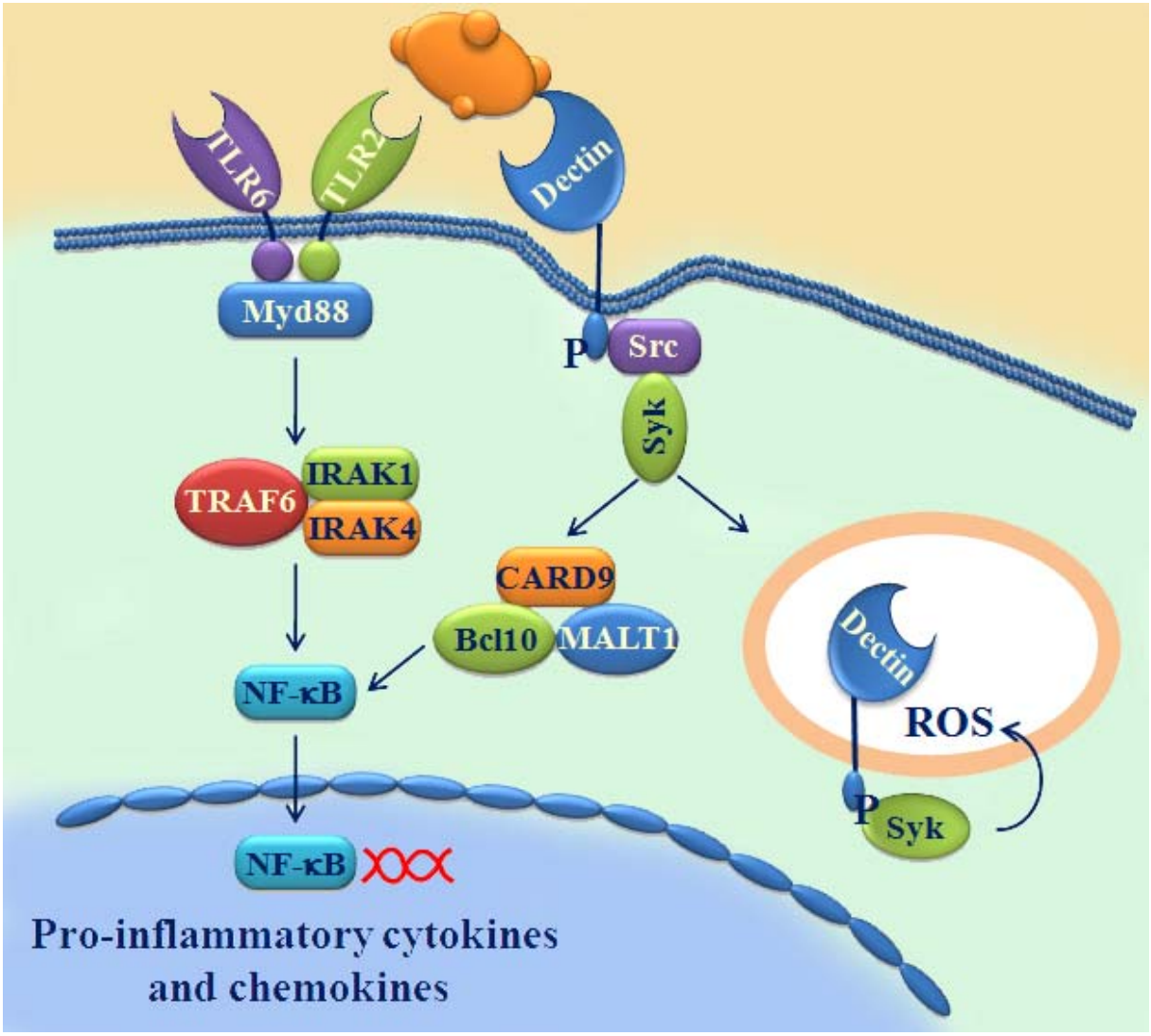

Figure 2. Pathogen recognition receptors (e.g. Toll-like receptors (TLR) or Dectin receptors) are capable of the recognition of conserved molecular structures of various mictobial pathogens (pathogen associated molecular patterns - PAMPs). Their activation alerts the cells about the presence of a potentially harmful invader, and initiates active defense mechanisms in order to protect the organism.

inflammatory chemokines/cytokines (e.g. TNFα, IL-1β, IL-10, IL-6, IL-23, CCL2, CCL3) (Reid, 2009; Dennehy, 2009) is increased, which demonstrate a cell-specific nature. Their production is controlled by several signaling pathways which have extensive cross-talk with those activated by different PRRs, and this is essential for effective antifungal immunity (Netea, 2006; Ruland 2008).

Mice lacking either the C-type lectin receptor dectin-1, encoded by the DECT1 gene, or the intracellular adapter molecule Card9, which is essential for dectin-1 signaling, have impaired antifungal immunity (Vautier, 2010).

Apart from these results, a recent study reported a pedigree with four women suffering RVVC. In this family, affected individuals had been shown to carry an early-stop-codon mutation (p.Tyr238X) in DECT1, suggesting that this gene also seems to be important in fighting *Candida* infections in humans (Ferwerda, 2009). Due to the identified mutation, DECT1 could not mediate a proper β-glucan binding, and led to defective production of various cytokines (IL-17, TNFα, and IL-6) after stimulation with β-glucan itself, or with *Candida albicans*. However, fungal phagocytosis and fungal killing were normal in the patients, explaining why DECT1 deficiency was not associated with invasive fungal infections and highlighting the specific role of DECT1 in human mucosal antifungal defense (Ferwerda et al., 2009).

Experimental evidence also exists that highlights the important role of CARD9 in these processes, too. Recent investigation on a large, consanguineous Iranian family with multiple cases of chronic mucocutaneous candidiasis (CMC) revealed a homozygous mutation in CARD9 gene that results in a loss-of-function mutation due to a premature stop codon in the coding sequence (Glocker, 2009; Glocker, 2010).

Also, experiments in the murine CARD9 $^{-/-}$ model showed that only wild-type CARD9 could restore cytokine production in response to the triggering of dectin-1, a pattern-recognition receptor for fungal cell-wall antigens (Glocker, 2009; Glocker, 2010).

Based on all these features and the results of the investigations performed in animal models, it is feasible to imagine that inherited factors that modify the regulation, expression, structure and/or function of these genes (DECT1, CARD9) may play a role in the genetic predisposition to RVVC. Thus, we strongly believe that CARD9 and DECT1 genes can serve as promising targets of further studies, and we hypothesize that the common genetic variants (polymorphisms = SNPs) of these genes may be important factors that are responsible for the genetic susceptibility for microbial vaginosis.

In order to find out whether genetic polymorphisms of the DECT1 and CARD9 genes play a role in the genetic predisposition to RVVC in a Central European population, we plan to perform a comprehensive analysis. For that, 7 SNP-containing exons (out of the total of 13) of the CARD9, and 2 (out of the total of 5) in case of the DECT1 will be investigated by DNA sequencing. This allows not only the detailed analysis of several potential genetic variants, but also we can investigate their interaction and linkage in our study population by creating and analyzing the effect of haplotypes. The relation of the resulting genetic to various demographic, clinical and microbiological parameters of the patients' cohort will also be studied.

Healthy, immunocompetent individuals get exposed to *Candida* early in life. As a result, first innate immune events are induced that will subsequently lead to an adaptive immunity that is developed toward the microbe. These events finally generate a characteristic serum/mucosal antibody production, *in vitro* T-cell responses and related cytokine production (Fidel, 2002; Yano, 2012). The resulting *Candida*-specific host immune responses are generally considered critical to keep the otherwise commensal microbe from converting into an opportunistic pathogen (Yano, 2012). However, when the recognition and/or the early signaling events that are initiated in response to *Candida* goes wrong, it may lead to enhanced colonization, thus also susceptibility toward the microbe. Our results can lead to the greater understanding of the molecular pathomechanism of RVVC which subsequently may also result in the development of novel, more effective treatment options.

2.2 Creation of protocol with detailed description of applied genetic methodology, documentations requirements, evaluation and data processing principles

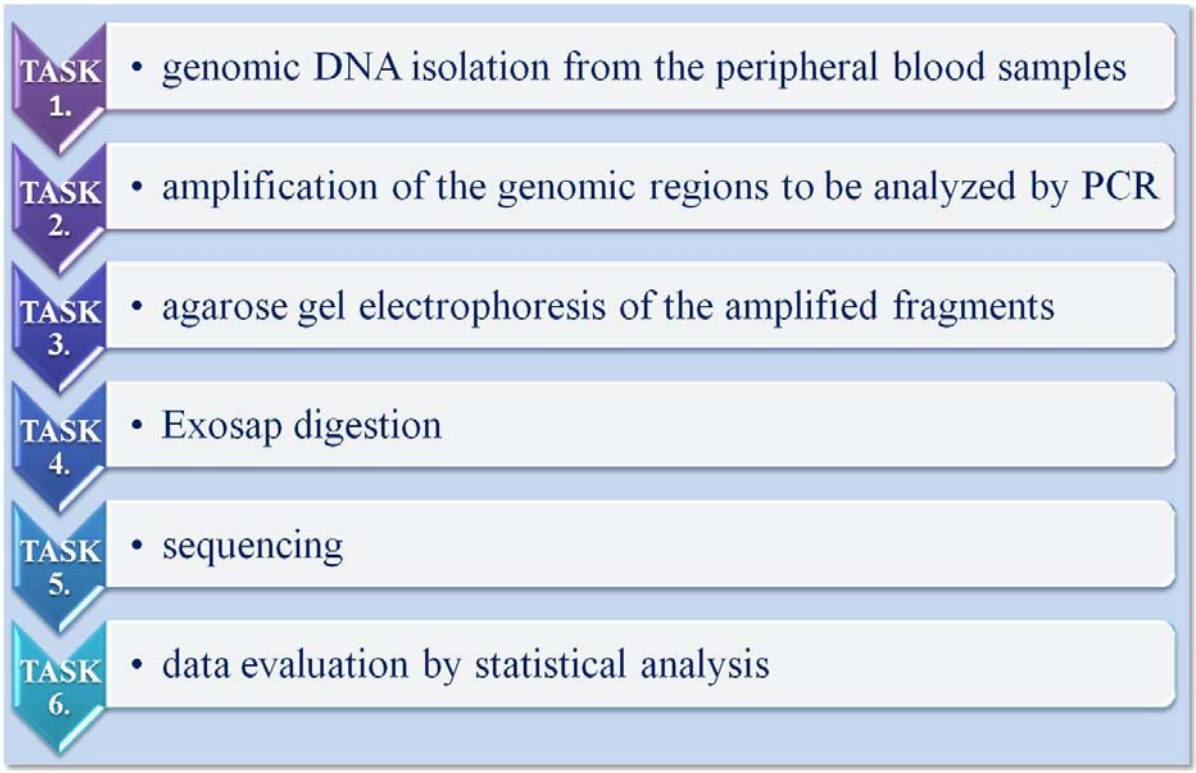

Figure 3. Flow-chart representing the sample processing from the arrival of the collected peripheral blood samples to the analysis of the obtained data.

In order to achieve our goals first we worked out a detailed protocol (summarized in Figure 3.) that included all the necessary tasks from the planning of the studies, through the acquisition of the necessary permits, all the way to the detailed protocol of the different tasks that is being performed.

2.3 DNA isolation

TASK 1. High-quality genomic DNA (gDNA) is isolated from the collected whole blood samples by the BioRobot EZ1 workstation at the Department of Dermatology and Allergology, Molecular Laboratory (University of Szeged). This robot is capable to the parallel processing of six samples at the same, which requires 15-20 minutes. The rapid speed of nucleic acid purification is achieved through magnetic particle technology: nucleic acids in sample lysates are isolated in one step through their binding to the silica surface of magnetic particles. Reagents are supplied in pre-filled EZ1 Reagent Cartridges, which ensures speed and convenience in loading the (Figure 4).

Figure 4. The isolation of gDNA from the patients' blood samples is done by using a BioRobot EZ1 workstation.

2.4 Progress of sample processing

TASK 2. When the gDNA samples are available, the previously determined parts of the DECT1 and the CARD9 loci are multiplied using a special biochemical technology which allows the amplification of a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of that particular DNA sequence. This method is called the polymerase chain reaction (PCR).

TASK 3. To visualize and to achieve a quality and quantity control of the performed PCR reactions agarose gel electrophoresis is being performed. This technique allows the separation of nucleic acid molecules by applying an electric field, which moves the negatively charged oligonucleotide molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones in a give time because shorter molecules migrate more easily through the pores of the gel (Sambrook, 2001). The number, size and intensity of the resulting bands provide a lot of useful information about the specificity and the efficacy of the PCR reactions.

TASK 4. The next step is the preparation of the amplified gene fragments for further processing. This step is achieved by an Exosap digestion of the samples, which removes all the impurities that would possibly interfere with the subsequent enzymatic reactions during the next steps.

TASK 5. The exact nucleotide composition of the amplified DNA fragments is determined by DNA sequencing.

TASK 6. The obtained sequence data of all the controls and patients are compared to each other and to the consensus sequences located in publically available genomic databases (e.g. NCBI; http://www.ncbi.nlm.nih.gov/), with a special care concerning the known polymorphic nucleotide positions.

2.5 Completion, summary, publication

After the carried genotypes at each polymorphic positions in case of all donors is determined, statistical analysis softwares (e.g. Vassarstat, SPSS) are used to determine the frequency of the different alleles of each SNPs in the control and the patient groups. If statistically significant differences are discovered, we will determine the contribution of the carriage of that particular polymorphism in the genetic predisposition to RVVC.

The results of these investigations will be presented at scientific conferences and published in international journals.

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3. Development of nanocomposite hydrogel for controlled drug delivery

A hydrogel is a network of hydrophilic polymers that can swell in water and hold a large amount of water while maintaining the structure (**Fig. 1. A.**). The porous structure hydrogels (**Fig. 1. B.**) can protect the drug from hostile environments, e.g. the presence of enzymes and low pH in the stomach. Due to the properties of this material it has a number of advantages for drug delivery applications. The active agent is dispersed or dissolved in an inert polymer (**Fig. 2**).

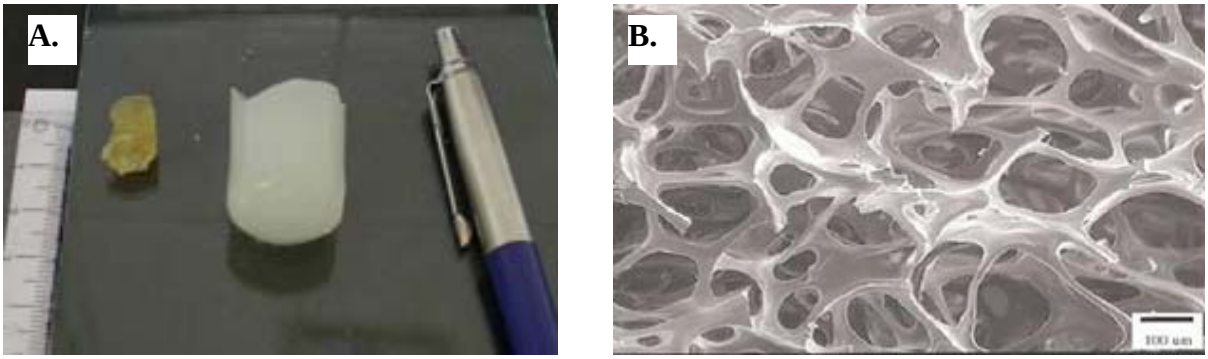

Fig. 1. Comparison of hydrogel's swelling in dry state and under physiological conditions (A.) and the porous structure of gel (B.)

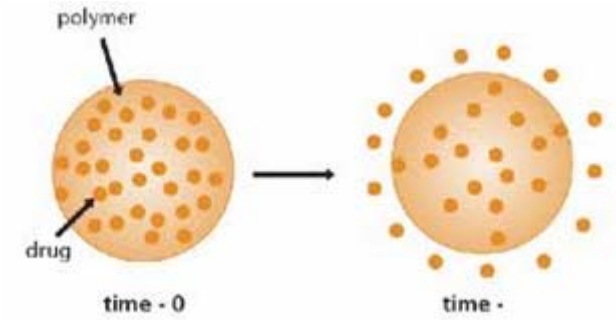

Fig. 2. Schematic representation of a hydrogel- based drug delivery device

3.1 Selection of polymers disposing with optimal physical and chemical characters

Hydrogels can also control drug release by changing the gel structure in response to environmental stimuli. These materials are also termed "intelligent gels", because, depending on their composition, they perceive changes in one or several environmental parameters (temperature, pH, light, magnetic field etc.) and respond with a functional reaction (swelling, shrinking, sol-gel phase transition) (**Fig. 3**). Many physical and chemical stimuli have been applied to induce various responses in smart hydrogel systems. Environment-sensitive hydrogels are ideal candidates for developing self-regulated drug delivery systems.

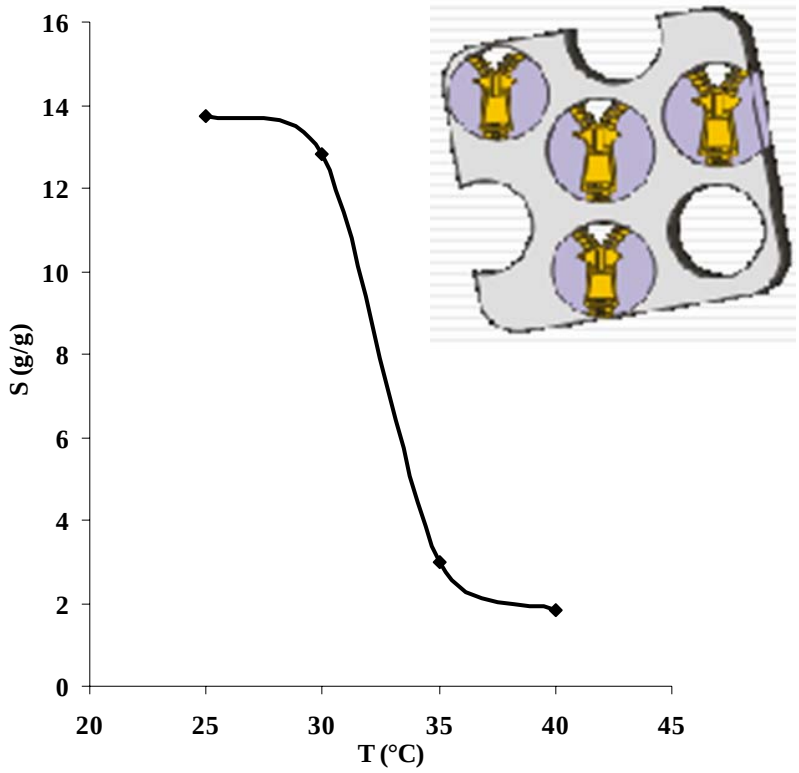

Fig. 3. The swelling values of thermosensitive poly(N- isopropyl- acrylamide) hydrogels as a function of temperature

3. 2 Development of synthesis of hydrogels and nanoparticle composites: preparation of nanohybrid materials

Hydrogels loaded with dispersed clays are a new class of composite materials which combine elasticity and permeability of the gels with high ability of the clays to adsorb different substances. Many clay minerals are able to absorb/ desorb organic molecules, and so are

widely studied as carriers or support of pharmaceuticals. The filler containing hydrogel samples can be fabricated in a variety of shapes and geometries (**Fig. 4**).

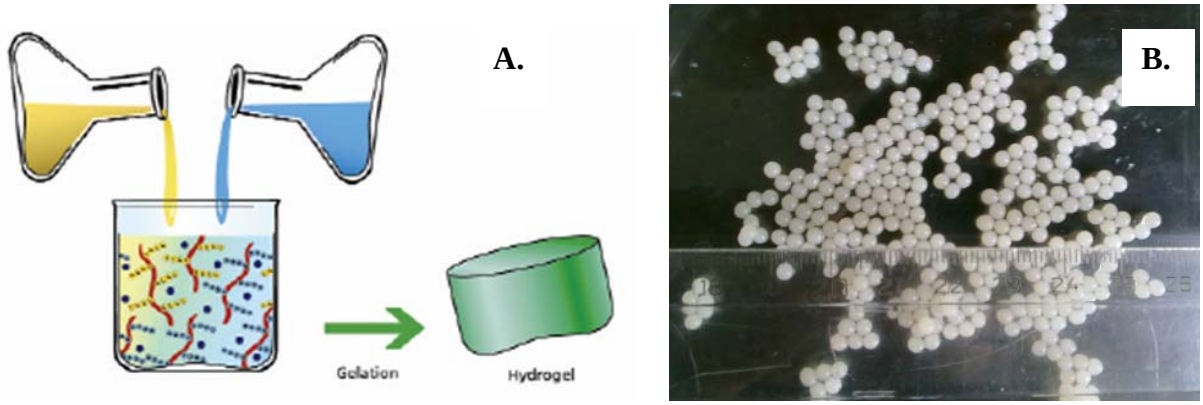

Fig. 4. Schematic representation of the synthesis of hydrophilic polymers (A.) and the hydrogel based beads containing clay minerals as polymer filler (B.)

3.3 Analysis of pH sensitive characters of hydrogel nanoparticles composites

pH-sensitive hydrogels are probably the most commonly studied class of environmentally sensitive polymer systems in drug delivery research. There are two types of pH-responsive polyelectrolytes; weak polyacids and weak polybases. For polyanionic hydrogels, swelling is minimal at low pH and maximal at higher pH (**Fig. 5. A.**). Polycationic hydrogels show the opposite effect: swelling is minimal at high pH and maximal at low pH (**Fig. 5. B.**). With the desired application in mind, a reasonable choice must be made between polyacids and polybases.

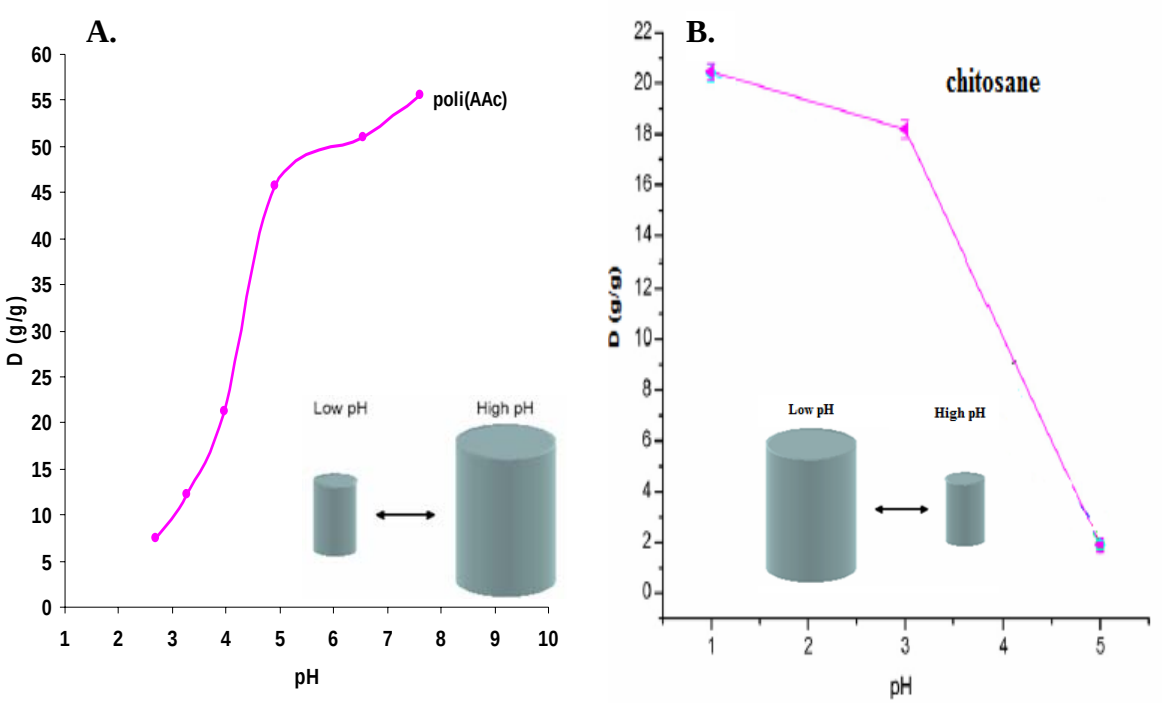

Fig. 5. The swelling values of poly(AAc) (A.) and chitosan (B.) based hydrogels as a function of pH

3.4 Development of the fixation of drug molecules on nanocomposite carrier

One of the objectives of our project is to encapsulate active agents into biopolymers and/or proteins, because various active agents functionalized in e.g. human serum albumin (HSA) or encapsulated into a HSA layer can easily be carried by the blood flow to different parts of the body. HSA is a transport protein localized in the blood plasma, thus its utilization enables the delivery of active agents of various philicities into the organism. Placing the resulting, protein-encapsulated active agent into polymers sensitive to different pH values, a system "opening up" at the desired pH is obtained, allowing pH-dependent delivery and subsequent absorption of the encapsulated agent (**Fig. 6.**).

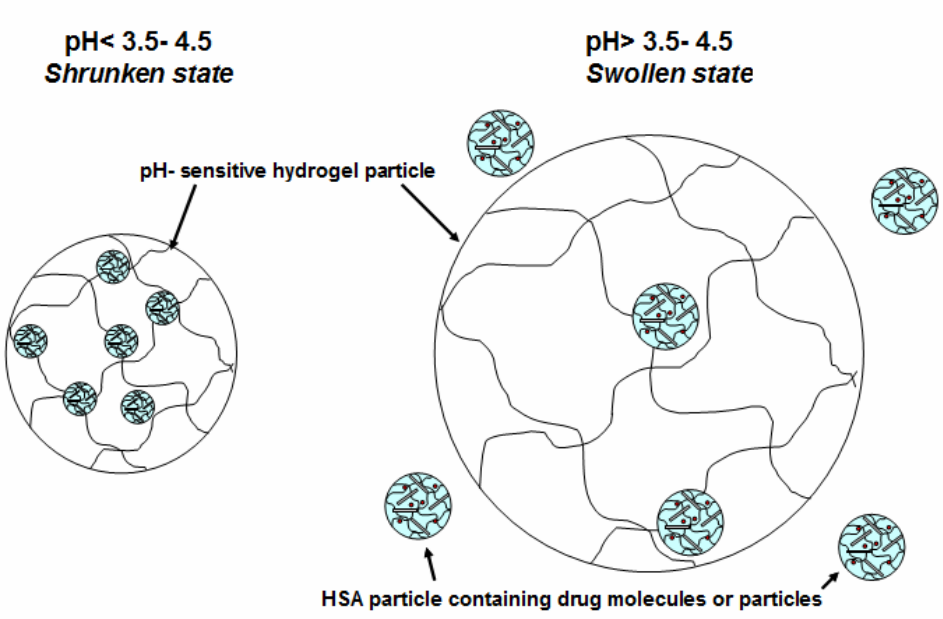

Fig. 6. Schematic representation of HSA- encapsulated drug molecules in pH- sensitive hydrogel particles

3.5 Examination of liberation of nanoparticles from polymer composite matrixes

The drug or antibacterial- agent content of hydrogel matrix is adjustable (**Fig. 7**) and the level of released drug is very important for medical application. To achieve and maintain the drug concentration in the body within the therapeutic range required for a medication, it is often necessary to take this type of drug delivery system several times a day. One of the most simplest method to measure the drug release is the dialysis experiments.

Dialysis experiments were carried out in dialysis tubes filled with either solution or dispersion of drug molecules or nanoparticles and immersed within the 100 fold volume of distilled water or physiological saline (**Fig. 8**).

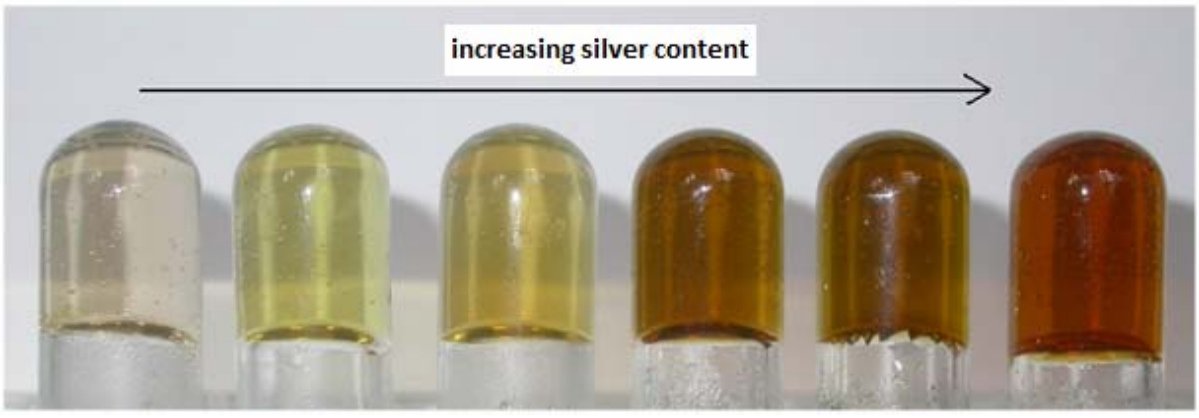

Fig. 7. Photos of silver nanoparticle loaded bulk hydrogel samples

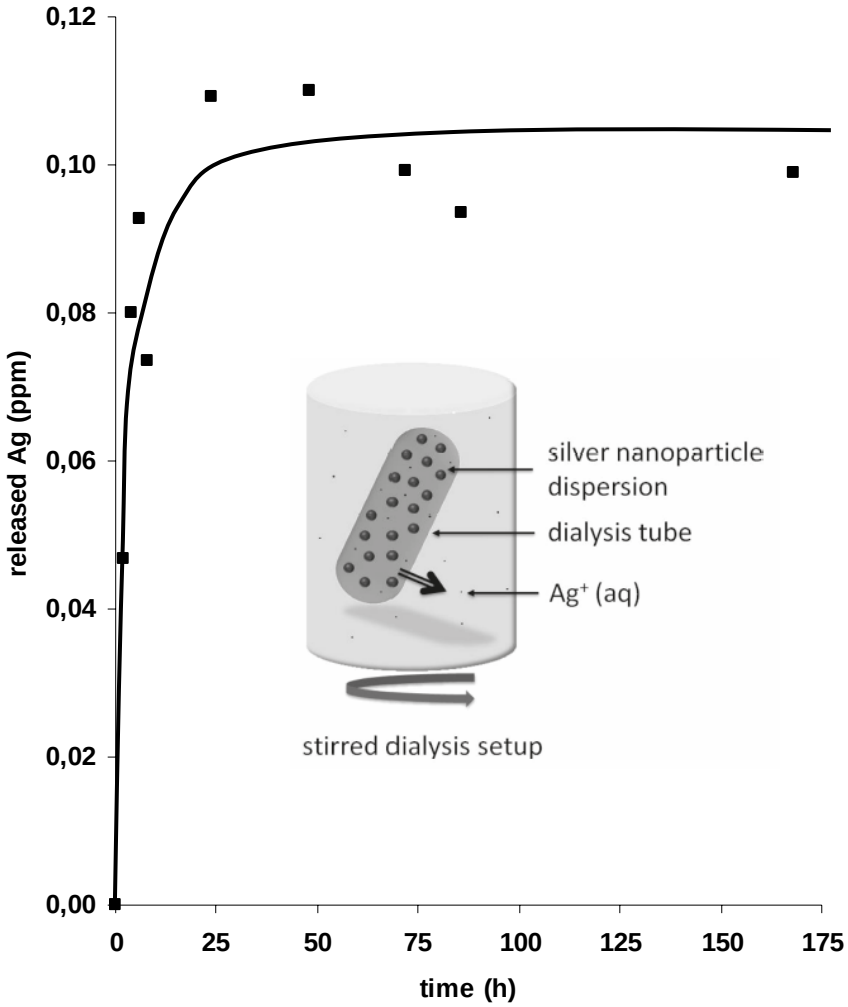

Fig. 8. The amount of dissoluted Ag^+ released from antibacterial silver nanoparticle dispersion

3.6 Analysis the in vitro antimicrobial effects of the developed nanocomposites

The different conditions affect not only the release of the drug from the polymer matrix but also the antibacterial potency of the released drug. In vitro test experiments must be carried out to measure the antibacterial properties of the released materials (**Fig. 9**).

Fig. 9. Inhibition zones of bacterial growth in the presence silver nanoparticles

Projekt leírás

A nemzetközi szakirodalmi adatok alapján a reproduktív korú női populációt érintő, recidiváló mikrobiális vaginitis hátterében genetikai faktorok állnak. Az akár meddőséghez vezető, visszatérő betegség célzott kezelésére az egyedi sajátosságokat is figyelembe vevő terápiás eljárás, készítmény nem áll rendelkezésre.

A projekt kutatási programjának célkitűzése a mikrobiális hüvelygyulladás következményeként definiált hatások (fogamzó képesség csökkenés, terhesség esetén koraszülés előidézés) megszüntetése, illetve mérséklése. Ennek érdekében a projektben zajló kutatási program tevékenységei

- hajlamosító genetikai tényezők beazonosítása, a fokozott kockázatú betegek kiszűrése

- egyedi adottságokat figyelembe vevő, ugyanakkor mellék-és teratogén hatásoktól mentes nanotechnológiai alapú terápia fejlesztése

- nanotechnológia, nőgyógyászat, bőrgyógyászat, mikrobiológia és genetika kutatási aspektusainak integrálása, határon átnyúló innovatív kutatói kooperáció működtetése a koraszülés és a meddőség új kutatási irányvonalai feltárása

- mikrobiális hüvelyi fertőzések kezelési központjai létrehozásának megalapozása

Scientific meeting in Novi Sad, Faculty of Medicine Univesrity of Novi Sad