Properties of ghost bacterial cells obtained by hybrid material with silver nanoparticles (PVA / AgNps) as well as their opportunities for use in vaccine prophylactics

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“All for one and one for all, united we stand divided we fall.”



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Alexandre Dumas, The Three Musketeers

NANOTECHNOLOGY

- Has emerged as a rapidly growing field with **applications in science and technology** for the production of new materials with dimensions from the nanoscale.
- The word "**nano**" is used to refer to **one billionth of a meter** or 10^{-9}.

•**The improvement of the properties by decreasing the size of the particles** is attributed to the **increase in the number of active sites on the surface of the material.**

•**Larger particles have more inner active sites, which remain actually intact** under chemical reactions, or external forces.

•The **metal nanoparticles** - the most promising, because they showed among others **good antibacterial properties**.

SILVER NANOPARTICLES (AgNps):

• can be **produced by chemical or physical methods;**

• serve as a **reservoir for supplying dissolved silver ions** with **strong bactericidal effect;**

•because of their small size can potentially **pass through biological membranes;**

•**regardless of the shape** of the silver, essential characteristic is the **concentration** of released silver ions;

•**Incorporation in different polymer** structures allows **control over their size, shape and stabilization**.

It can be summarized that:

1. **The toxicity of nano-silver is greater than silver as a whole** (Pal S. et al., 2007; Elechiguerra J. L. et al.,2005).
2. **The silver is significantly more toxic than other heavy metals when they are in the form of nanoparticles** (Braydich-Stolle et al., 2005).

PVA/AgNps

•A hybrid material with **thermally reduced silver nanoparticles**, stabilised in **polyvinyl-alcohol**;

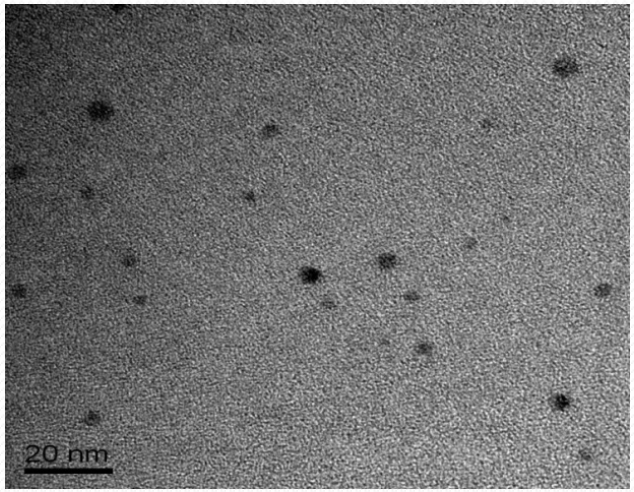
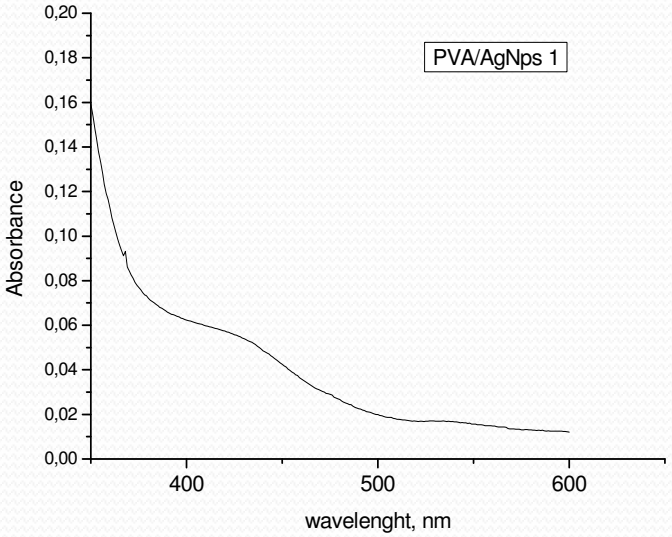


Fig . TEM analisis of silver nanoparticles, stabilized in PVA

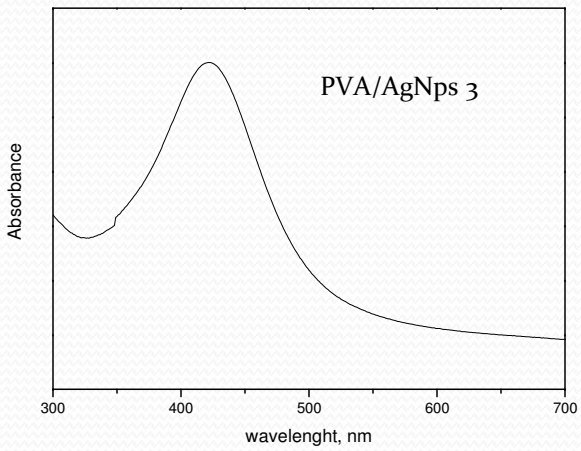
The silver nanoparticles in a PVA/AgNps are well-defined with **average diameter 5-6 nm.**

Using **UV-vis spectroscopy** of different synthesized samples PVA/AgNps is detected **peak at 420 nm**, characteristic of **the formation of the nanoparticles**.

a)



c)



b)

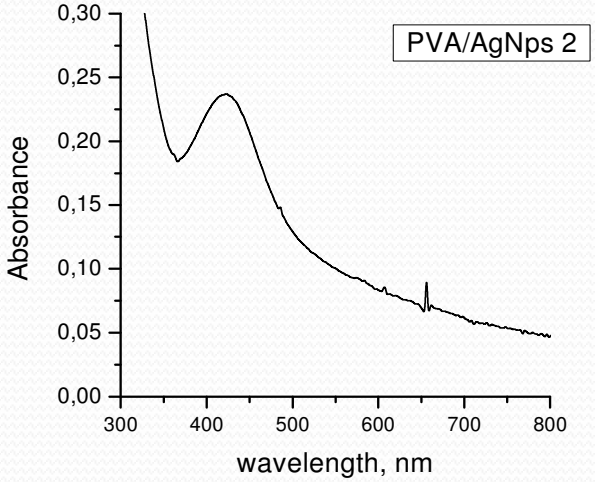


Fig .: UV-vis spectrum of AgNps stabilized by PVA; silver concentration : a)175.9 mg/L; b) 2032 mg/L c) 156.9 mg/L.

PVA/AgNps :

• well characterised with experimental results from **physicochemical, microbiological and cytological tests**;

•**tested on nearly 150 bacterial and fungal strains** with proved previously resistance to two or more antibiotics / antimycotics;

•can be used for **produce of "ghost" candidate vaccine cells**.

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GHOST BACTERIAL CELLS

Wikipedia: A **ghost** is the soul or spirit of **a dead** person or animal (way not also bacteria) that can appear, in visible form or other manifestation, to the living.

The use of "bacterial ghosts" as a candidate vaccine is a **new and progressive approach for introduction of safe, non-living, active vaccines** for prevention of a wide range of infectious veterinary diseases (Szostak P. et al, 1996).

"Ghost" vaccines retain its **natural outer membrane** with strong **immunostimulatory lipopolysaccharide structure**.

There is scientific evidence for the **preparation of bacterial "ghost cells" by a non-denaturing process through controlled expression of plasmid PhiX174 of lysis gene E in gram-negative bacteria.** The result is a **tunnel formed by specific protein E,** which is limited to a **small fraction of the total cell surface** (Szostak M. et al, 1996).

1. **Killed, containing whole cells vaccines** have **the advantage of representing the full range of antigenic determinants** of the immune system.
2. **Disadvantage of the traditional production of non-living (killed) vaccine** by heat treatment, irradiation or chemical treatment of the pathogen often is the **denaturation of significant structural components of the cell wall:**
    - •loss of important immunogenic epitopes;
    - •lack of a complete immunity (Jalava K. et al , 2002).

There are a lot of publications for **lethal action of silver nanoparticles on bacteria, fungi, viruses and parasites** (Clement, J. and Jarrett P., 1994; Silver S., 2003; Hurst K., 2006; Rai M. et al., 2009; Galdiero S. et al., 2011, Rai M. et al, 2014).

•**Silver nanoparticles** impact on the target object by **multiple mechanisms**, but a way of killing cells is the **formation of 'pores' on their membranes** (Sondi, I. and Salopek-Sondi B., 2004; Lee J. et al, 2010).

This is what gives a reason to assume that in **the treatment of bacterial cells with the hybrid material PVA/AgNps will produce "ghost" candidate vaccine cells.** (Pencheva D. et al. Testing of...,2010; Pencheva D. at al. Is there a presence...., 2011; Pencheva D. et al, Poly(vinyl alcohol)/Silver nanoparticles...., 2012)

•On the one hand **the positively charged silver cations** associate with **negatively charged components of the bacteria** (cell wall and membrane).

•Another mechanism of action is **penetration of the silver cations inside of the bacterial cell** binding to the negatively charged proteins, enzymes, DNA or RNA, to interfere with electron transport, cell division and cell replication.

•AgNps have **activity against fungi and viruses by attaching in an analogous mechanism** with the negatively charged parts (Sondi and Salopek-Sondi, 2004).

Dimorphic transition of **C. albicans** from yeasts to the micellar form is considered to be responsible for pathogenicity.

•AgNps **inhibit the extension and the formation of mycelium** by attacking their membranes, and thus **distort the membrane potential**.

•The AgNps stirred membrane lipid bilayer, causing **outpouring of ions and other materials**, and also **formation of pores and distribution of the electric potential of the membrane**.

•Ag- nanoparticles cause **disturbances in the normal process of budding**, which correlates with damage to the membrane (Lee J. et al., 2010).

•**AgNps enter in the fungi, forming space with a small molecular weight in the center of the fungus attached to the respiratory chain and eventually stop the cell division,** which results in cell death (Nasrollahi A. et al., 2011).

At **TEM** is found that they cause (Lee J. et al., 2010):

•formation of holes in the cell walls

•pores in the cytoplasmic membrane.

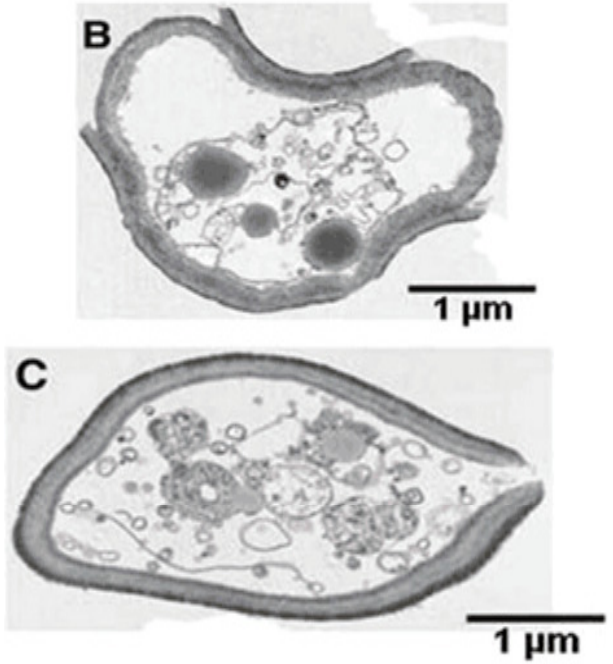


Fig. 5. Transmission electron micrograph (TEM) of *C. albicans* cells. *C. albicans* were incubated in the presence of different amounts of Nano-Ag for 24 hrs at 28 °C. The bar marker represents 1 µm. (A) Control, (B) Cells treated with 170 µg/ml of Nano-Ag, (C) Cells treated with 400 µg/ml of Nano-Ag.

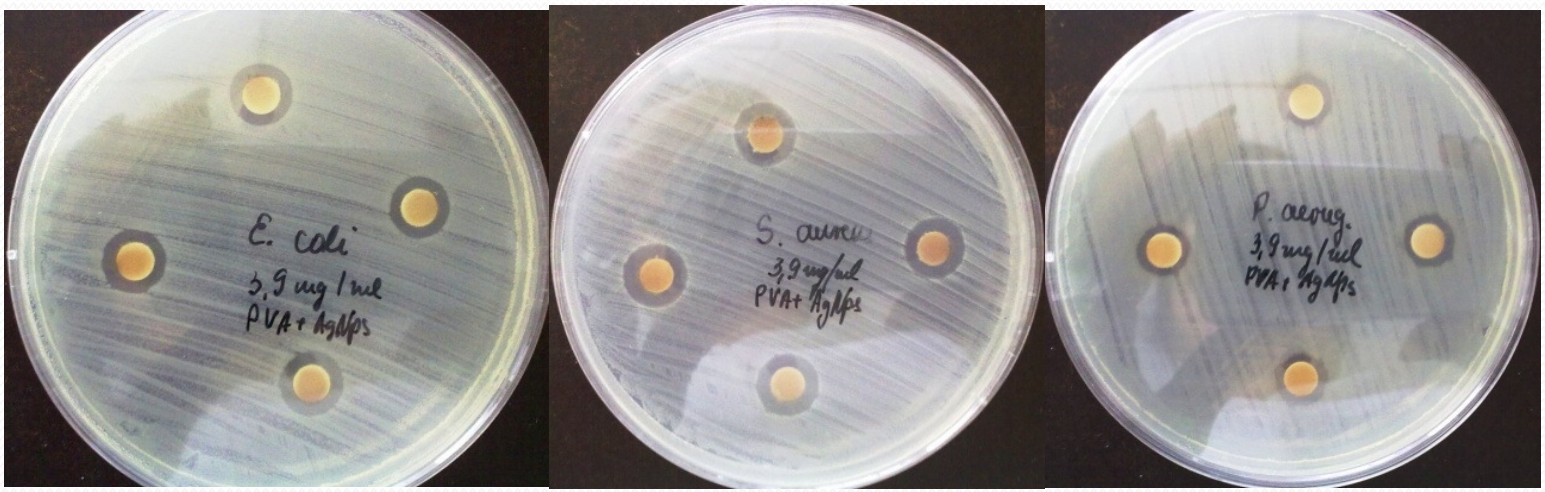
(Lee J., Kim K.-J., Sung W.S., Kim J.G. and Lee D.G., 2010. The Silver Nanoparticle (Nano-Ag): a New Model for Antifungal Agents, Silver Nanoparticles, David Pozo Perez (Ed.), ISBN: 978-953-307-028-5, InTech, Available from: http://www.intechopen.com/articles/show/title/the-silver-nanoparticle-nano-ag-a-new-model-for-antifungal-agents)

IN VITRO MICROBIOLOGICAL TESTS

For testing the antimicrobial properties of the synthesized samples PVA / AgNps following methods are used (Pencheva D. et al. Testing of...,2010; Pencheva D. at al. Is there a presence...., 2011; Pencheva D. et al, Poly(vinyl alcohol)/Silver nanoparticles...., 2012) :

1. **DDM** (Disk Diffusion Method);
2. MIC by the **agar dilution method**;
3. **Methods with the macro dilutions** (MBC, MFC);
4. **Chess-board method** for testing the presence of synergism of PVA/AgNps and Pi, Ce and Cz ;
5. **Modified method for testing the presence of synergism** of the material to antimycotics;

Bactericidal properties of hybrid materials PVA / AgNps established initially through testing to **control bacterial strains** over a **DDM**.

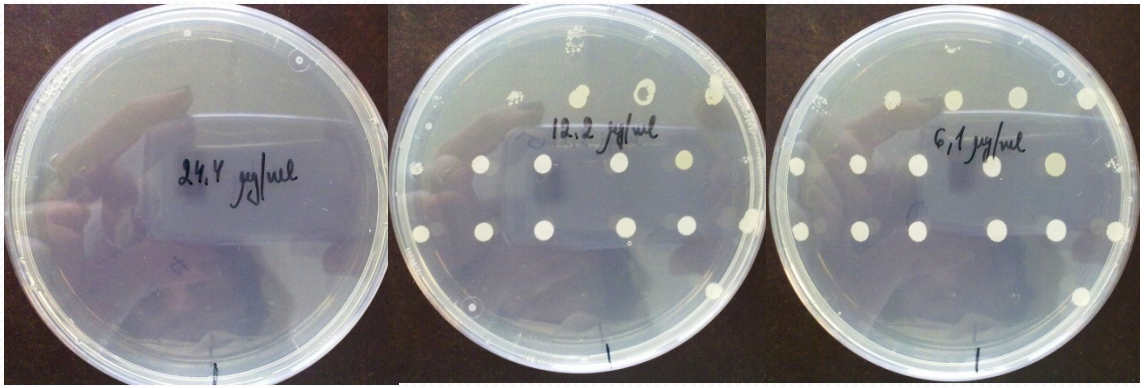


•**The Agar Dilution Method** is very convenient for the simultaneous **determination of the MIC of a large number of strains.** In the experiment were used:

- 21 clinical isolates from *Staphylococcus sp*.,
- 24 clinical strains *E.coli* and
- 26 clinical strains *P.aeruginosa*.

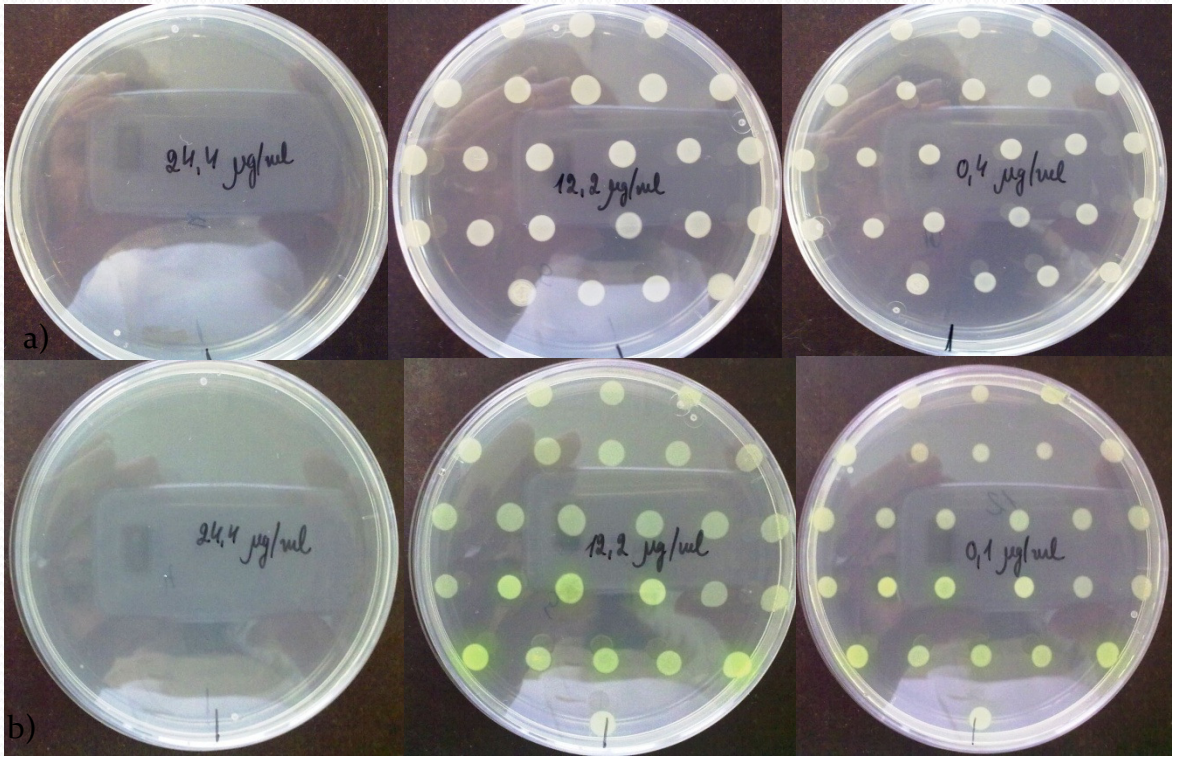
The tested clinical strains of *S.aureus* and *S. saprophyticus* were with established resistance to 6 antimicrobial substances.

MIC for all staphylococci was ≥24,4 µg/ml with the **exception of four strains, in which it was lower**.



Agar dilution method for testing the MIC of the PVA/AgNps to gram-positive clinical strains

The tested clinical strains of ***E. coli*** were **resistant to 11 antibiotics**, and the explored clinical ***P.aeruginosa*** **strains - to 8 antibiotics**. **MIC** for all tested Gram negative bacteria was **≥ 24,4 µg / ml.**

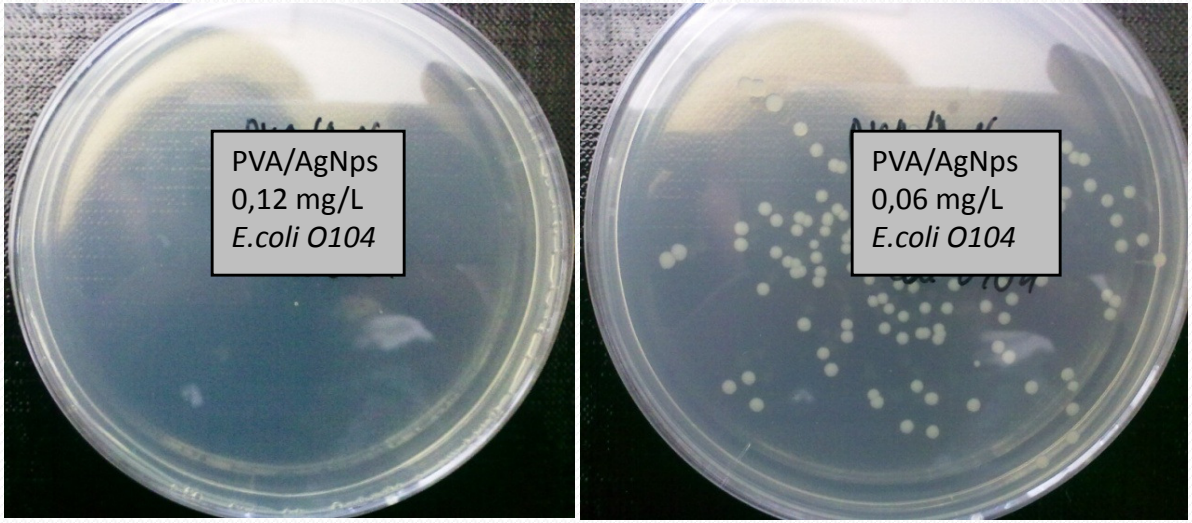


Agar dilution method for testing the MIC of the gram-negative clinical strains: E.coli a) and P.aeruginosa b)

For the determination of the **MBC (MBC ≥ 0,12 mg / L)** with the **method of macro dilution** were selected at first **four multiresistant clinical isolates** :

- **two strains *P.aeruginosa*, isolates from humans**
  - ✓ *P.aeruginosa* 1773, resistant to Pi, Cz, Ct, Ce, Azt, I, G,Cp
  - ✓ *P.aeruginosa* 1570, resistant to Pi, Ct, Cz, ,Ce, Azt,G,Cp
- **one strain *E.coli*, human's isolate**
  - ✓*E.coli* № 5, resistant to A, A/S, AmC, Cx, Cz, Ct, Cm, Cft,Ce, Azt, G,Cp
- ***A.baumanii*-isolate from an ear infection in a dog**
  - ✓*A.baumanii*, resistant to Pi, A/S, Cz, Ct, Cft, Ce, I, G, Tb, Am, T, D, Cp, S/T

MBC of the **control strain *E. coli* O104 Kopenhagen: ≥ 0,12 mg/L.**



Other **30 Pseudomonas sp., Klebsiella sp.** and **Salmonella sp.** (Iliev M., 2013) **clinical strains**, tested with other synthesized sample PVA/AgNps (ICP 175.9 mg/L) were with established **MBC ≥ 0.5 mg/L.**

The MBCs of synthesized samples (AAA 174 mg/L) were determined also for ***E. coli* O 149, *E. coli* O 157 H7 and *S. Typhimurium*** (common pathogens in farm animals with huge losses for animal farming) as followed:

- ***E. coli* O 149** and ***S. Typhimurium* - 0,05 mg/L**
- ***E. coli* O 157 H7 - 0,03 mg/L**.

This values were even less than the determined for other bacterial strains – **0,12 mg/L**.


PVA/AgNps 0,03 mg/L
E.coli O157H7
PVA/AgNps 0,014 mg/L
E.coli O157H7
PVA/AgNps 0,007 mg/L
E.coli O157H7
PVA/AgNps 0,05 mg/L
E.coli O149
PVA/AgNps 0,03 mg/L **E.coli O149**
PVA/AgNps 0,014 mg/L
E.coli O149
PVA/AgNps 0,05 mg/L
S.typhimurium
PVA/AgNps 0,03 mg/L
S.typhimurium
PVA/AgNps 0,014 mg/L
S.typhimurium

Determination of MFC of the tested yeasts.

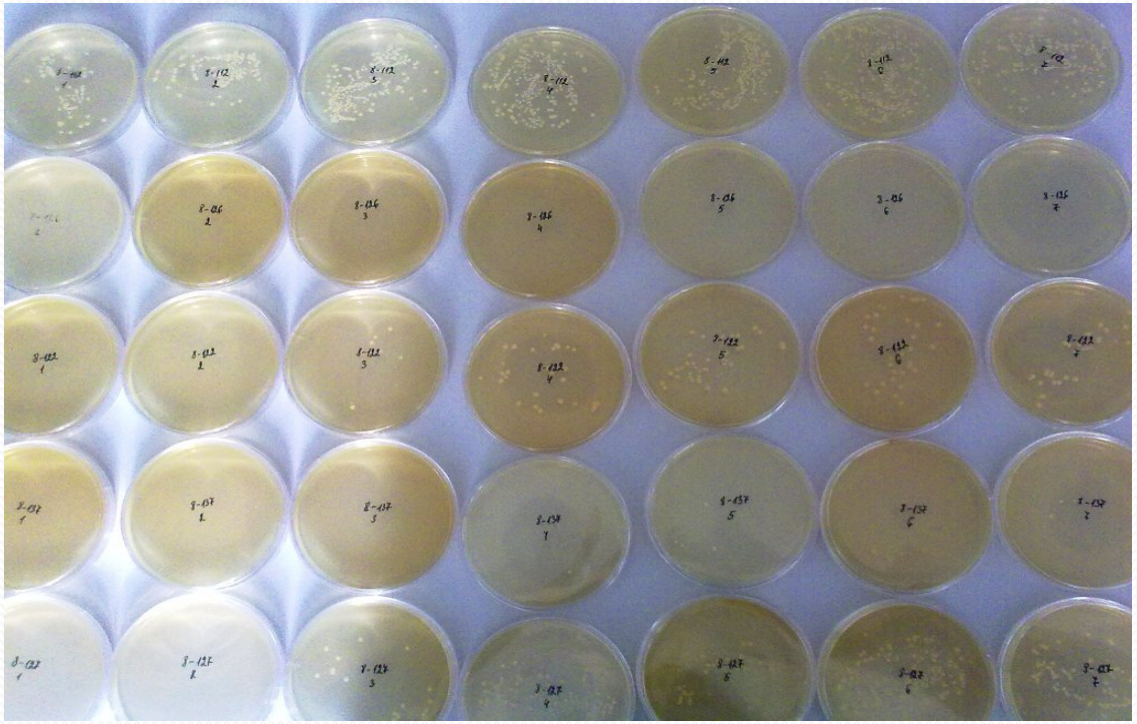
The MFC for the **control strains** was determined onto modified CLSI method (Pencheva D. et al, Poly(vinyl alcohol)/Silver nanoparticles…., 2012):

- *Candida albicans* and *Candida krusei* - lower than **14.5** mg/L
- *Candida tropicalis* -**28.99** mg/L
- *Candida glabrata* – **115.98** mg/L
- *Aspergillus brasiliensis* - **927.81** mg/L.

The fungicidal activity of PVA/AgNps was observed against **five clinical strains** with proven resistance to one or more antimycotics. The established values **for MFC differ from the results obtained for the control strains** (Pencheva, D.et al., Comparison....., 2010):

- *Candida albicans* 8-127 and *Candida glabrata* 8-122 - **463.91** mg/L,
- *Candida albicans* 8-137 - **231.95** mg/L,
- *Candida krusei* 8-126 MFC - **lower than 14.5** mg/L.
- *C. krusei* 8-112 -**no fungicidal activity** of the tested PVA/AgNps was observed.

Further, ***C. krusei* 8-112 strain was found to be resistant to silver in PVA/AgNps** respectively at as high as **1960 mg/L Ag concentration,** which is indicative for the **presence of silver resistance strain.**



Determination of MFC of PVA / AgNps for clinical *Candida* spp.isolates.

Other four clinical Candida strains (***C. krusei* 8-48, *C. parapsilosis* 0-115, *C. glabrata* 0-73, *C. nivariensis* 383**) were tested **using another validated method**, by adding such a quantity of the suspension to each tube of the reaction system, which **guarantees the submission of 10^5-10^6 Cfu / ml**.

The MFC for all of them was defined as **less than 0,27 mg/L** (Pencheva D., Ivanova Z. et al., 2014).

A chess-board method was used for testing the presence of synergism in combining the hybrid material with:

- **piperacillin (Pi)** and **cefepime (Ce)**: **self-administered, the hybrid material has a higher antibacterial activity** than in combination with antibiotics;
- with **ceftazidime (Cz)** on *Klebsiella* 2494 (Iliev M. , ESBL..., 2013) and on *Salmonella* Paratyphi B 176 (Iliev M., Antimicrobial's...., 2013) showed that according to EUCAST –the combine effect can be reported as **sinergism**.

Further, **the presence of synergism** was investigated **combining PVA/AgNps with antifungal agents**, using a commercial product ATB™Fungus.

A **synergistic effect** was observed when PVA/AgNps material with decreasing concentrations of antifungal agents was used.

Similar effect was **observed even for *C.krusei* 8-112 strain** which was **resistant to PVA/AgNps** when it was used **singularly** (Pencheva D.et al., Presence of synergism ....., 2011).

Specified **MBC** and some evidence of **therapeutically efficiency** (**TE**) can determine **the nearest appropriate range of their application** at given silver concentration.

The growth of all tested with this sample cell lines was suppressed in a **dose-dependent manner**.

These sample **PVA/AgNps** was established as **not cytotoxic**, on two cell lines (MDCK and EBTr) tested at concentrations ranging **from 0.0005 mg/L to 1 mg/L** and **TE = 90 x 10^3** (Pencheva D., Ivanova Z. et al., 2014).

IN VIVO EXPERIMENTS



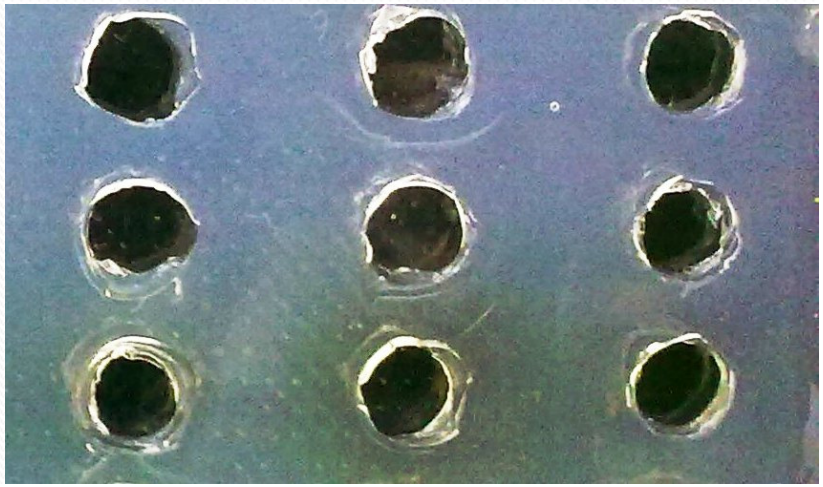
•**Dermal cytotoxicity test** and **subcutaneous injections** on **white mouse-** PVA/AgNps was a **non-toxic** in the enclosed silver concentration (30 mg/L);



•**as inactivator of a bacterial strain *E. coli* O 104** with final concentration of silver **30 mg/L** -for the preparation of **antigen for immunisation of rabbits**;

•**as preservative** of the obtained in consequence of immunisation, **hyperimmune *E. coli* O104 rabbit antiserum.**

Before in the produced agglutinating rabbit antiserum E.coli O104 to be added as a preservative the hybrid material PVA / AgNps, it was enrolled **in antigenicity studies** in the **diffusion on Uhterloni** onto 2% agarose plate.



Linear immunodiffusion on Uhterloni of E.coli O104 of an experimental antisera and PVA/AgNps.

This indicates a **certain antigenic activity of the hybrid material**, as a result of which they **form soluble antibodies** against the used for the inactivation of the strain hybrid material PVA / AgNps.

Given that it is a complex compound having **a molecular weight of PVA only over 20 000 daltons** (molecular weight above 6 000 daltons, for the most part are immunogenic) can be questioned whether the hybrid material **possesses adjuvant properties**.

•**Killed vaccines** induce a strong **polyclonal immune response and immunity tense** as a result **of "build in" adjuvants.** Such effects have less purified vaccines.

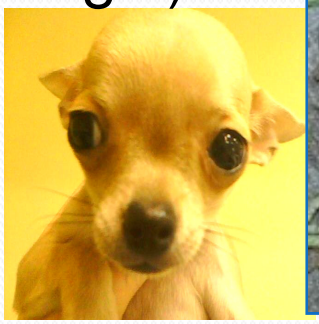
•**The more purified they are, the weaker the immune response is and stimulate greater number of required immunizations, which raises the cost of the vaccine.**

•In highly purified peptides and carbohydrates is essential to add an adjuvant to fail to immune tolerance to them (Spickler A. and Roth J., 2003).

VETERINARY EXPERIENCE

The hybrid material PVA / AgNps was administered in different Ag -concentration by a veterinarian for **treatment agent in dogs** with:

- **purulent wound** from awns (30 mg/l);
- **wound in the ears** (30 mg/l);
- **cough** (200 mg/l);
- **recurrent otitis** (600 mg/l).









MULTIDISCIPLINARY APPROACH FOR "ONE HEALTH"