UNIVERSITY OF PARDUBICE

Faculty of Chemical Technology

Department of Analytical Chemistry

THE STUDY OF EFFECT ON CONTROLLED RELEASE OF IODINE ON BACTERIAL BIOFILM ERADICATION IN WOUNDS

Katarzyna Maria Szuszkiewicz

DOCTORAL THESIS

2016

Advisor: **Prof. Ing. Karel Ventura, CSc.**
Head of the Department of Analytical Chemistry,
Faculty of Chemical Technology, University of Pardubice

Advisor – specialist: **Ing. Pavel Klein, Ph.D.**
Head of Experimental Animal Facility,
Biomedical Centre, Faculty of Medicine in Pilsen, Charles University in Prague
former employee of Contipro Pharma a.s. (Head of Wound Healing Research Group)

Student: **Ing. Katarzyna Maria Szuszkiewicz**
Department of Analytical Chemistry,
Faculty of Chemical Technology, University of Pardubice
former employee of Contipro Pharma a.s. (Wound Healing Research Group)

ABSTRACT

The purpose of the research was to specify the influence of the kinetics of iodine release from wound care products containing iodine on the biofilm eradication. The disruption of biofilm should concern both killing the bacteria in already formed biofilm and protecting the wound against its re-formation. The research was divided into two parts: preparation of samples with its analyses of iodine releasing, and the *in vitro* study.

The main objectives were to determine the influence of the kind of active compound and its concentration, auxiliary compounds, the form of their sources, and their arrangement in the wound dressings on the kinetics of iodine release and simultaneously on their abilities to inhibit wound biofilm. The selection of the prototype of the anti-biofilm wound dressing that could have been tested on the *in vivo* porcine model with infected wounds covered with biofilm should have been the outcome from mentioned aims.

The wound dressing was based on the two-component Iodine Generator. Several application forms of this medical device were prepared and the kinetics of iodine release from the bandage was defined. The dressing may have contained different active layers placed inside the bandage. From three chosen principles of iodine formation the further research was conducted with the prototypes impregnated with potassium iodide where suspension of organic peroxides (dibenzoyl peroxide or carbamide peroxide) was applied on it to trigger the iodine release.

The kinetics of iodine release from the dressings was tested on the "starch gel" model and subsequently with the use of a microanalytical method (sensors with platinum electrodes working in a biamperometric mode). The efficacy of bandages on biofilm eradication was confirmed using the modified Lubbock biofilm model that was prepared from the blend of following bacteria strains previously isolated from human chronic wounds: *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli* and *Streptococcus agalactiae*.

Finally, there was chosen a prototype of a dressing that was directed for the *in vivo* study on the chronically infected wounds in minipigs which constituted the next part of the TACR (Technical Agency of Czech Republic) project: *TA 03011029, New wound dressings with programmed release of active substances for biofilm inhibition*. This work was mainly done at Contipro Pharma, Dolní Dobrouč, under the auspices of doc. RNDr. V. Velebný, CSc.

AUTHOR´S STATEMENT

I hereby confirm that I have written this paper independently. All the reference literature and information used in the paper are quoted in the list of reference literature.

I hereby acknowledge that all the rights and duties resulting from Act N. 121/2000 Sb., the Copyright Act, apply to my written work, especially that the University of Pardubice has the right to make a license agreement of use of this written work as a school work pursuant to § 60 section 1 of the Copyright Act. On the condition that the written work shall be used by me or a license shall be provided to another subject for the use hereof, the University of Pardubice shall have the right to require from me a relevant contribution to reimburse the costs incurred for the making of such work including all relevant costs and total overall expenditure and expenses incurred.

I express my consent with making the work accessible in the University Library.

Dated in Pardubice on 20 May 2016

Katarzyna Szuszkiewicz

Katarzyna Szuszkiewicz

ACKNOWLEDGEMENTS

I would like to thank professor Ing. Karel Ventura, CSc. who gave me the opportunity to study and supported me in the long way of reaching a peak of these studies.

I would like to thank my supervisor and leader, Ing. Pavel Klein, Ph.D. for his willingness of showing me a world of research, for advice in each step of work and time that he offered me, even at the expense of his family. I would also thank to his wife and three daughters for the patience and words of support.

I would like to thank doc. RNDr. Vladimír Velebný, CSc. for the possibility of working and studying simultaneously and for the allowance of using data from my work in Contipro Pharma in this dissertation.

I would like to thank to my co-workers in Contipro who helped me both in scientific and laboratory work, mainly to Veronika Hekrlová, RNDr Martin Sojka Ph.D., Ing. Zuzana Valentová Ph.D., Rastislav Slavkovský Ph.D., Ing. Michal Zavadil, Ph.D.; Mgr. Vojtěch Pavlík, Bc. Jan Němec, Martin Pravda, Ph.D., Mgr. Jan Kučera, MUDr. Jana Matonohová, MVDr. Zuzana Satinská and PharmDr. Lukaš Palek, Ph.D..

I would like to thank Ing. Radovan Metelka, Ph.D. and Ing. Martin Bartoš CSc. for their support in development of the system for the continuous monitoring of iodine determination and a professional collaboration during TACR project.

I would also like to thank Ing. Petra Bajerová Ph.D. and Eva Karamazovová for their help in complementing of formalities during studies.

Lastly, I would like to thank my family for all their love and encouragement. For my parents, Elżbieta and Tomasz, who raised me with a love of science and supported me in all my pursuits. Especially for my Mother who motivated me every day to reach the goals. For my husband Piotr who taught me how to be a stronger person and how to use the achievements of information technology. For my daughter Anna Emilia who was a driving force in passing my final exams and is a motivation in being a well-educated and creative mother.

Katarzyna Szuszkiewicz

ANNOTATION

The work deals with the determination of the influence of the kinetics of iodine release from wound care products containing iodine on the biofilm eradication. The kinetics of iodine release of commercial formulations and own-made wound dressings with two-component Iodine Generator were compared with the use of an *in vitro* starch gel model and subsequently with the use of sensors with platinum electrodes working in a biamperometric mode. The efficacy of bandages on biofilm eradication was checked using the modified Lubbock biofilm model that was prepared from the blend of following bacteria strains previously isolated from human chronic wounds: *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli* and *Streptococcus agalactiae*.

KEYWORDS

microbial biofilm, iodine, chronic wounds, biamperometry, screen-printed platinum electrodes

NÁZEV

Studium efektu řízeného uvolňovaní jódu na eradikaci bakteriálního biofilmu v ránách

ANOTACE

Práce se zabývá stanovením vlivu kinetiky uvolňování jódu z krytí na rány obsahujících jód na eradikaci biofilmu. Kinetika uvolňování jódu z komerčních formulací a vlastně vyrobených krytí s dvousložkovým Generátorem Jódu byly porovnány s využitím *in vitro* modelu škrobových gelů a následně s využitím senzorů s platinovými elektrodami pracujících v biamperometrickým režimu. Účinnost krytí na eradikaci biofilmu byla kontrolována pomocí upraveného modelu Lubbockého biofilmu, který byl připraven ze směsi následujících bakteriálních kmenů dříve izolovaných z lidských chronických ran: *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli* a *Streptococcus agalactiae*.

KLÍČOVÁ SLOVA

mikrobiální biofilm, jód , chronické rány, biamperometrie, tištěné platinové elektrody

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

ABBREVIATIONS

AUC	area under the curve
BF	biofilm
BPW	buffered peptone water
BS	*Bacillus subtilis*
CFU	colony forming unit
CMC	carboxymethylcellulose
DBP	dibenzoyl peroxide
DFU	diabetic foot ulcers
DSS	dioctyl sulphosuccinate sodium salt
EF	*Enterococcus faecalis*
EPS	extracellular polymeric substance
EVA	ethylene vinyl acetate
I_2G	iodine generator
kGy	kiloGray
KI	potassium iodide
McF	McFarland
PA	*Pseudomonas aeruginosa*
PAD	polyamide
PE	polyethylene
PES	polyester
PHMB	polyhexamethylenbiguanid
PMNs	polymorphonuclear leucocytes
PTR	peptone water (buffered) – abbreviation on the graph
PVP	polyvinylpyrrolidone
SA	*Staphylococcus aureus*
TA	Technical Absorbents (means: the textile 2678)
VS	viscose
VS/PES	viscose + polyester
XTH	xanthan

1 BACKGROUND

Wound healing is a complicated cascade process that includes resurfacing, reconstruction and restoring the tensile strength of injured skin. The process of healing is divided into four stages: haemostasis, inflammation, proliferation and maturation. In the first phase, coagulation factors are activated and fibrinogen is transformed to insoluble fibrin. Then, two to four days after the formation of the wound, the inflammatory phase appears where the inflammatory cells move to the injured place to release chemotactic and mitogenic factors to avoid infection and promote regeneration. Proliferative phase includes the migration of fibroblasts to fibrin matrix and its proliferation directed to the formation of collagen. In this third phase also occur epithelialization and angiogenesis. And finally comes the maturation phase that begins from the third week of healing and may last for an indefinite phase, where the newly produced collagen molecules are cross-linked with the already existed collagen and protein molecules. (Chang, 2013)

The above described cascade process may be affected by changes in pH at each phase. It was shown in several studies that pH of chronic wounds is in the range of 7.15 to 8.9. These highly alkaline conditions lower the healing rate when compared with wound having a pH closer to neutral. It is suggested to use pH values as a diagnostic tool being an indicator of wound healing. (Percival, 2014) In the Greener's work it was supposed that the pH level of wounds is an effective way of reducing protease activity and promoting wound healing. (Greener, 2005) Besides, when Roberts et al. performed a randomized study on 25 patients with venous ulcers, the measurements of pH and the temperature on the surface of the wound directed them to the conclusion that lowering wound pH and a higher wound temperature seems to be favourable to healing. In detail, the wound surface was significantly lower for a mean pH 6.91 against 7.42 in case of non-healed patients ($p=0.01$). (Roberts, 2005)

Due to the positive results with products lowering the pH of the wound some clinicians prefers to use honey as a wound dressing as its typical pH ranges from 3 to 4. Acidification of the wound bed has been shown to promote healing by releasing more oxygen from haemoglobin which in turn reduces the hydrostatic pressure in the interstitial fluid, thus allowing improved circulation in the tissues. (Molan, 1999)

However, it has been shown that a strong acidification of wound environment results in a reduction of phagocytosis and diminishes migration and chemotaxis of neutrophils.

In other words the acidic pH may attenuate neutrophil function. It was also mentioned that lymphocyte activity can be affected in an acidic microenvironment, but only a small number of studies have been undertaken. (Trevani, 1999)

The objective of the research was to find a wound dressing that could be effective in biofilm eradication. Ability to form biofilm is a virulence factor in the survival of pathogens in a diverse environment (Costerton, 1987; von Eiff, 1999). In case of planktonic bacteria usually a narrow range of pH enables them to live, for instance: pH (optimal growth of bacteria) in the range of 5.6 to 8.0 (6.6 - 7.0) for *Pseudomonas aeruginosa*, 4.4 to 9 (6.0-7.0) for *Escherichia coli* and 4.2 to 9.3 (7.0 – 7.5) for *Staphylococcus aureus*. (Todar, 2014) However, bacteria in the biofilm community can be exposed to pH which normally has an inhibitory effect on them and no negative effect is observed. (Percival, 2004) That is why the searches in this work were focused on finding a bandage that will decrease the pH of the wound and simultaneously will not be irritating for the surrounding skin. There may be a visible benefits of maintaining an infected wound to a slightly acid environment. This action is expected to enhance antimicrobial effect of antimicrobials (mainly silver) and reduce the wound bioburden in a shorter time. (Percival, 2014)

1.1 BIOFILM IN WOUNDS

A biofilm has been defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton, 1999). Interest concerning the presence of biofilm in chronic wounds has been growing and the new methods of treatment have been invented. But still there exist difficulties in proving the persistence of biofilm in treated wound. The presence of slimy material within a wound should never be interpreted as the presence of biofilm, since the arrangement of microbial cells within that slime can only be discerned with high power magnification techniques rather than the naked eye. Slough in a wound is not an indicator of a biofilm, because slough can be relatively easily removed by conventional debridement, while biofilm cannot. Nowadays, the detection of biofilm is based on the use of scanning electron microscopy and confocal laser scanning microscopy. The other method of using peptide nucleic acid fluorescence *in situ* hybridization (FISH) with epifluorescence microscopy gives the ability to recognize specific bacteria. (Cooper, 2010)

The widely used and probably the most efficient treatments of a biofilm infection are based on the mechanical debridement of the infected area. This method is mainly used

in case of a catheter, an implant or an infected organ eligible for the transplantation. However, it may be painful or cause the risk of complications for patients. The other way of fighting with biofilm is a blending of several antibiotics used in high doses. Treatment of biofilm with the minimal doses of drugs for a short period may induce further resistance and tolerance. (Bjarnsholt, 2013) That is why the Iodine Generator (wound dressing being developed during this work) is designed to show germicidal properties during the first 24 hours of the treatment (release of a high level of the active component) and to continue with bacteriostatic properties during at least next 48 hours.

1.1.1 Formation of biofilm

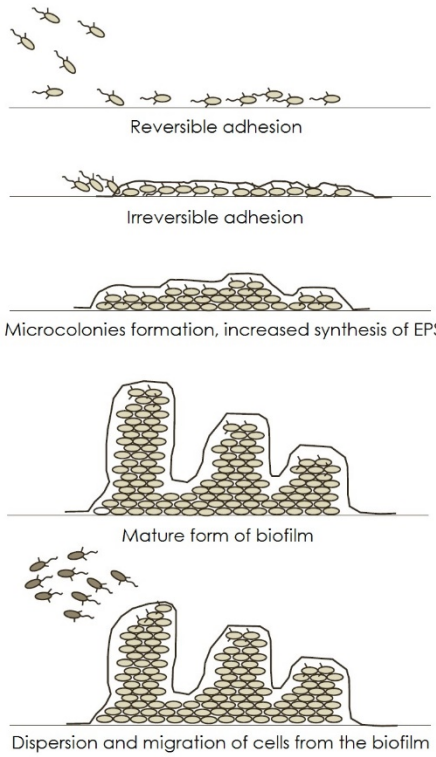


Figure 1. Formation of a mature biofilm. (Kolwzan, 2011)

Biofilm formation is a multistage process that is based on the kind of the bacteria strains and the construction and properties of the surface where the biofilm is attached (figure 1). The main role in adhesion play extracellular polymeric substances (EPS) produced by biofilm bacteria. EPS are formed by lipopolysaccharides and the proteins from the bacterial cell walls, and also extracellular structures, e.g. cilia, fimbriae. The colonization of biofilm bacteria is facilitated by the structure of the surface, its damages and roughness. The process of biofilm formation has not been well known, but there are visible the following phases: reversible adhesion of microorganisms, irreversible adhesion, maturation of biofilm and its dispersion. (Kołwzan, 2011)

As mentioned earlier, attachment is the initial phase in biofilm formation. Secondary, colonizers bind to the primary colonizers and the sequential bacterial binding results in the formation of nascent surfaces that bridge with the next co-aggregating bacterial cells. Wound biofilms may form in a manner similar to oral biofilms with initial colonization of skin flora and secondary colonization of wound pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. (Mancl, 2013)

It has been suggested that biofilm is involved in up to 80% of all infections in the human body including skin and genito-urinary tracts. The most often specified benefits of the bacteria living in a biofilm are improved protection from host immune response, superior resistance to antibiotic treatment and more accessible gene transfer leading to sharing of profitable attributes such as increased virulence. In case of wound healing, biofilms are considered to interfere mainly with the inflammatory process by the following mechanisms - first: evasion of the immune response by the matrix of extracellular polymeric substances (EPS) and second: by induction of a chronic non-healing inflammatory phase. (Thomson, 2011)

1.1.2 Biofilm resistance

Based on the literature basis, Costerton and col. suggest three hypotheses of biofilm resistance to antimicrobial agents. First mechanism is based on the failure of an agent to penetrate the full depth of the biofilm. EPS are considered to retard the diffusion of antibiotics and solutes at slower pace within biofilms than they behave in water. The second explanation suggests that at least some of the cells in a biofilm experience nutrient limitation which results in a slow-growing or starved state. And the last one, the most speculative hypothesis concerns that at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype. The phenotype is not a response to nutrient limitation. However, it is a biologically programmed response to the growth on a surface. (Costerton, 1999)

Staphylococcus aureus and *Pseudomonas aeruginosa* are traditionally recognized as major wound pathogens. There exists a hypothesis that many chronic ulcers do not heal because of the presence of biofilms. (Bjarnsholt, 2007) It is supposed that certain biofilms of *P. aeruginosa* produce a factor that shields the bacteria from the phagocytic activity of invading polymorphonuclear leucocytes (PMNs), thus preventing them of bacteria clearance. *S. aureus* has also been shown to display similar properties. This kind of mechanism may explain the failure of antibiotics to clear infections and allow wounds to heal. (Singh, 2008)

1.1.3 Common bacterial strains present in wound biofilms

It is possible to state that the nature of biofilm is polymicrobial. One of the studies showed that chronic wounds contain multiple bacteria species from which the common ones are *Staphylococcus aureus* (presented in 93.5% of ulcers), *Enterococcus faecalis* (71.7%), *Pseudomonas aeruginosa* (52.2%), coagulase-negative staphylococci (45.7%), *Proteus* species (41.3%) and anaerobic bacteria (39.1%). (Gjødsbøl, 2006)

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium with virtually no specific growth requirements. It is a non-fermentative organism that is able to grow either in the presence or without the presence of oxygen. 10% of its genes encode proteins involved in regulatory processes, which makes it a very diverse and adaptable organism. *P. aeruginosa* forms a highly differentiated biofilms composed of mushroom structures when glucose is used as a carbon source, whereas the addition of citrate as a carbon source makes from the same strain a flat and undifferentiated biofilm. (Bjarnsholt, 2013)

Staphylococcus aureus ("golden staph"), that is a facultative anaerobic Gram-positive coccal bacterium, is the most common cause of wound infection – e.g. from accidental injury or surgery. The obvious signs are that the wound is red and inflamed, seeping yellow pus, and may easily break open or fail to heal. Moreover, a wound abscess may develop. (HPA, 2009)

Enterococcus species often form biofilms or large communities of bacteria living on a solid surface within a self-produced matrix. These bacteria may infect humans and domestic animals because of their many virulence factors associated with biofilm formation including gelatinase, aggregation substance, capsule formation and enterococcal surface protein. *E. faecalis* (a Gram-positive, facultative anaerobic, commensal bacterium inhabiting the gastrointestinal tracts of humans and mammals) is the most common cause (80-90%) of infection followed by *E. faecium*. (Oli, 2012)

Escherichia coli is a Gram-negative, facultative anaerobic bacterium that is commonly found in the gastrointestinal tract and is a part of the normal bacterial flora. However, some *E. coli* strains are able to produce toxin that can cause serious infections. The main source of these strains is grass-feeding animals (cattle in particular). One of the toxins produced by *E. coli* is shiga toxin that has a high potential to cause severe enteric and systemic disease in humans (haemorrhagic colon and haemolytic uraemic syndrome. (ECDC, 2011)

Streptococcus agalactiae is a beta-hemolytic Gram-positive streptococcus that is mainly known as a strain that causes sepsis (blood infection) and meningitis (brain infection) in new-borns. Most bacterial pathogens are equipped with long filamentous structures such as pili or fimbriae that are often involved in the initial adhesion of bacteria to host tissues and in interactions between bacteria that result in biofilm formation. Characterization of the pilus locus in *S. agalactiae* showed that it encodes a major pilin and two minor pilin subunits that are covalently polymerized due to the action of two enzymes from the sortase C family. One of the pilins' functions is the responsibility for the adhesive property of the pilus. Konto-Ghiorghi (2009) revealed that pilus integrity is critical in adherence assays under flow

conditions. They also added that *S. agalactiae* can form biofilms and that pili play an important role in this process.

1.1.4 *In vitro* models of biofilm

There exist many different *in vitro* models of biofilm from which two of them will be presented in this chapter (third one is described in Methods).

Percival et. al (2007) introduced the *in vitro* biofilm model based on the poloxamer hydrogels. Poloxamer hydrogels are diblock polymers of polyoxymethylene and polyoxypropylene that display thermoreversible gelation properties (liquid below 15 °C). In this study the mentioned hydrogels were prepared by mixing Mueller-Hinton broth together with poloxamer to get the final concentration of 30% (w/v) poloxamer. The solutions were chilled to allow the poloxamer to go into solution and then autoclaved (121 °C, 15 minutes) and subsequently stored at 4 °C. The solutions were mixed and poured to the Petri dishes. Finally, the dishes were incubated at 35 °C to allow the poloxamer to solidify, again.

In this model, each microbial isolate (e.g. *Pseudomonas aeruginosa, Enterococcus faecalis*) was cultured overnight (concentration approximately 1 x 10^5 cfu/ml) and subsequently 1 ml of the microorganism suspension was inoculated on the surface of Mueller-Hinton agar and poloxamer gel plates in duplicates. The plates were gently agitated to get the equal distribution of the inoculum on the agar or gel surface. The tested dressings were cut to have a round shape and were put on the plates. After 24 hours of incubation, the plates were observed and the zone of inhibition (ZOI) around each sample on both agar and poloxamer gel plates were measured to assess the anti-biofilm properties of tested formulations.

Constant Depth Film Fermentor (CDFF) system is another one *in vitro* model used by researchers. The cultivated strains of bacteria (both aerobic and anaerobic) were recirculated through the specially designed fermenters for 24 hours to “seed” the system. After this time, the inoculum was disconnected and the fresh uninoculated medium was directed into the CDFF. The growth medium was transmitted at rate of 30 ml/h with the use of a peristaltic pump (Watson-Marlow). To quantify the bacteria counts, each of five plug inserts present in a single pan were removed aseptically and the biofilms from two plug inserts were resuspended in phosphate buffer by vortexing for 2 minutes. Then, the serial dilutions of re-suspensions were prepared and applied on the blood agar no. 2 (BA, aerobic isolates) and on fastidious anaerobe agar (FAA, anaerobic isolates), both supplemented with 5% defibrinated sheep blood. Plates were incubated at 37 °C for 2-3 and 7 days, respectively. (Hill, 2010)

1.2 TREATMENT OF INFECTED WOUNDS

Products for treatment of biofilm – infected wounds present on the market can be divided into three groups. First group concerns products with silver which are directed to the highly infected wounds. It is said that dressings with ionic silver are safer than products with colloidal silver. Some of the products are Debrisoft, Sorbion, Kerramax or Aquacel Ag. The second group consists of products used as rinsing formulations from which mainly Prontosan (BBraun) with PHMB and undecylenamidoprophylbetaine is going to be advertised as an anti-biofilm formulation (approved by *in vitro* studies). Also Octenidin (Schulke Mayer) is attending to be an anti-biofilm formulation (while studies) with no toxic effects on human cells.

PHMB compounds recently introduced to clinical practice have a broad spectrum of biocidal activity and have a range of uses. They have been used as contact lens cleansers, in mouth washes and as an antiseptic including use in wounds. PHMB is regarded as being safe to use following its inclusion as a preservative in cosmetics, where it has a low frequency of sensitization. (Cutting, 2010)

Therefore, the third group that is more and more visible on the biofilm market is the group concerning the presence of PHMB but in the form of solution or dressings. PHMB is a fast-acting biguanide compound composed of a synthetic mixture of polymers. It is structurally similar to the antimicrobial peptides (AMPs) produced by many cells within the wound, such as keratinocytes and inflammatory neutrophils, where they are thought to help as protection against infection. (Butcher, 2012) For instance, Suprasorb X+PHMB (L&R, Activa) is a dressing made of cellulose and 0.3% PHMB. Due to its gelling consistence it is not suggested for exuding wounds.

Nowadays iodine is not put to the group of anti-biofilm formulations but in the following years it may have appeared as one of the main biofilm-killer. In accordance to the theme of this work there will be presented information concerning wound care products containing iodine.

1.2.1 Iodine in wound healing

Iodine denatures proteins and inactivates enzymes, phospholipids and membrane structures and seems to be one of the most effective antimicrobial compound available in the wound treatment. (Schwartz, 2012) Iodine can be cytotoxic to eukaryotic cells at high concentrations so that there appeared the idea to design the dressing producing iodine *in situ* that will show germicidal properties during the first day of the treatment (release of high levels of iodine) and to continue with bacteriostatic properties during at least next two days.

The dressing should show a high absorption capacity for fluids, release iodine slowly and be non-toxic to tissues *in vivo*.

1.2.1.1 Wound care formulations with iodine

Several methods have been used to release iodine slowly to ensure non-toxic concentrations. From them the best known is povidone-iodine (e.g. Betadine from Egis). This formulation is well tolerated for a short period of time; even in diluted form it can be used as eye drops. However, the formulations (aqueous or gel solutions) of PVP-iodine run off and dry easily that cause the unequal distribution of iodine on the wound surface. In one part the concentration of the antimicrobial is really low and in the second one too concentrated and cytotoxic. Moreover, the manufacturers use different auxiliary agents, such as detergents, that also show their own toxicity. (Hunt, 1995)

On the chronic exudative wounds market there is presented an effective product containing iodine as an antimicrobial compound. Cadexomer iodine (Iodosorb, Smith&Nephew; currently not available in Czech Republic) is composed of a three-dimensional lattice formed into spherical microbeads. It has an extremely high absorption capacity for fluids, releases iodine (0.9% w/w) slowly, is non-toxic to tissues *in vivo*, and finally has an additional antiseptic property of trapping microorganisms.

In vitro study concerning the treatment of skin biopsies of chronic exudative wounds with cadexomer-iodine have demonstrated no evidence of cell necrosis, displayed re-epithelisation, and revealed bacteria within the cadexomer beads. Previously, there was also performed a test with 10% PVP-iodine from which it was visible that this preparation could not kill *Staphylococcus aureus* cells covered with glycocalyx.

Bacterial glycocalyx is highly hydrated (>99% water) and tends to collapse during dehydratation. The authors of the study suggest that cadexomer-iodine soaks up *S. aureus* cells encircled by glycocalyx, directly destroys biofilm structures and collapses glycocalyx during dehydratation and subsequently kills *S. aureus* cells within biofilm. Cadexomer iodine seems to be a promising treatment to clear *S. aureus* cells within biofilm from skin lesions of exudative or infectious wounds and to prevent wound exacerbation. (Akiyama, 2004)

Schwartz et al. (2012) performed a study with a use of two commercially available forms of cadexomer iodine (both from Smith&Nephew): a gel application (Iodosorb) and an absorbable pad (Iodoflex). There was no statistical difference noted between the used preparations of cadexomer iodine across all of data points. Therefore the results were

not differentiated in this work. These dressings are indicated to be replaced every 72 hours or as needed. However, the participants in this study had more frequent dressing changes. After 6-weeks study period, most patients dropped below the critical 10^5 bacterial level, which would indicate that they should proceed to an adjunctive wound closure. However, it would be good to continue in the study to see if the completed healing was achieved. While iodine has been compared to other wound treatments in the past, it has never been analysed and compared for success of decreasing wound bioburden and biofilm in DFU (diabetic foot ulcers). The ability of cadexomer iodine to reduce the bioburden of DFUs and to lead to a wound healing progression is really promising, but does require broader future *in vivo* studies.

On the other hand, Philips and col. used an *ex vivo* model of the mature *P. aeruginosa* biofilm on porcine skin explants for assessing the efficacy of several commercially available antimicrobial formulations. The model showed that antibiotics, silver and even PHMB are the least effective in killing mature biofilm. Whereas cadexomer iodine (Iodoflex) appeared to be an effective agent in *P. aeruginosa* biofilm eradication. (Philips, 2014)

1.2.1.2 Cytotoxicity of the iodine containing compositions

The continuous use of povidone-iodine formulations has the therapeutic reduction of the number of microorganisms, but several *in vivo* studies show their cytotoxicity. However, Nieder (1997) holds that PVP-I_2 that is used correctly does not cause any cell damage, even in a 10% concentration. He suggests that PVP-I_2 should not be spread on the wound together with detergents, e.g. liquid soaps.

Zhou and col. (2002) tested the cytotoxicity of cadexomer-iodine and found it to be non-toxic to fibroblasts *in vitro* when used at concentrations up to 0.45%. They did not observed any changes in viability, morphology, cellular proliferation, ability to produce collagen and cell outgrowth from explants. Moreover, *in vivo* skin biopsies of chronic exudative wounds that were treated with cadexomer-iodine showed no evidence of cell necrosis in dermis and epidermis.

1.2.2 Dibenzoyl peroxide as an active compound for the treatment of skin disorders

Food and Drug Administration (FDA) classifies benzoyl peroxide (DBP, $C_{14}H_{10}O_4$) as a generally recognized as safe and effective (GRASE) active ingredient in over-the-counter (OTC) topical acne drug product. This rule became effective on March 4, 2011.

When looking to the history, on 15th January 1985 Federal Register published a rule in which 2.5 to 10 percent DBP was proposed GRASE for the topical treatment of acne. But on 7th August 1991 this compound was classified to category III (e.g. "more-data-needed") instead of category I (GRASE). This category came after considering new safety data and information suggesting that DBP may initiate tumour formation and promote tumour development in animals. Finally, in 2010, after reviewing the data, it was stated that published studies in mice showed no evidence of DBP being carcinogenic and photocarcinogenic and concluded that DBP has to be adequately labelled to minimize the risks associated with DBP while delivering effective acne treatment. Consequently, DBP was classified as category I in the final rule.

However, some of the studies suggest that DBP is a tumour promoter with chemical initiators in animals, and three studies demonstrate that DBP is not carcinogenic or photocarcinogenic in animals. For instance, in the rat study DBP was applied at doses of 5, 15 and 45 mg per rat once daily to 12 cm^2 on the dorsal skin. The rats were scarified at 52 weeks or 104 weeks, and complete necropsies were performed. This study showed that DBP had no effect on survival, body weight, food consumption, or gross pathology, and neither produced any evidence of systemic toxicity. The dosing used in the study, that was 0.17, 0.83 and 2.5 mg per cm^2 every day, probably represented the doses used by humans under actual use conditions. *(In comparison to bandage with Iodine Generator: there is the same daily amount of DBP per day that is 45 mg on 12 cm^2 per 1 day when the strongest variant of tested bandages with 5% Akneroxid stays on the wound for only 48 hours.)* On the other hand, the tumour promotion studies indicated that DBP is a tumour promoter in the presence of a chemical initiator. It is unclear from the studies whether DBP is a tumour promoter in the presence of UV radiation (as an initiator) because two studies are contradictory. Therefore, the Committee believes these three studies are more meaningful than the conflicting tumour promoter studies. (FDA, 2011)

Benzoyl peroxide in a form of lotion can be used as a topical agent for treating chronic wounds. It is considered to have antimicrobial, antifungal and antipruritic properties. In 1985 in Sweden there was performed an experiment where the effects of different dilutions of DBP lotion compared with saline were tested. Both substances were impregnated on a wound dressing sponge which was then secured using an elastic support bandage. Most patients had leg ulcers of venous and arterial aetiology. Each patient had at least two leg ulcers and acted as their own control. The lotion with both 10% and 20% concentrations of DBP proved to be significantly more effective than saline in reducing ulcer area (~30% increase of healing

in comparison to saline). There was little difference in healing rates between the two concentrations of the lotion, indicating that lower strengths can be used with similar effect. It was reported that three patients had severe local irritation after using 10% solution.

The second study described in the same review was based on the comparison of a 20% benzoyl peroxide pack with collagen gel in patients with leg ulcers of various aetiologies (8 patients with venous insufficiency, 3 with arterial insufficiency and 9 patients with both). Before the treatment, all patients received topical trichlorocarbanilide solution and polymyxin in order to resolve any signs of infection. Similarly to the previous study, patients acted as their own controls; there were compared only ulcers on the same leg. The researchers reported a statistically significant difference of wound area decrease between the collagen gel and benzoyl peroxide pack (0.5% versus 15.9%, $p<0.01$). (O'Meara, 2000; 2001)

In 2008 there were compared several active products used for chronic wounds healing regarding their properties of being allergens. From all tested compounds, PVP-iodine and Balsam of Peru showed the highest value of contact sensitization (20% and 15.6%, respectively). However, benzoyl peroxide caused a sensitization only in one patient (2.2%). (Freise, 2008)

De Cuellar (1984) presented a patent that concerns a composition for treating skin lesions, comprising silver particles and benzoyl peroxide dispersed within a carrier such as kaolin powder. The composition includes finely ground metallic silver particles (around 5% of the total composition) ranging in size from 1 to 10 microns, benzoyl peroxide (1.5%), lidocaine hydrochloride (5%) and kaolin (88.5%). It can be applied to lesions by simply dusting the lesion with the formulation. But it can also be sprayed on the lesions if the composition is in a form of a spray (e.g. with the addition of a freon). Because of bactericidal properties of the composition, the invention may be used in dermo-epidermic wounds with infections and/or exudate, such as:

- Accidents (burns of different degrees, open wounds, dermal and epidermal skinning),
- Surgery when infected post-operative wounds appear (e.g. cholecystectomies, gastrectomies, laparotomies),
- Reconstructed vascular surgery (donor zones for free grafts),
- Peripheral vascular system (sluggishly healing ulcers, pressure ulcers),
- Orthopaedics (drying up of secretions due to friction and chafing),

- Dermatology (primary infections of the skin, e.g. Gangrenous Pyoderma),
- Secondary skin infections (e.g. infected eczematous dermatitis, purulent acne, sluggish ulcers, and prevention of infections of surgical and traumatic wounds).

One of the examples showed the use of the spray as a composition of invention used for eradication of germs presented in wounds (the most frequent bacteria was *Escherichia coli*). The composition was applied twice a day and the recovery was seen in all cases (11 patients) with the appearance of granulation yolks between the 4^{th} and 5^{th} day, and the complete healing between the 8^{th} and 14^{th} day.

1.3 WELL-DESIGNED WOUND DRESSING

One of the crucial parts of achieving a well-designed dressing is a construction of the bandage corresponding to its aim of use. A lot of patents describe different application forms of dressings and several of them are interesting from Iodine Generator's point of view.

1.3.1 Construction of the dressing

The most similar to I_2G (double-wings bandages saturated with the suspension of benzoyl peroxide just before the application) applications are mentioned in patent WO0128600 (Green, 2001). The said patent describes two forms of application of a wound dressing having an anti-infective activity. The monolayer dressing is made of a sheet comprising an oxidant generating formulation contained in a cross-linked polymeric matrix. The polymeric matrix (polyacrylamide) is impermeable to reactants (e.g. bacteria, catalase, heme proteins) which could be capable of reacting with hydrogen peroxide and oxygen in the sheet. The oxidant generating formulation is stable at least until the contact with a substrate (which is permeable into the polymeric matrix) from the patient's body fluid.

The oxidant generating formulation comprises an oxidoreductase (glucose oxidase) which reacts with the substrate (glucose) from the body fluid. The contact of oxidant generating formulation (iodide, glucose oxidase and peroxidase entrapped in the polymeric matrix) with glucose from the patient's body fluid results in the formation of hydrogen peroxide at first (glucose + glucose oxidase). Then if the sufficient amount of hydrogen peroxide is present, the iodide is oxidized and the elemental iodine is produced. The claimed amount of iodine that should release from the sheet at the wound site is from about 5 ppm to about 100 ppm. The oxidant generating formulation may also comprise an iodide, an oxidant, glucose oxidase, and catalase entrapped in the polymeric matrix. Glucose from body fluids reacts

with the glucose oxidase to produce a proton donor, and then the iodide is oxidized by the oxidant to form elemental iodine. The component of the oxidant generating formulation which reacts with the substrate is selected from the group consisting of a proton donor generator and an oxidant generator. The proton donor generator is selected from the group consisting of an oxidoreductase, a lipase, and an esterase.

The second application form, bi-layer dressing, is made of two sheets. The first sheet has an iodide, an oxidant or oxidant generator impregnated therein and the second one has a proton donor (e.g. iodate). The first sheet comprises a hydrophobic polymer having a microcannular structure releasably containing the iodide and oxidant or oxidant generator, while the second sheet comprises a polymeric material having the proton donor impregnated therein. Moreover, at least one of the first and second sheets comprises a lyophilized hydrogel.

1.3.2 Polyvinylpyrrolidone as a condensator of iodine

For the effective kinetics of iodine release from the dressing, the kind of capacitor might be needed. Some from the searched patents include that kind of solution. Patent US5928665 (Cercone, 1999) describes a dressing that is based on the sponge material with polyvinyl acetal. The soft and dry composition contains the complex of iodine and acetylated polyvinyl alcohol. The dressing is flexible to provide the easier application on the wound while the dressing is dry prior to absorbing biological fluids from the body. The other interesting idea of the form of the iodine's storage is showed in patent US2006078628 (Koman, 2006). The wound treating composition contains a zeolite-iodine inclusion compound. The complex may be granulated, pelletized, granular or powdery, but the best coagulation results were achieved for an average grain size of the zeolite-iodine inclusion compound of from 0.5 to 2 mm, especially 1 mm. The wound treatment agent may be applied in a loose pad or a pad having a porous side.

But the most common capacitor of iodine is PVP. PVP-I_2 is commonly used in the liquid bandages. For instance, the invention from a patent US0072251 (Hymes, 1983) shows a flexible, liquid-absorbent, adhesive bandage which includes a backing element and a substrate attached to the backing element. The substrate comprises a homogeneous, hydrophilic, stable matrix that includes a solid phase formed of a synthetic polymer and/or a long chain polysaccharide or its combination. The liquid phase of the matrix consists of a hydric alcohol, carbohydrates and/or proteins in an aqueous solution, and/or its combination. The matrix contains an active compound (e.g. povidone-iodine) for release to the affected areas.

PVP may also be used in the impregnated form. The composition described in patent WO2011085356 (Gilman, 2011) contains povidone-iodine mixed with sucrose (ratio 1:3 to 1:4) and gelling agent (sodium alginate) impregnated on the gauze or other material. The gelling agent is sufficient to thicken the composition and to substantially prevent the composition from running and release a desired amount of sucrose and povidone to a wound when applied on the wound. The dressing can be used for both exuding and non-exuding wound (difference in the amount of sodium alginate). The following composition that can contain PVP-I_2 in the impregnated form is shown in patent CN1810298 (Huang, 2006). Here, one kind of porous bacteriostatic polyelectrolyte material suitable for stopping bleeding and dressing wound is disclosed. The material is prepared with chitosan solution in 1-5 wt% concentration and sodium alginate solution in 1-5 wt% concentration. The final form is prepared through mixing and freeze drying. The material includes also sulfadiazine silver or PVP-iodine. The bacteriostatic agent exists in the amount of 3-10 wt% of chitosan and sodium alginate. Moreover, the porous material has no toxicity, relatively great pores, high water absorption rate and short bleeding stopping period.

1.3.3 Bandages prepared by lamination

Lamination is a method of a thermic strengthening or bonding of textile layers. The principle of this method is the application of the thermoplastic binder, its melting by increasing the temperature, formation of binding places and reinforcement of the binder by cooling.

Binders are used in a form of powders, fibers and bicomponent binding fibers prepared by spinning, foils and grilles. The grain size in powders usually is between 0,1 and 0,5 mm. They are prepared by mechanical crashing of the granulate in special mills. In case of copolymers with low melting temperatures the special crushers have to be used. Their functional units are cooled by liquid hydrogen. Due to the increased formation of heat during crushing, the considerable costs for cooling medium have to be taken into consideration. Foils are prepared by calendering or extruding the melt by the nozzles in the shape of annulus. Their use is limited due to the uneven distribution of binder in the volume of fabric. Grilles are produced either by spinning with the use of special spinning nozzles or by pruning of the film and its subsequent expansion in width. From the chemical point of view there are presented the binders in a form of homopolymers (polypropylene, polyamide) and copolymers (copolyesters, copolyamids, polyvinyl acetate, polyvinyl ethylene or ethylene vinyl

acetate, etc.). The criteria of the polymer choice is based on the requirements for the temperature and chemical tolerance of the product and on the hardening rate that is given by the adhesion of the binder to the fiber.

Powders are applied on the textile layer with the use of the device that consists of a hopper resting on a rasterized roll or cylinder having a helical discharge groove on its surface (figure 2). To the surface of the cylinder there is mounted a rotating brush from which release adhering grains. The powder falls on the fiber sheet by gravity. The spot application of the powder is performed by discharge drum (figure 3) or coarsely perforated rotating stencil with a spatula, into which the powder is supplied by worm dosing device (figure 4). Typical dimensions of bonding sites is about 2 mm.

The advantages of the thermoplastic binding are:

- hygenic harmlessness products,
- ecologically low level of hazardous products (not used cross-linking agents, no storage of chemicals),
- simple machinery,
- high speed of heating (fast production),
- low energy consumption in comparison to other methods of binding, e.g. dispersions.

On the other hand, the disadvantages of this method are nobler and more expensive form of binders, higher intensity of mixing the fibres with a binder, lower contact surface of fiber-binder and higher problems with the stability of the product during maintenance.

The continuous lamination may be done with the use of a device such as calender machine (figure 5). In this device the textile with the binder are binding together by going through the gap between two rollers from which one or both are heated. Both layers are bonded due to the exact temperature to melt the binder or to provide its viscoelastic form that means deformable by external force. Due to pressure the binding places are formed. The binder and the textile are hardened after cooling.

The calenders can usually heat up to 250 °C with the downforce of the roller up to 300 000 N/m and the working speed up to 150 m per minute. The binders are usually in a form of a powder that is applied on the textile or binding fibres mixed together with basic fibres. Generally, the bounded layers have the basis weight on the level 10 - 100 g/m^2. If there is a need to bind heavier textiles (from 1,5 to 3,0 kg/m^2) it is required to use the calender with the preheating infrared or hot-air equipment or to use a calender with more rollers.

The rollers can be plain or rasterized. In the first case the whole area of the textile is bonded, however in the second case the textile is bonded in bonding places in the optional shape and size.

Basic parameters of bonding with the use of calender are a type and a concentration of the binder, temperature, pressure, the pace of entering the textile layer and weight of the textile. Due to the mentioned variables there occur spilling of the binder and formation of connection between basic fibres. There is generally presented the agglomeration structure with different degrees of continuity of particles of the binder.

The typical products manufactured with the use of calender are textiles for hospitals, hygienic products, filters, cleaning textiles and so on.

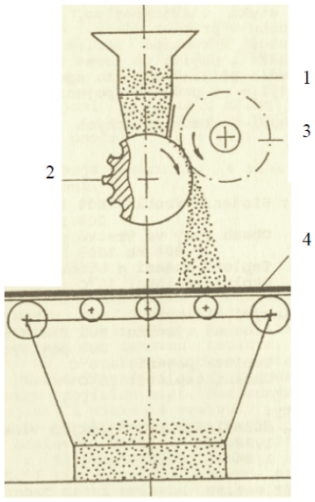


Figure 2. Device for the application of powder: 1 - powder magazine, 2 - cylinder with helical discharge groove 3 - rotating brush, 4 – textile layer (Jirsák, 2014)

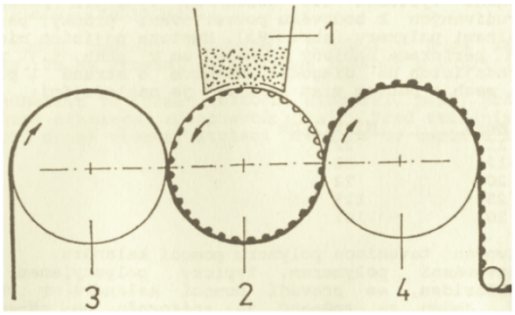


Figure 3. Device for a spot application of the powder: 1 - powder container, 2 - discharge drum, 3 - transfer drum, 4 - heated pressure drum. (Jirsák, 2014)

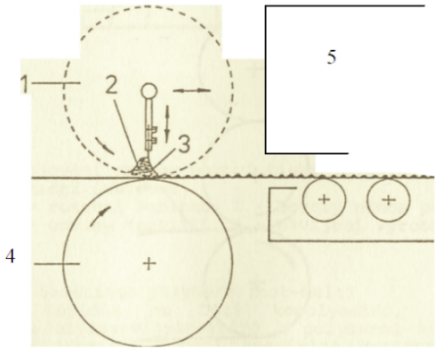


Figure 4. Rotary template for spot coating: 1 - rotary template, 2 - binder, 3 - blades, 4 - supporting roll, 5 - heating container for hardening of binder. (Jirsák, 2014)

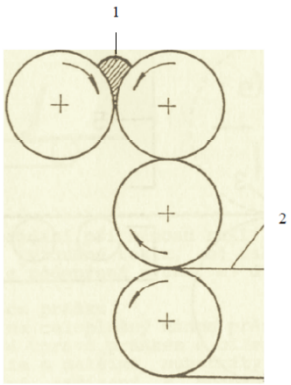


Figure 5. Application of polymer on the textile with the use of calender: 1 – binder, 2 - textile layer. (Jirsák, 2014)

Moreover, there exist also different ways of binding, such as hot-air bonding and bonding by ultrasound and infra-red rays. In case of hot-air bonding there is usually used a blend of basic and binding fibres but also foils or grid put between two textile layers. The condition for this kind of bonding is a permeability of layers. The principle of this method is to transfer the textile layers with the binder through a binding chamber with circulating hot air. The hot air that temperature is maintained by the appropriate heating control system is kept on the level sufficient for melting the binder and is passed by a ventilator though the fibrous layer. The mentioned method allows to get a really fast heating of the layer.

Ultrasound is based on a metal sonotrode that transfers vibration from generator of ultrasound. Characteristic frequency is about 18 000 Hz. The fibrous layer is fed between the sonotrode and a pad that is usually formed by transfer drum. The textile is bounded due to the adjustable pressure. The sonotrode oscillating energy in a fibrous material is transformed to the oscillating motion of molecules that causes the increase of temperature of the material. The reached temperature has to cause the softening or melting of the polymer but should not lead to the thermal or oxidative destruction of material. The advantages of the ultrasound binding are high manufacturing speed (even 100 meters/minute), the use of basic thermoplastic material simultaneously as a binder, the possibility of using a patterned pressure drum for a model or discontinuous bonding. On the other hand, the disadvantages are the need to use expensive materials (e.g. titanium alloy) for the manufacture of sonotrode, the limited life of these materials and the inability to process infusible materials.

The infra-red radiation is a part of the electromagnetic spectrum characterized by wavelengths 10^{-6} – 10^{-4} m. This kind of bonding fits to dark materials that have higher absorbing coefficient. Dark to black materials absorb greater part of infra-red light that light materials. Infrared heaters are mainly ceramic elements embedded by resistance wires that are electrically heated to 550 – 950 °C. The textile layer that has to be dried or bonded is passing at a specific speed and at a specific distance from the element that is emitting radiation. Part of the radiation changes to heat so that the liquid from the layer is evaporated or the thermoplastic share in textile material is changed to a form of a binder. The lower the rate of material and the bigger size of emitters, and smaller distance from the emitter, and grater absorbing coefficient, the reached temperature of the layer is higher. A technical problem with this method is the high temperature of the radiator. Because of this, the rate of material heating is relatively fast but in case of failure the material may be overheated above the flash point and may easily cause a fire. (Jirsák, 2014)

1.4 GAMMA RADIATION VS DIFFERENT WAYS OF STERILIZATION OF MEDICAL DEVICES

There exist five types of sterilization procedure for medical devices acceptable by authorities that are:

- steam sterilization (heating in an autoclave, minimum 121ºC for 15 min) that is preferred for aqueous preparations,
- dry heat sterilization (minimum 160ºC for at least 2 h),
- ionizing radiation sterilization that is exposure to gamma radiation (from a radioisotopic source, such as ^{60}Co) or beam of electrons (from an electron accelerator),
- gas sterilization (ethylene oxide, formaldehyde) that can be used only if there is no suitable alternative,
- filtration through a nominal pore size of 0.22 μm or less. (Marciniec, 2008)

From all of the mentioned types of sterilization, gamma radiation was chosen for the sterilization of developed dressing with Iodine Generator. Selection of the mentioned method of sterilization was based on the following reasons:

- the assurance that the dressing will be totally penetrated by the sterilant and no area of the product and packaging will be left uncertain sterility after treatment, even in case of high-density materials;
- EVA adhesive (used in the developed dressing to link KI to the textile) is reasonably resistant to radiation sterilization (Sherman, 1998);
- gamma radiation eliminates the need for permeable packaging materials (developed wound dressing has to be stored in dry conditions, humidity causes the loss of the stability of KI impregnated on the textile); the possibily of using the impermeable packaging materials (such as aluminum sachets used for dressing with I_2G) provide a strong, long-term sterile barrier;
- gamma radiation ensures a rapid processing, the immediate availability of the dressings after processing and short time of delivering the sterilized products;
- gamma rays destroy chemical bonds by interacting with the electrons of the atomic constituents and are highly effective in killing microorganisms;
- gamma rays do not initiate residuals (as in the case of ethylene oxide sterilization) and do not produce suffient dose of energy to impart radioactivity in the processed equipment;

- finally gamma processing provides fast, flexible and cost-effective sterilization. (Sterigenics, 2015)

The most suitable gamma radiation sources are ^{60}Co and ^{137}Cs. They have the relatively high energy of their gamma rays and fairly long half-life (5.27 years and 31.1 years, respectively). Currently, all industrial processes employ cobalt as the gamma radiation source. Standard dose used in gamma sterilization of medical devices is 25 kGy (kiloGray – unit of absorbed radiation dose of ionizing radiation). (Mehta, 2008)

During the sterilization process a highly penetrating gamma radiation is used that enables bombarding and killing the bacteria in products that are sealed in their final packaging. The energy from radiation is transferred to the irradiated product by collisions between the radiation and the atoms of the product. The collisions of atoms lead to the loose of bound electrons from atoms (called ionization). This process causes the irremediable damage of living organisms and the initiation of crosslinking or main chair scission in polymeric materials. The described reactions generate free radicals that aid in breaking chemicals bonds. Radiation disrupts microbial DNA and causes their disability to survive. (Silva Aquino, 2012)

Moreover, gamma radiation is characterised by significant advantages over other methods using for sterilization of medical devices. The methods mentioned below are either not applicable or destructive for the given kind of the bandage.

For instance, the recommendation for steam sterilization are heating in an autoclave for 15 minutes at 121-124 °C (200 kPa). Minimum time of sterilization depends on the moment when all processed materials have reached the required temperature throughout. Moreover, it is essential to monitor the physical conditions within the autoclave during sterilization. Steam sterilization would be destructive for the given kind of dressing due to the loss of stability of KI (high humidity and high temperature) and the change of the dressing's appearance (creases of the textile due to high humidity).

The next way of sterilization, that is dry-heat process, requires a higher temperature than moist heat and a longer exposure time. The developed product (textile part of the wound dressing) is in a non-aqueous form that satisfies one of the conditions but is not heat-stable: both due to KI and EVA adhesive. Materials destined for dry-heat sterilization have to be filled in units that are either sealed or temporarily closed for sterilization. The given kind of dressing contains EVA (ethylene vinyl acetate) as a compound adhering KI to the textile. The use of dry-heat sterilization can cause melting of EVA and its linking to the package.

The last destructive method of sterilization for the developed dressing is gas sterilization. The whole process of sterilization is difficult to control and should be taken into consideration

only if no other sterilization methods can be used. Although EVA is compatible with gaseous sterilization (Sherman, 1998), the method is not suitable for the dressings used in this work. The sterilizing efficiency of ethylene oxide depends not only on the concentration of the gas and time of the exposure, but also on the sufficient humidity and the temperature. The last two parameters could cause the loss of the stability of the active compound that is KI. Moreover, it is necessary to ensure that the packaging will allow the gas exchange, so the aluminium sachets (being one of the points of dressings protection) could not be used. Furthermore, the records of gas concentration, temperature and humidity should be fixed for each cycle. And the appropriate process conditions have to be determined experimentally for each type of sterilized material. Finally, after sterilization, specified time should be devoted for the elimination of residual sterilizing agents and other volatile residues, which should be confirmed by specific tests. (Ph. Int., 2015)

On the other hand, sterilization by filtration is employed mainly for thermolabile solutions so it is not applicable to the solid state of the developed wound dressings. That method was only used for the initial work with wound dressings where the textiles were saturated with the water solution of potassium iodide. The KI solution filtered by filters having 0,22 μm pores was applied on the previously autoclaved textiles. The process was done with the possibly high aseptic conditions (laminar box).

1.5 SELECTED METHODS OF DETERMINING IODINE

Many studies have shown that iodine and iodide can be quantitatively and qualitatively specified by variety of methods including the cheapest ones: titration and rapid test kits. Furthermore, gas chromatography-mass spectrometry, UV-VIS spectrophotometry, catalytic spectrophotometric methods, atomic absorption spectrometry, inductively coupled plasma mass spectrometry (ICP-MS), radiochemical neutron activation analysis and capillary electrophoresis might also been used to evaluate the trace amounts of iodine. Most of these techniques are either very expensive or require many several stages of manipulation for routine analysis. But it is advisable to find a method that is rapid, simple, reliable, affordable and flexible.

Titration is the most frequently used method to determine quantities of iodine in salt due to its accuracy, relatively easy way of use and low cost. This method is offered to be used at various levels of quality control. Depending on the form of iodine (iodide or iodate), different analysing methods are needed to be used. The content of iodine can be evaluated by liberating

iodine from a salt sample and subsequently by titration of iodine with sodium thiosulphate using starch as an external indicator. However, this method is not recommended for routine monitoring because of time-consuming process. The second mentioned method is the employment of rapid test kits that are easily applied in a field setting and do not require any training of the chemistry laboratory personnel. These spot tests can be either used at sites of production and distribution or at household. The spots are based on the common reaction mechanism. A starch based reagent solution produces a blue colour when iodine is present in a salt sample. In case of this method, there may occur overestimates of iodine quantity and false-positive results if contaminated instruments (e.g. spoons and test plates) are used. (Khazan, 2013)

On the other hand, the assessment of urinary iodine is evaluated mainly by one of two methods: an age-old kinetic spectrophotometric method called the Sandell-Kolthoff reaction and the ICP-MS method which permits great sensitivity and sometimes allows direct sample analysis following dilution such as in urine samples. (Khazan, 2013) In Sandell-Kolthoff reaction iodide catalyses the reduction of ceric ammonium sulphate (yellow) to the colourless cerous form in the presence of arsenious acid. In this method it is required to rid urine of impurities (commonly by acid digestion) before the main analysis. (Gnat, 2003) However, in recent years, the inductively coupled plasma mass spectrometry (ICP-MS) has been marked as a fast, accurate, robust and specific method of the reliable determination of iodine in urine.

Most iodine in biological sample is covalently bonded and certain substances interfere with the reaction and can affect the results. For instance, the mineralization step in milk samples is necessary before analysis because of high content of lipid that may cause problems in spectrophotometric readings. In case of milk there are mainly used two instrumental methods for iodine evaluation. First one includes the instrumentation such as X-ray, fluorescence, polarography, ICP-MS and atomic absorption spectrometry, whereas the second one uses colorimetric methods in association with Sandell-Kolthoff reaction. (Khazan, 2013)

Continous monitoring of iodine

Continuous monitoring of an analyte with the use of the electrochemical detection requires high demands for the execution of a detector, the stability of electrode material and its resistance against a possible passivation of the electrode surface due to products of electrochemical reactions. Biamperometry seems to be the most advantageous for monitoring the presence of redox substances. The mentioned electrochemical technic uses the principle of measurement of current on two polarized electrodes, which are supplied with a low voltage in order of tens

of millivolts. The current flows between two electrodes when two forms of red-ox pair are present in the solution and can oxidize and reduce under given potential difference (Reilley, 1951) or when two different redox systems with close half-cell potentials are present (Song, 2002).

Instrumental configuration in biamperometry is fast and easy to set the conditions of the experiment. The method is sufficiently sensitive and its selectivity can be modified by the magnitude of the potential difference that is applied on electrodes. (Song, 2002) With increasing the voltage on the electrodes the measuring current increases, too. Its magnitude also depends on the area of electrodes. Electrodes are constantly polarized with a small constant voltage to limit the corrosion of electrode materials in long-term experiments (Rahimi, 2011) and to remove the influence of charging current. In case of planar arrangement of biamperometric detector the possible set of working electrodes designed to improve the sensitivity of the measurements are restricted. (Lichtig, 1993)

The increase of the measuring sensitivity can be reached when two interdigitated electrodes are used. These electrodes have the shape of a comb and they are locked to each other. (Rahimi, 2011) The bands and gaps between them can be produced by lithographic methods in a width of tens of nanometers to hundreds of micrometers. In case of small sizes of electrodes that are close enough to each other exhibits the redox effect of cyclic reaction. The reversible product of electrochemical reaction changes backward between neighbouring polarized electrodes to the default substance and conversely. (Anderson, 1965) Limiting current of redox cyclic reaction increases even by several orders with decreasing width of electrodes and gaps between them. (Bard, 1986) Nevertheless, the simulating researches do not imply its further reinforcement for spaces smaller than 100 nm. (Yang, 2007)

2 MATERIALS AND METHODS

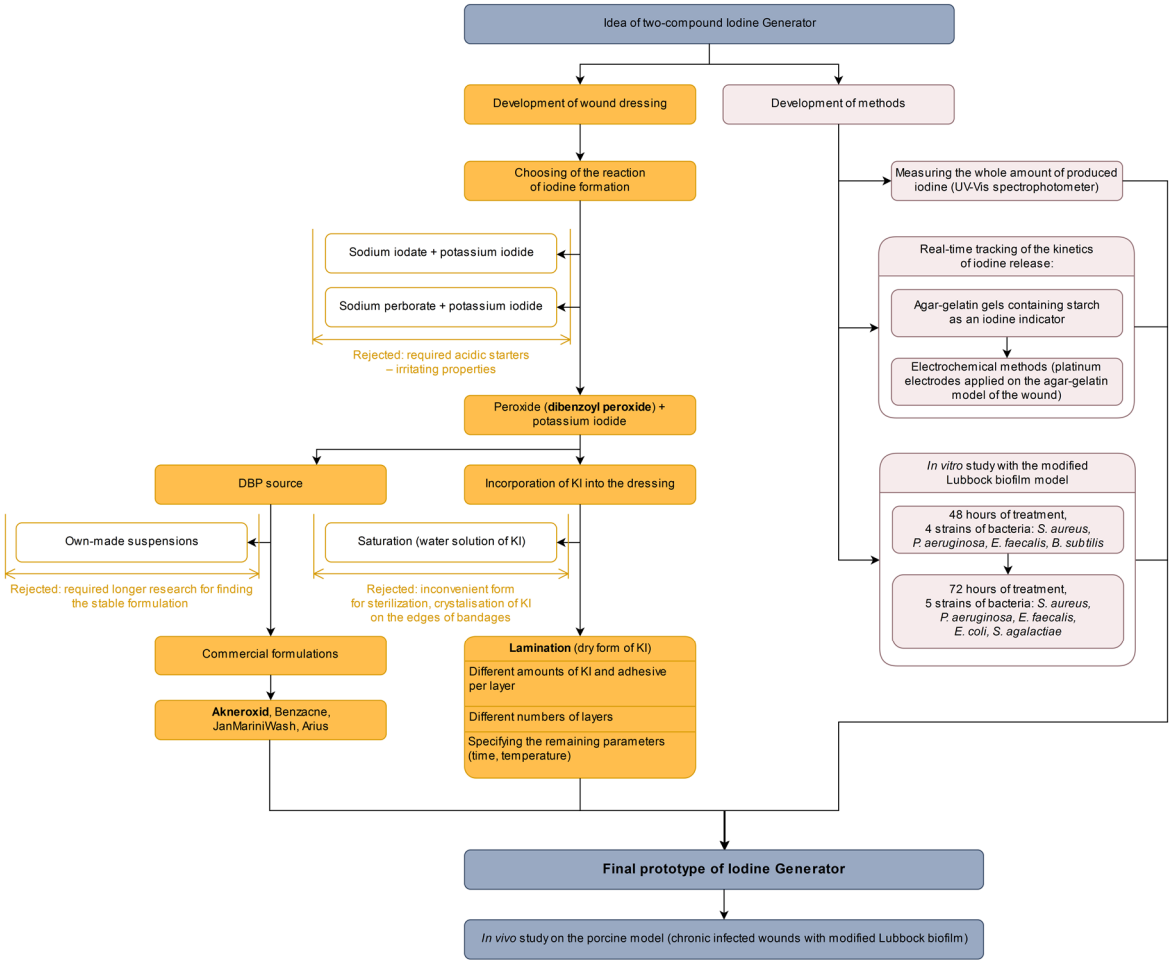
2.1 MATERIALS AND EQUIPMENT

Material/device	Lot number	Producer
Agar - agar ultrapure, granulated	VM256013 104, VM556313 320	Merc spol. s.r.o.
Akneroxid 5% Gel	226164, 304202	Almiral
Akneroxid 10% Gel	129722, 201732	Almiral
Alfadin	475119A	Bioveta, a.s.
Arius – creams: 2.5% & 5% DBP	*	Arius (India)
Arlasolve DMI-LQ-(AP)	0000701500	CRODA
Betadine – ointment	2736PO613	Egis
Betadine - solution	2951 NO113, 2961NO313, 2965PO413	Egis
Blanose (Carboxymethylcellulose) TYPE 7HF Pharm	90707	Herkules
Bile Esculin Azide Agar	BCBK 7999V	Fluka
Bolton Broth Base	BCBB7257V	Fluka
Butylene glycol	BCBC9648V	Fluka
Carbomer	1510D3A	Dr. Kulich Pharma
Cetrimide Agar	1256287, 1316804, 1375028, 1413185, 1393090	Oxoid
Citric acid ($C_6H_8O_7 \cdot H_2O$)	1511291110	Penta
Columbia CAP Selective Agar with Sheep Blood	1249934, 1271068, 1313490, 1337481, 1366451, 1369296, 1380974, 1389693, 1401304	Oxoid
Dioctyl sulphosuccinate sodium salt	S5B C4665V	Sigma Aldrich
Endo Agar Base	1154717	Oxoid
Ethanol	PP/2011/10265	Lach-Ner
Gelatine	1511020610FC	Penta
Glycerol anhydrous g.r	PP/2012/10636	Lach-Ner
Guar gum	030M0173	Sigma Aldrich
Hialuronic acid	160811-03	Contipro
Inadine	1328/2/2/1	Systagenic
Iodine	PP/2007/2181	Lach-Ner
Iodosorb (cadexomer iodine gel)	BTB 081, BTK 261, BTH 021	Smith&Nephew
JanMarini Wash	22442D	JanMarini
Kollidon 25	BCBH7443V	Sigma
Lauryl alcohol	STBC5455V	Sigma Aldrich
Luperox A75 (benzoyl peroxide)	MKBG7172V	Sigma Aldrich
Luperox A75FP (fine particles of benzoyl peroxide, USP grade)	MKBK2717V	Sigma Aldrich
Lyocell (50% Lyo, 30% PET, 20% PET) 185g/m^2	*	Norafin
MediPAD	*	Magic
Parafinum perliquidum ČL 2009	127962	Penta

PAD 40 (monofil, 40g/m^2)	*	Silk&Progress
Peptone water, buffered	VN397128 2121	MERCK
PES 30 (monofil, 30g/m^2)	*	Polytex
PES 140	*	Polytex
Polyethylene glycol, PEG 400	BCBC5976	Fluka
Polyvinylpyrrolidone K30	BCBH6921V	Fluka
Polyvinylpyrrolidone K90	xxx	Fluka
Potassium iodide (KI)	PP/2012/02467/150529051 a	Lach-Ner/Penta
Propylene glycol	424903/1 41802	Fluka
Sodium Carboxymethylcellulose	MKBF849ZV	Sigma Aldrich
Sodium hyaluronate	160811-3	Contipro
Sodium iodate ($NaIO_3$)	301005ZE	Penta
Sodium perborate ($NaBO_3 \cdot 4\ H_2O$)	1409200504F	Penta
Sodium phosphate ($Na_2HPO_4 \cdot 12\ H_2O$)	300606F	Penta
Sodium phosphate ($NaH_2PO_4 \cdot 2\ H_2O$)	221204F	Penta
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5\ H_2O$)	PP/2008/06332/0	Lach-Ner
Starch soluble (from potatoes)	PP/2011/02856	Lach-Ner
TA (70% viscose, 30% polyester), 80 g/m^2	2678	Technical Absorbents
Thioglycollate medium	1215607	Oxoid
Tryptone Soya Agar	1378737	Oxoid
Tryptone Soya Broth	1228459	Oxoid
Tween 20	A0235351	Acros Organics
Tween 80	A0245809	Acros Organics
VS 140 g/m^2	*	Polytex
Xanthan (pharmaceutical grade)	890.235	Donau Chem
XyliPure (Xylitol)	18248E8 5/10	Jarrow Formulas
Analytical scales	XS 204	Mettler Toledo
Autoclave	VX-150	Systec GmbH
Camera	EOS 60D	Canon
Impulse sealer	CD – 4	Dea Lun Co. Ltd.
Incubator	K110835 30306317	D-Lab a.s. Jouan
Laminar box	41321451	Thermo-scientific
Magnetic mixing and heating device	EKT HeiCon	Heidolph
pH-meter	inoLab pH 720	WTW GmbH
Shaking incubator	11N205 164	N-Biotek
Transiluminator	UVT-20MV	Herolab
TissueLyser II	85300	Quiagen
UV-VIS spectrophotometer	Cary 100 Conc	Varian Australia Pty Ltd.
Software PalmSens	PSTrace 4.0	PalmSens
Software concerning splitting data from .txt format (one channel after another) to Excel (separating data from one channel to one sheet)	SplitToWorksheets	Szuszkiewi.cz

2.2 METHODS

The flowchart (graph 1) that is located below presents the story of I_2G dressing development and the methods that were used during this work.



Graph 1. Chart presenting the summary of the development of the dressing with I_2G.

2.2.1 Preparation of bandages

The two kinds of dressings with Iodine Generator were tested in this work. First variant was based on the saturation of the sterile textiles with water solution of potassium iodide (possibly with the addition of polysaccharides and the second layer containing sodium iodate). The second one concerned the use of potassium iodide in a dry form. All the steps of the bandages preparation used for the microbiological tests were done in the clean room.

Just before the tests, the bandage was supplemented with water or the second active compound. The solution of sodium perborate, the suspension/gel containing dibenzoyl peroxide or carbamide peroxide was used as an activator of *in situ* iodine production. The gels containing

DBP that were used in this work were 5% and 10% Akneroxid gel (Almirall), 5% Benzacne Gel (Nykomed/Takeda), 2.5% JanMarini Wash (Jan Marini) and 5% and 10% gels from Arius (India).

2.2.1.1 Saturation of the bandage with a solution containing KI

The aqueous solution (possibly containing xanthan or other gelling agent) containing the required amount of KI (or KI and PVP) was applied (through a 0.22 μm filter for the *in vitro* microbiological test with biofilm) on the sterile absorbing textile having the specified dimensions. The formed covering was located on the glass Petri dish and inserted to the drying device at 40 °C for 3 hours. Finally, the impregnated textiles (often inserted into PES-PAD sachet) were ready to be used for the laboratory tests.

2.2.1.2 Powdering and lamination of the bandage

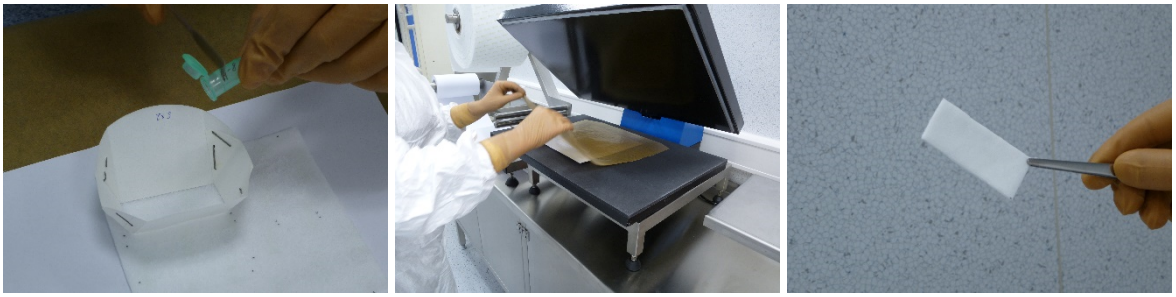


Figure 6. Preparation of bandages with the use of a laminating device.

There was used a manual powdering after the incorporation of KI and/or PVP to the textile's covering. Firstly, the defined amounts of powder compounds (KI/PVP and EVA) were weighted on the analytical scales. Secondly, by shaking, there was formed a homogenized blend of KI and EVA, respectively PVP and EVA. The resulting blend was uniformly applied using a paper template having the width of 0.5 mm and the hole of an appropriate size on the absorbing textile. The EVA sheet and/or some thin textile were deposited on the said fabric, depending on the required construction. The resulting "sandwich" was carefully transferred to the laminating device (put on a plain paper and covered with a backing paper) and laminated under 120 °C for 20 seconds (a semi-covering) or under 150 °C for 30 seconds (completion of individual semi-coverings). Finally, the upper heated plate was raised and the samples were removed from the device and let to cool down at room temperature. After cooling, the protective papers were taken off and the coverings were cut to the proper size (figure 6).

Strength test of laminated joints

The strength of laminated joints were found out by measurements on the tensiometer. During preparation of samples the application of EVA was not done on the whole area of the bandage. At least 0,5 cm at the edges was left without EVA to get at least one unjointed corner. This corner was gripped between upper jaw of tensiometer and subsequently the layers of the bandage were torn apart each other with definite speed. From the measured data there was calculated the total force required for tearing of two layers (integration of area under the curve).

2.2.2 Determination of iodine

2.2.2.1 UV-Vis spectrophotometry

The determination of iodine produced *in situ* in the bandages containing Iodine Generator was done with the use of spectrophotometric method. The bandage (4 cm^2) was put to the Erlenmeyer flask and poured with 2.5 ml of water, the flask was closed with a stopper and Parafilm and subsequently located in the incubator for the defined time at 37 °C. After this time 8.5 ml of 96% ethanol was added to stop the reaction of iodine formation. The stirrer was also put to the flask and the extraction of iodine to ethanol started (30 minutes). Each solution was diluted with the solution of 74% ethanol and the UV-VIS measurement was done. The absorbance at 278 nm was used for the following comparisons of samples and calculations of the amount of produced iodine. Serial dilutions of Lugol's solution were used as a calibration curve.

2.2.2.2 Tracking the kinetics of iodine release („starch gel model")

In the first phase of work, the kinetics of iodine release from the bandages was determined by the use of an *in vitro* starch gel model that is comparative to the real conditions in wounds. The gel was made of gelatine and agar with the addition of 0.2% starch as an indicator (table 1).

4 ml of the prepared gel was poured to the Petri dish (diameter = 4 cm). The gels were left to coagulate at room temperature (around 30 minutes) and then directed to the incubator (37 °C) for 15 minutes before the application of tested dressings.

The bandage that was directed for testing on starch gel model was put on the gel and after a certain time of incubation in the thermostat (37 °C, e.g. 5, 10, 30, 60 minutes,

depending on the concentration of released iodine) was subsequently moved to the next Petri dish and so on till the exhaustion of the source of iodine in tested sample (no formation of blue colour on the surface of the gel). Each dish with the gel, after the application of the sample, was put on the transilluminator and the photo was taken. All conditions had to be identical for each tested sample and for the preparation of the calibration curve (Lugol's solution in the concentration in the range of 0.05 to 0.5 mg I_2/ml). The obtained pictures were analysed with the use of software ImageJ.

Table 1. Composition of the "starch gel".

Compound	**Demineralised water**	**Additional information**
Agar (0.6 g)	50 ml	Boil around 5 minutes
Gelatine (6 g)	40 ml	Heat up and dissolve
Starch (0.2 g)	10 ml	Boil till getting transparency

Due to the fact that the kinetics of iodine release lasted for more than 24 hours (in final variants) it was necessary to put the Petri dishes with the "starch gel" and with tested bandages to the closed box with a guaranteed high humidity. In this case there was a break while taking photos during night and in the morning next day a high amount of released iodine was visible as a dark blue/black colour on the gel (figure 7). In most cases it increased the rate of mistake and the result was presented with a quite big approximation. But all results from the starch gel tests were comparable to each other and the range of mistakes should have been the same in each case.

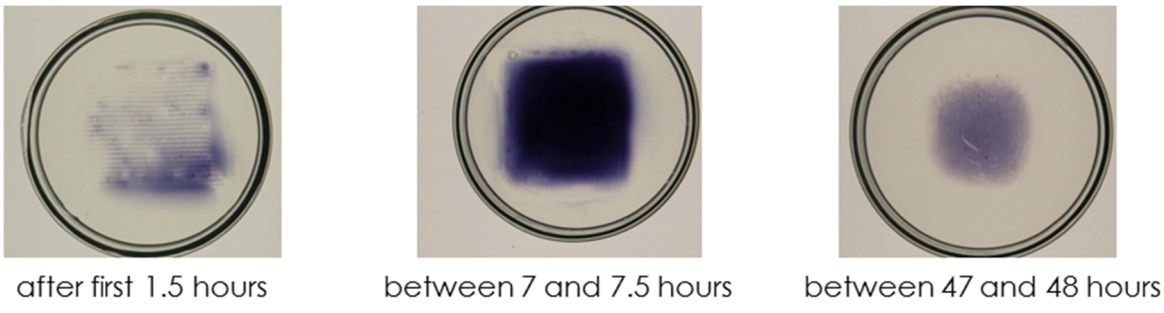


Figure 7. The photos of three chosen Petri dishes with starch gel after the application of the bandages with Iodine Generator.

2.2.2.3 Tracking the kinetics of iodine release (electrochemical methods)

2.2.2.3.1 Choosing the proper sensor for the continous monitoring of iodine release from wound coverings

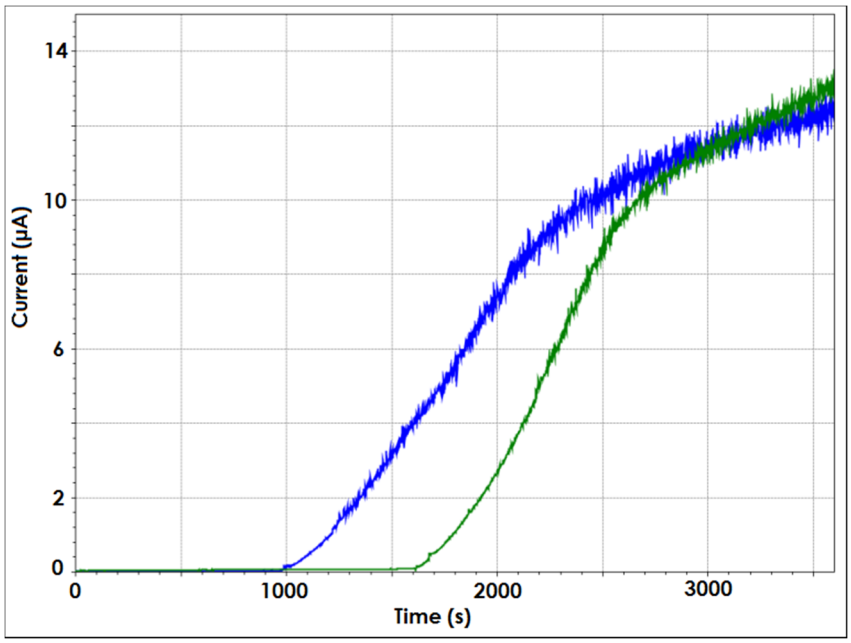
Due to the nature of the analyte, analysed environment and requirements of analysis from a series of published instrumental methods for the determination of iodine the biamperometric technic was selected as the most promising method for our purposes. (Edmonds and Morita, 1998) The principle of biamperometry is monitoring the current response at two polarizable platinum electrodes to which a constant voltage is applied in the order of tens of millivolts. Size of the current flow is dependent on the ratio of the concentration of the reduced (I^-) and oxidized forms (I_2, respectively I^{3-}). If only one form of the substance is present, the flowing current is almost zero. The redox system iodine / iodide is electrochemically reversible and therefore there can be applied a small constant voltage to the electrodes, which limits the current contribution of other substances in the analysed area. Using electrochemical technique allows monitoring the formation of iodine and using miniaturized instrumentation (e.g. PalmSens, The Netherlands) in the longer term (e.g. 6 days as during this research).

The effect of various configurations of working electrodes on biamperometric current response of redox species in continuous monitoring was examined. Three different designs of platinum electrodes ranging from simple band electrodes screen-printed on corundum ceramic base to interdigitated platinum arrays formed on the glass substrate by the lithographic techniques were tested. The iodide/iodine redox couple in triiodide solutions was selected as a model system with reversible electrochemical behaviour.

Model tests with two platinum electrodes showed the usefulness of biamperometry for continuous monitoring of iodine after the addition of Akneroxid gel containing dibenzoyl peroxide (graph 2). In control measurements without KI Akneroxid was added to the basic electrolyte such as potassium chloride (KCl). There was observed only a very small increase in the background current that was significantly lower than the currents observed for the solution with the least content of KI. Akneroxid by itself did not disturb the biamperometric measurements of iodine and it was possible to lead the research under conditions with high amount of iodide.

For tracking iodine that excludes from wound dressing it was required to perform the analysis in planar arrangement. Planar printed sensors, where the platinum electrodes are formed on the ceramic substrate by the thick film layer technic, met this requirement.

During the development of a system there were firstly used screen-printed sensors AC1.W2.RS (figure 8) with circular arrangement (BVT Technologies, CR) and there were involved only platinum working electrode (diameter: 1 mm) and auxiliary electrode (from three available electrodes in this sensor). Control measurement confirmed the usefulness of these electrodes for the proposed method of determining the presence of iodine, even after two days of continuous measurement there was seen no evidence of the infringement or isolation of the electrode material. However, after a few days of performing the experiment the unbounded silver reference electrode was melted due to the corrosive action of iodine. It was the reason of not using this kind of sensors in further tests.



Graph 2. Current response of iodine formation on biamperometric sensor Metrohm with two platinum electrodes in the course of 1 hour. Two experiments, basic electrolyte: 250 mg KI in 5 ml of demineralised water, additive: 0,02 g of 5% Akneroxid gel, mixing of the solution, E=40mV, sampling interval: 1s.

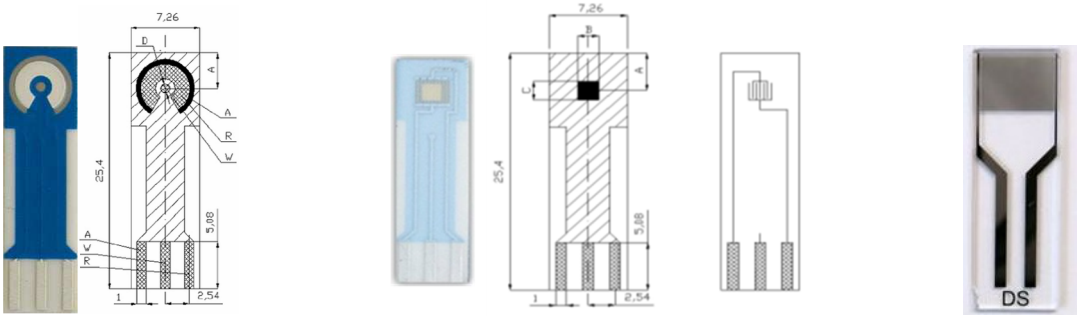
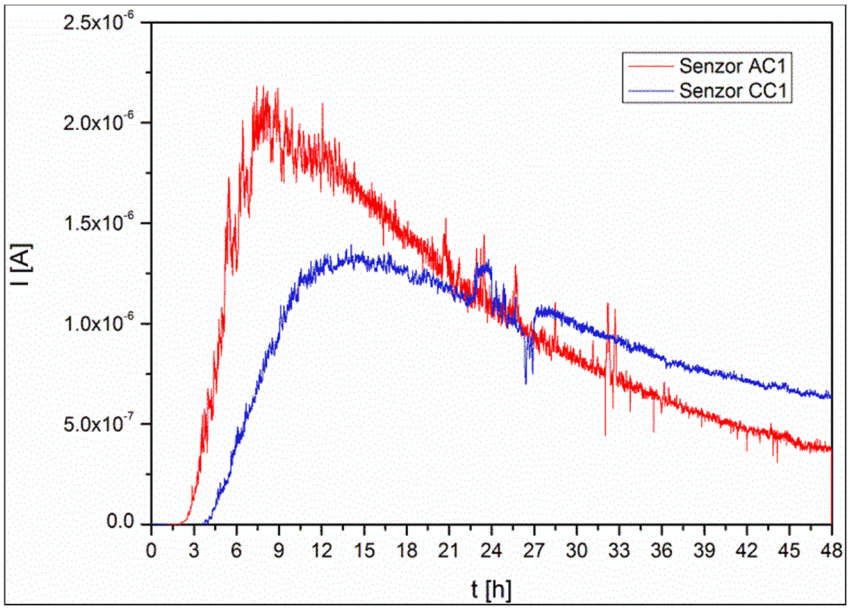
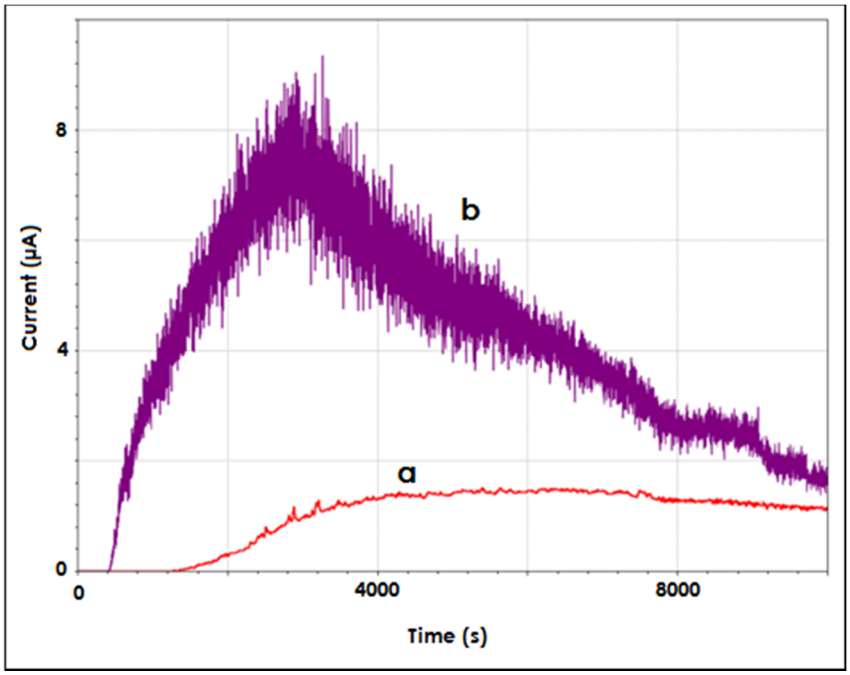


Figure 8. Screen-printed sensors with platinum electrodes AC1 (on left) and CC1 (on the middle) from BVT Technologies (Czech Republic); thin-layer sensor DRP-IDEPT10 (on right) with entwined platinum electrodes with a width of bands and gaps having 10 µm (DropSens, Spain).



Graph 3. Monitoring of the content of iodine during two days on different printed sensors. Basic electrolyte 125 mg KI in 5 ml of demineralised water, addition of about 0,02 g of Akneroxid, E=40mV, sampling interval: 20 s, without mixing, analyser: PGSTAT30 (EcoChemie, Netherlands).



Graph 4. Influence of mixing of the solution on the current response: a) without stirring, 130 mg KI, b) with stirring, 126,4 mg KI. Addition of about 0,02 g of Akneroxid in 5 ml of distilled water, E=40mV, sampling interval: 10 s.

For further experiments the other type of the printed sensor was used. Sensor CC1.W2 (BVT Technologies, CR, figure 8) is formed on a corundum ceramic base. Onto this surface two interdigitated structures of electrodes of working area 2 x 2 mm (geometric area of one comb electrode: 1,09 mm^2) are applied. Due to the bigger total area of the electrode the higher current responses than in case of sensors AC1 were expected. After checking the stability of the sensor materials exposed to iodine for two days (graph 3), the influence of stirring

of the solution of potassium iodide with Akneroxid (source of dibenzoyl peroxide) was checked (graph 4). In first case Akneroxid was carefully added to the solution in the meantime before the reading of current. It was done to protect from the unwanted mixing of the solution that could cause the increase of current response due to the faster flow of the analyte to the electrodes. In the second case, the increased mass transfer to electrodes improved the sensitivity of measurement but also the measurement noise increased significantly. The noise was caused by local changes in concentration of analytes at the surface of electrodes. Moreover, the transmission of iodine from the surface of Akneroxid gel to the solution and the observed current response were hasten by mixing. In case of experiments with wound coverings the analysis were planned to be performed in a steady-state, so that the interfering effect of fast variations of the measured currents should have been suppressed. On recordings without stirring there were also presented various current fluctuations that were probably caused by the movement of the solution during the disintegration of the piece of Akneroxid in water environment during the experiment.

After first trials on the *in vitro* model of a wound it was observed that sensors from BVT Technologies were very sensitive to iodine and were giving tricky results. The addition of more KI did not cause the increase of the current response. Moreover, it was necessary to exchange the sensor to the new one after one measurement (5 days). Finally, for the target measurements, there were used screen-printed sensors with two entwined platinum electrodes (type G-IDEPT, DropSens, Spain, figure 8) which are formed on the glass substrate with the use of thin film techniques (evaporation). Individual types of sensors vary between each other in the width of electrode bands and gaps (5 μm for DRP-G-IDEPT5 and 10 μm for DRP-G-IDEPT10). The size of working electrode is 7 mm x 5 mm and the geometric area of one comb electrode is 8,75 mm^2. The material of chosen sensors is made of homogenous metal in contrast to heterogeneous screen-printed electrodes and shows higher current response due to the bigger area of electrodes. This material is more durable in contrast to screen-printed counterparts that can be disrupted by long-term effects of iodine release.

2.2.2.3.2 Adaptation of the system - measurements with the use of model wound

Several variants of the *in vitro* model (gel) imitating a chronic wound was checked from which the one described below was chosen for the further experiments (figure 9).

Figure 9. *In vitro* model of a wound used for the determination of iodine with the use of sensors.

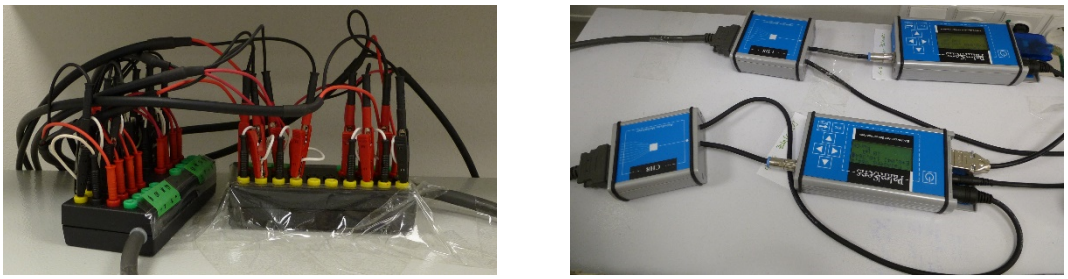


Figure 10. Instrumentation used for the measurements: analysers (PalmSens) and external multiplexers.

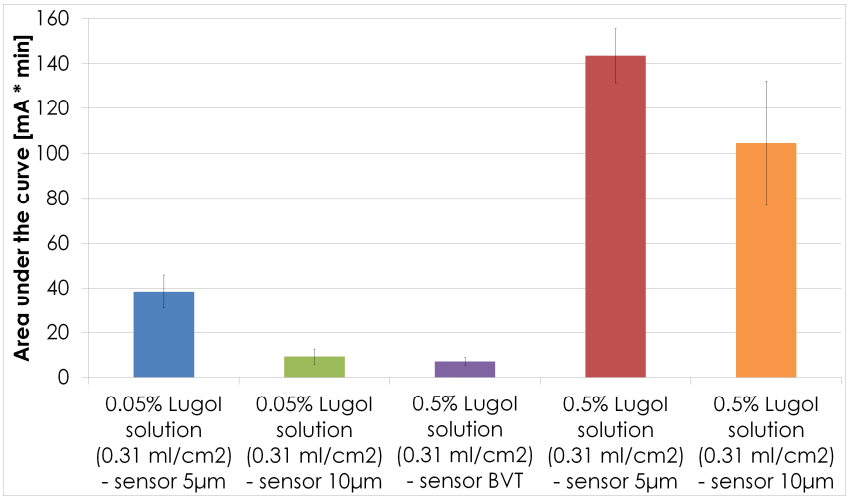
The sensor was put on the thick (~ 1.5 cm) layer of the agar-gelatine (1.2% and 6% w/v, respectively) gel and subsequently covered with a thin layer (~0.2 cm) containing potassium iodide (0.67%). Everything was placed on the beaker and therefore inserted into the dish with some amount of water needed for maintaining humidity of the bandage. Addition of potassium iodide (I^- ions) to the model was required for the electrode functioning mainly in case of products that do not contain KI in their formulation. I^- ions were needed for working of electrodes that show the response when both iodine and iodide ions were available. It was required to analyse several samples simultaneously. Eight sensors were connected to the electrochemical analyser (PalmSens) with the use of the external multiplexer (figure 10). The data were analysed with the use of PalmSens software (PSTrace 4.0) that formed a .txt file and subsequently with the use of the prepared software SplitToWorksheets that divided the data from 8 channels to separate sheets in Microsoft Excel where results were finally elaborated.

2.2.2.3.3 Comparison of sensors and a calibration curve for the chosen one

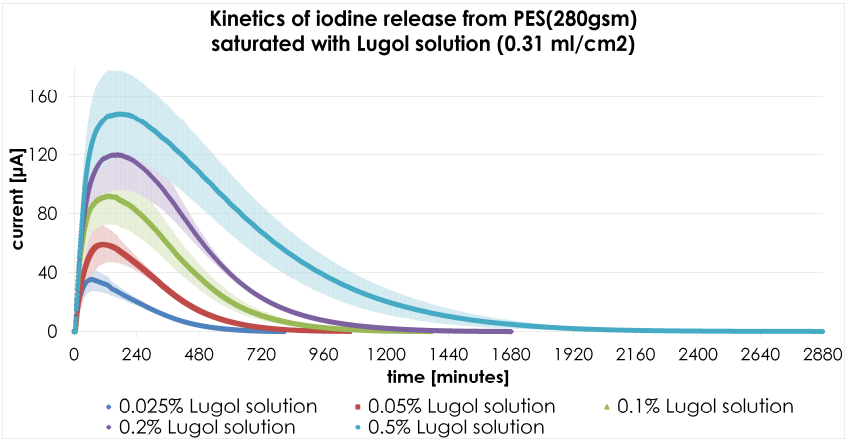
After choosing the appropriate gel model, the calibration curve with the use of several dilutions of Lugol's solution was prepared. The area under the curve formed by the current responses was used as the dependence to the concentration of iodine.

There were available two kinds of glass DRP sensors from DropSens that were compared to a ceramic sensor from BVT Technologies. It was observed that with increasing geometric area of the sensors, the total measured currents were increasing, too. The current responses

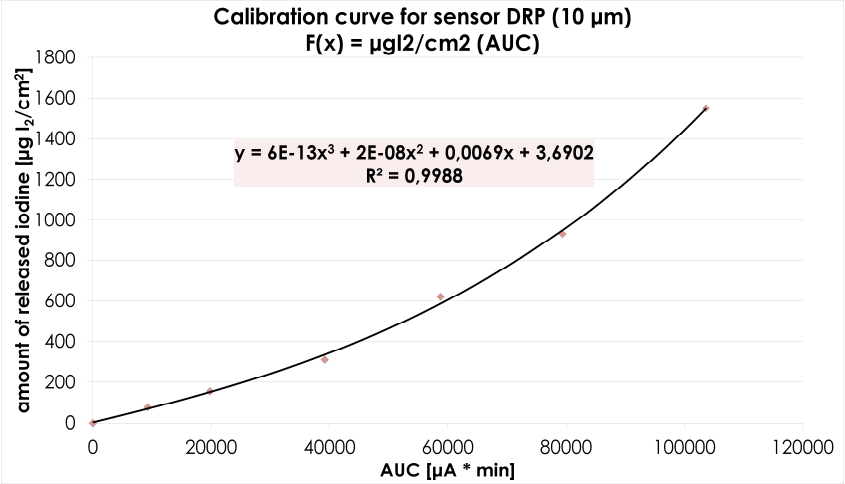
from BVT sensors (1,09 mm^2) were drastically lower (at least 10 times) in comparison to DropSens ones (8,75 mm^2). That is why the final experiments were performed with the use of these glass sensors (graph 5). From two sensors from DropSens with a width of bands and gaps having 5 and 10 µm, the second variant was used for the further measurements. The reason of this was the proper ratio received after testing 0.05%and 0.5% Lugol solutions that was 1:11 (in case of 5 µm the ratio was 1:3) that almost corresponded to the 10-fold dilution. In case of entwined platinum electrodes with small width of bands and gaps there exist the effect of redox cycling of electrochemically reversible analyte. Finally, sensors DRP-IDEPT10 were used for the preparation of the calibration curve for further analysis (graphs 6 and 7). There was used the inverse calibration because it predicts better than the classical one (Centner, 1998). Each measurement was done at least in four repetitions.



Graph 5. Comparison of BVT and DRP sensors.



Graph 6. Presentation of the kinetics of iodine release from the textiles saturated with exact dilutions of Lugol solution that were subsequently used for the preparation of the calibration curve (brighter colour = standard deviation).



Graph 7. Calibration curve of the sensor DRP10 (relation of the amount of the released iodine to the AUC of the current).

2.2.3 Checking the sterility of bandages after gamma radiation sterilization

Ten Duran bottles containing Tryptone Soya Broth (1000 ml) and ten Duran bottles containing Thioglycollate medium (1000 ml) were prepared. The surface of the Thioglycollate medium was additionally covered with 10 ml of paraffin oil. The media were sterilized in a steam autoclave at 121 °C for 15 minutes.

From each batch there were tested at least two samples for the determination of the aerobic bacteria (TSB medium) and two for anaerobic (TG medium). During the processing of coverings, the area of laminar box was monitored with gravity bowls. Samples with TSB medium were cultivated at 25 °C and samples with TG medium at 35 °C for 10 days. There was performed a continuous control of the microorganisms growth in two-day intervals. At the same time there were cultivated negative controls with TSB and TG medium.

2.2.4 Testing the efficacy of bandages on the *in vitro* Lubbock biofilm model

2.2.4.1 Preparation of the *in vitro* Lubbock biofilm

Bacteria originally isolated from the chronically infected wounds of patients at the University Hospital, Hradec Kralove, Czech Republic were used in this experiment: *Staphylococcus* aureus, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Bacillus subtilis* (in the first part of tests) or *Escherichia coli* and *Streptococcus agalactiae* (in the second approach).

The biofilm was cultivated (16 – 24 hours) in the sterile polystyrene tubes filled with the media of the following composition:

- 3 ml of Bolton Broth Base with 2% solution of gelatine,
- 2.7 ml of the filtrated (0.22 μm) porcine plasma,
- 0.3 ml of the filtrated freeze-disrupted erythrocytes.

The bacterial mixture used for the inoculation was prepared in the way described below. 5 ml of the buffered peptone water was poured to the sterile plastic tube. There was added one of the used bacteria strains and it was diluted to get the optical density on the level of 1 +/-0.1 McF (that is approximately 3 x 10^8 CFU/ml) and subsequently was diluted 100-times with peptone water. Each of five solutions containing chosen bacteria strains were mixed together in ratios 1:1:1:1:1 to form a bacterial blend containing approximately 10^6 CFU/ml that was then used as an inoculum. 10 μl of the inoculum was taken with the use of the 200 μl tip and put together to the media. The tip played a role of the platform for biofilm formation. That is why it was indicated not to let the inoculum off from the tip to the media but to leave it in the tip and insert them together to the media. The biofilms were incubated at 37 °C in orbital shaker (200 rpm) for 48 hours. (Kučera, 2014)

2.2.4.2 Testing bandages on the *in vitro* Lubbock biofilm

Petri dishes with two-layer nutrient medium composed of Bolton broth supplemented with 1% (w/v) gelatine and 1,2% (w/v) agar were prepared as follows. 2 mm thin layer of medium was poured into Petri dishes and subsequently after its solidification one sterile PTFE-coated magnetic stirring bar (20 mm x 8 mm) was put onto the agar in each dish. Afterwards, the second layer (2 mm) of the medium was applied. After the medium was gelled enough, stirring bars were aseptically removed from the agar. The resulting wells were used as the "bed" for biofilm replantation.

Mature biofilm after 48 hours of incubation was taken from the polystyrene tube with the use of tweezers, slightly dried with sterile gauze and put to the well present on the Petri dish with a nutrient medium. The biofilm was covered with the dressing (2 cm x 5 cm) containing Iodine Generator wetted with circa 4.5 ml of peptone water or a cotton gauze (2 cm x 5 cm) wetted with commercial product containing iodine or with peptone water (control). Subsequently, the Petri dishes were incubated at 37 °C for 24, 48 and 72 hours. In the specified time points, the biofilm was removed from the Petri dish with the use of the sterile tweezers and homogenized for 10 minutes in 900 μl of peptone water using TissueLyser II (Quiagen, Germany). The homogenization process was performed in 2 ml microcentrifuge tube with 5mm diameter stainless steel bead where the high-speed shaking was

set to oscillation frequency of 15 Hz (900 oscillations per minute). Finally the weight of biofilm was determined. (figure 11)

2.2.4.3 Quantification of bacteria

Bacterial counts were determined as follows. Homogenized biofilms were initially diluted 1:10 in peptone buffered water and vigorously vortexed for 2-3 minutes. The suspended cells were then diluted 10-fold up to 10^{-8}, and 10 µl aliquots of each dilution and undiluted homogenate spotted onto agar plates. The respective dilutions (10 µl) were applied on the selective and diagnostic media (Cetrimide Agar for the determination of *Pseudomonas aeruginosa*, Bile Esculin Azide Agar for *Enterococcus faecalis*, Columbia CAP Agar for *Bacillus subtilis*, Columbia CAP Agar with sheep blood for *Staphylococcus aureus*, *Streptococcus agalactiae* and MacConkey Agar for *Escherichia coli*) and subsequently CFU were counted after 16 hours of incubation at 37 °C. The Petri dishes were incubated in the direction of bottom on top. The viable bacterial counts were subsequently expressed as CFU per 1 mg of original biofilm. (figure 11)

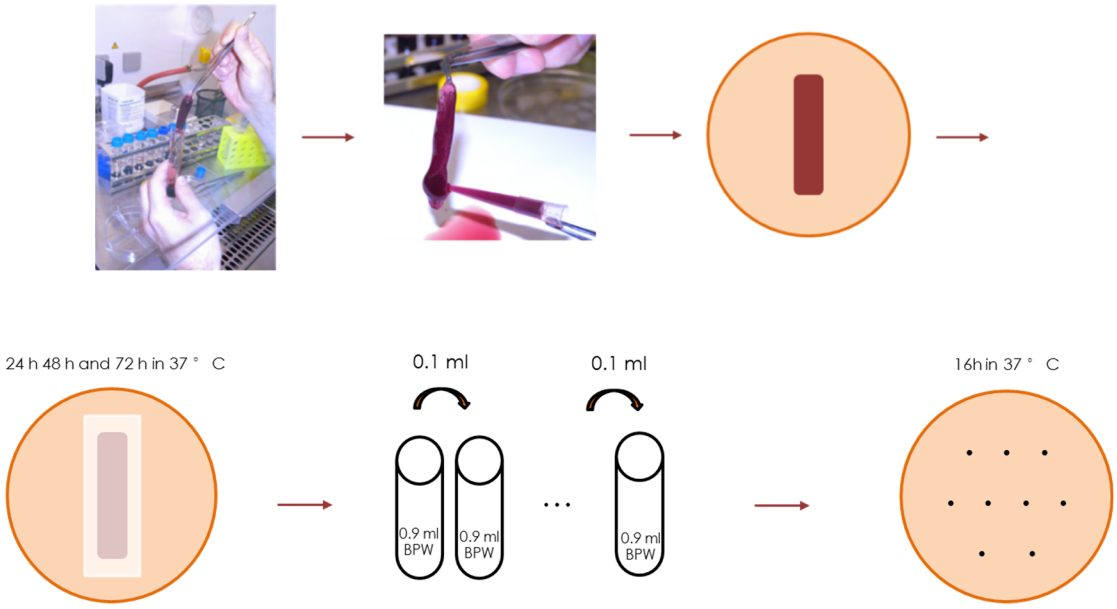


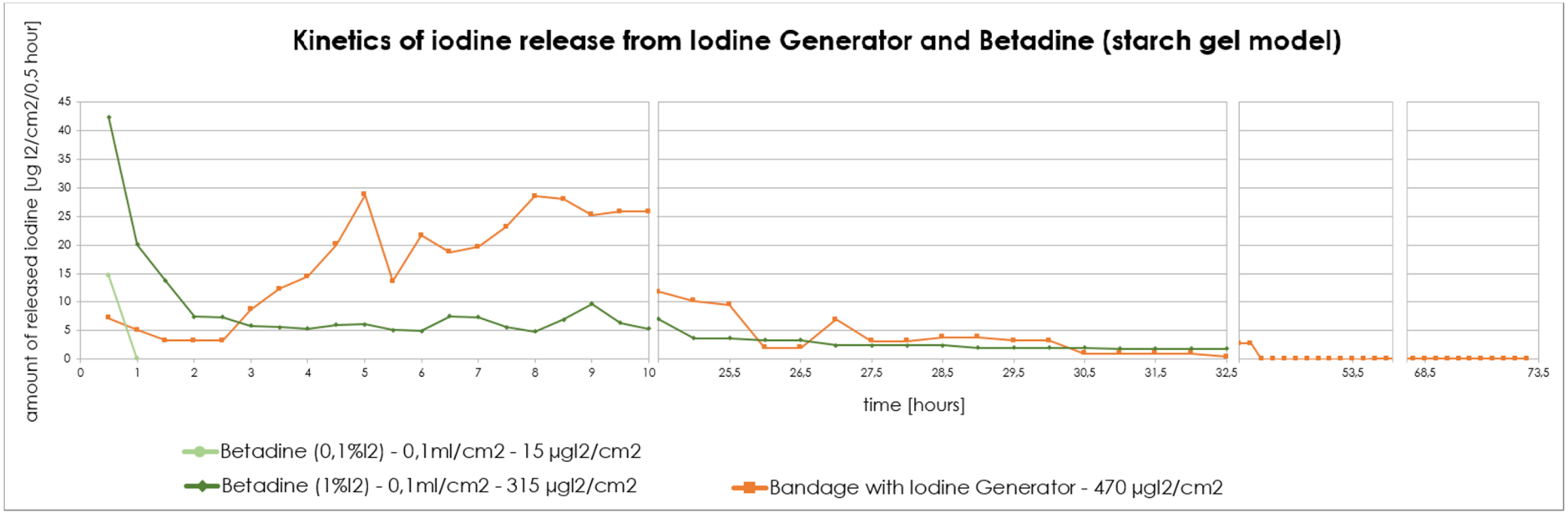
Figure 11. Picture presenting the manipulation with the *in vitro* Lubbock biofilm model: removal of the mature biofilm (after 48 hours of incubation) from the polystyrene tube with the use of tweezers; application of the biofilm to the well present on the Petri dish with a nutrient medium; application of the wound dressing on the Petri dish and its incubation at 37 °C for 24, 48 and 72 hours; preparation of 10-fold dilutions up to 10^{-8} of the previously homogenized biofilm taken from the Petri dish; application of the respective dilutions (10 µl) on the selective and diagnostic media and subsequent transfer of Petri dishes for the 16 hours incubation at 37 °C.

3 RESULTS AND DISCUSSION

During this work there were tested several variants of wound care products containing iodine or producing iodine *in situ*. The influence of the kinetics of iodine release from the wound dressings on the eradication of the *in vitro* Lubbock biofilm was inspected. In this thesis there will be presented data from over a dozen of various constructions of wound dressings (Iodine Generator) having different manners of iodine production (e.g. iodide with iodate or iodide with peroxide) in comparison to the commercially available compositions with PVP-iodine (Betadine from Egis, CZ) or cadexomer-iodine (Iodosorb from Smith&Nephew, USA).

Most of wound care formulations containing iodine shows a fast kinetics of its release, during a few hours or even minutes. These formulations could not be used for the effective biofilm eradication. That is why the idea of Iodine Generator was based on searching the kinetics that showed the established secretion of iodine: quite steady release of iodine during several days with increased values during first few hours to receive the eradication of wound biofilm in the first phase of treatment and preserving from its reappearance. At the beginning three ways of the iodine formation were checked. When the third principle with dibenzoyl peroxide met the expectations, the wound coverings were subsequently modified and tested on the developed *in vitro* Lubbock biofilm model.

First experiments determining the kinetics of iodine release were done with the starch gel model. In the graph no. 8 there is presented the kinetics of iodine release from Iodine Generator and Betadine in non-diluted (1% iodine) and diluted (0,1% iodine, clinically relevant) form. It was clearly visible that diluted Betadine released iodine only for 1 hour while the two remaining formulations for more than 24 hours. Betadine showed a high peak (possibility of skin irritation) of released iodine at the beginning of treatment and a fast decrease of its amounts in the next hours. On the other hand, the Iodine Generator started releasing iodine slower than expected, but then maintained a stable high level of released iodine for several hours.



Graph 8. Kinetics of iodine release from dressing with Iodine Generator and textiles saturated with the solution Betadine (PVP-I_2) in two concentrations.

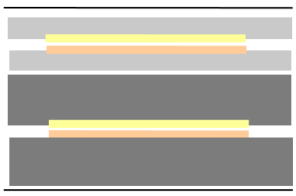
Due to the fact that the "starch gel" model did not give the opportunity for the continuous tracking of the kinetics of iodine release from wound coverings, the micro-analytical method was taken into consideration. This method was the confirmation of the previous analyses concerning iodine release and hasten the work with further variants of coverings. Moreover, the kinetics of iodine release from the competitive product, Iodosorb (cadexomer iodine) could be measured and became the positive control in *in vitro* tests with biofilm. Finally, the efficacy of the iodine release was proved on the *in vitro* Lubbock biofilm test where Iodine Generator showed a better biofilm-fighting properties than Betadine. Additionally, the use of the diluted form of Betadine did not show any inhibition of biofilm that makes it an useless formulation for fighting with microbial biofilm (graph 55).

3.1 PRINCIPLES OF IODINE FORMATION

During the research there were suggested three types of reactions for the *in situ* iodine formation. Two reactions were based on the oxidation of potassium iodide by another inorganic reagent, first of them was sodium iodate and the second was sodium perborate. Both required the addition of acidic starters to reach the expected kinetics of iodine release. The third reaction was catalysed only by the increase of the temperature to 37 °C and the addition of organic reagent such as benzoyl peroxide.

3.1.1 Bandages with potassium iodide and sodium iodate

Initially, based on the Takahashi work (2000), the dressing had a form of a bandage which contained two active layers: first with potassium iodide and second with sodium iodate. The reaction of iodine formation started after the saturation of the bandage with some liquid (e.g. water or phosphate buffer). There were prepared several variants of bandages with different amounts of active sources and initiating agents (citric acid and phosphate acids) impregnated on the textiles together with gelling agent, such as xanthan. The structure of the dressing (the number of textile layers (from 2 to 9) and their kinds (PES,PAD, VS/PES, MediPAD, Lyocell, TA) was changed in the dependence on the amount of probable exudates and on the expected kinetics of iodine release. Final structure for this inorganic version of I_2G is presented in the figure 12.



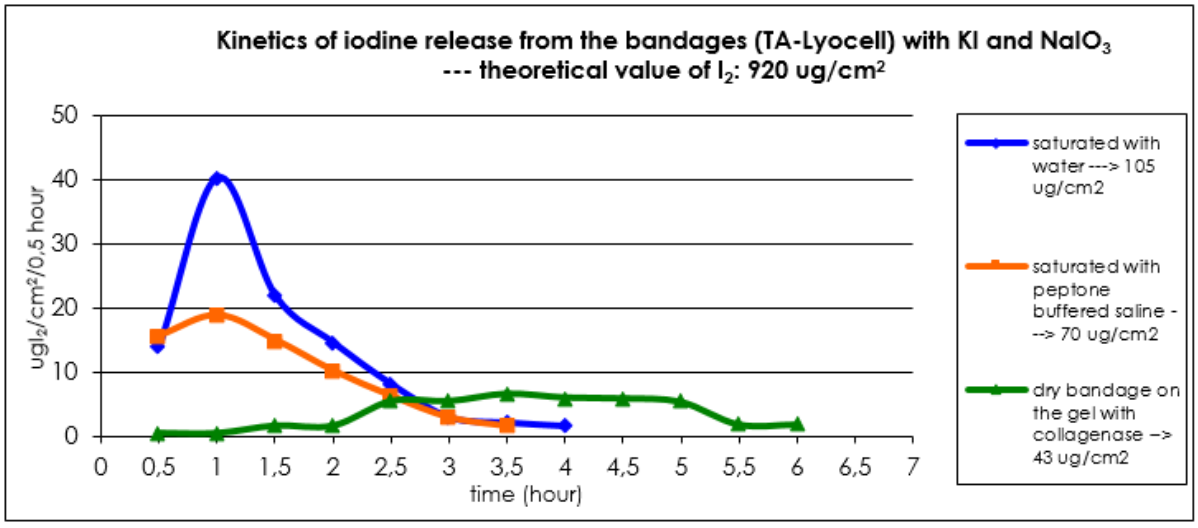
PAD - contact layer
TA (+ layer with KI)
TA (+ layer with $NaIO_3$)

Lyocell (+ layer with KI)

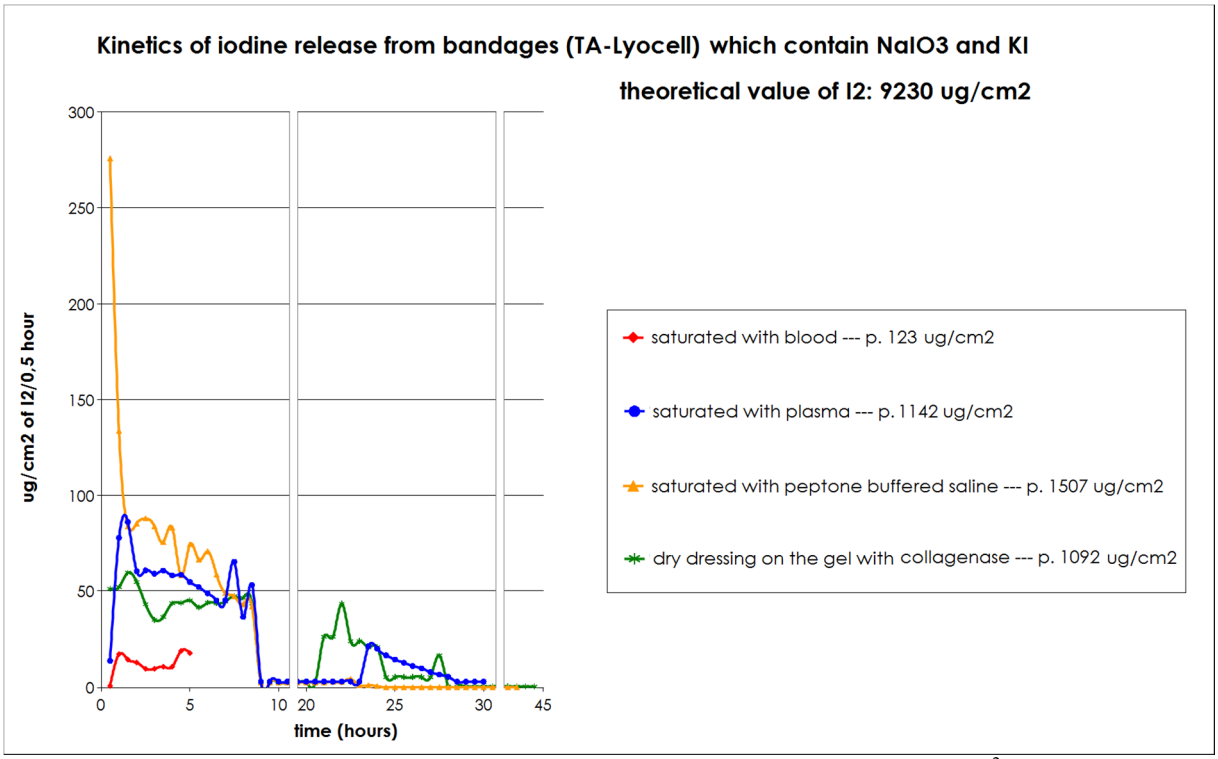
Lyocell (+ layer with $NaIO_3$)
PES - covering layer

Figure 12. Structure of the "tea-bag" dressing with Iodine Generator.

Due to the fact of achieving a short kinetics (4 to 8 hours, graph 9) and no abilities of that generator to produce iodine in the presence of plasma or blood (strong alkali buffers), the amount of iodine and acidic starters were increased (graph 10). The 10-times increase of the amount of active substances (and acidic sources) allowed the bandage to prolong the time of iodine release (even more than 24 hours) and was effective even in the presence of plasma (30% of loss in comparison to PBS) and blood (90% loss in comparison to PBS; only 5 hours kinetics). But in this case there was a huge possibility of high skin irritating properties of the bandages (pH ~3.7, table 3). Moreover, this kind of application had a low stability during the storage – strong yellow (or even brown) colour was visible after few weeks (product highly dependent on the humidity; even little amount of water initiated the reaction while storing).



Graph 9. Kinetics of iodine release from the bandage with $NaIO_3$ and KI (0.9 mg I_2/cm^2) in the presence of water and peptone buffered saline.

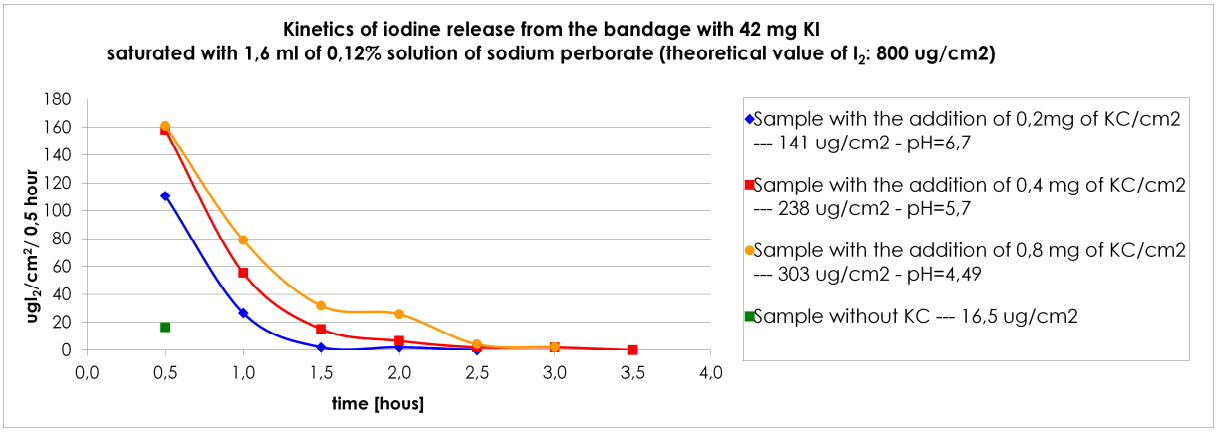


Graph 10. Kinetics of iodine release from the bandages with $NaIO_3$ and KI (9.2 mg I_2/cm^2) in the presence of plasma and blood or without saturation (on the gel with collagenase).

3.1.2 Bandages with potassium iodide and sodium perborate

Due to the fact of having not satisfied results with biofilm eradication in the pH higher than 5, the other reactions were taken into consideration. First option was the use of sodium perborate which in the presence of water forms hydrogen peroxide. Then, hydrogen peroxide reacts with potassium iodide (that is present in the bandage) to form expected iodine.

Tested bandages were impregnated with potassium iodide and just before the application on the wound model ("starch gel") the solution of sodium perborate was poured on the dressing. Some amount of citric acid had to be added to the dressing to increase the iodine production (to decrease alkali pH of the device). This reaction allowed producing iodine in the good efficiency in comparison to the theoretical value (40%), but the time of iodine release was not prolonged in comparison to the previous oxidizing compound, sodium iodate (graph 11). Moreover, the kinetics itself was not satisfactory - there was a really immediate decrease of iodine's level. What is more, this application also required the highly acidic conditions that may have enlarged the unpleasant feelings for the patients.



Graph 11. Kinetics of iodine release from the bandages impregnated with KI and subsequently saturated with the solution of sodium perborate.

3.1.3 Bandages with potassium iodide and peroxide

The next tested compound was dibenzoyl peroxide (DBP) that gave much more promising results in comparison to sodium perborate. The idea was based on the temperature dependent reaction of benzoyl peroxide with potassium iodide (used as a procedure of analytical method of specifying the amount of hydrous benzoyl peroxide, (USP, 2012)). Benzoyl peroxide breaks down in contact with skin, producing benzoic acid and oxygen, neither of which is significantly toxic (Piergallini, 2011). Then benzoic acid is excreted as benzoate in the urine (Daily Med, 2014). What is really important in the case of this application is the fact that the mechanism of the reaction is not dependent on the pH conditions. The pH of the dressing seems to be around 5 that should remove the suspicion of skin irritation.

The main part of the research was finally based on this peroxide and its reaction with potassium iodide. Most of variants of dressings with I_2G used this form of reaction. The further research concentrated on the development of the way of the wound dressing's preparation and on the methods allowing to obtain all required information to achieve the main goal – wound dressing with anti-biofilm properties.

The other initiating agent that was checked in this work was carbamide peroxide (urea peroxide, CPO) that is an adduct of hydrogen peroxide and urea. CPO dissolves in water to give free hydrogen peroxide. This oxidizing agent is used as a disinfecting and bleaching agent in cosmetics and pharmaceuticals. As a drug, this compound is used in some preparations for teeth whitening. It is also used to relieve minor inflammation of gums, oral mucosal surfaces and lips including canker sores and dental irritation, and to emulsify and disperse ear wax. A pharmaceutical company Elorac has performed clinical trials for the use of carbamide peroxide for the treatment of acne vulgaris. The idea of using carbamide peroxide in anti-acne

formulations was mentioned in early eighties. The patented formulation with 1% to 15% of CPO was directed to the treatment of both closed and open comedones. (Bernstein, 1986) And about 20 years later there appeared several new patents concerning the similar use of CPO that is present in gel, cream or lotion. The concentration of this peroxide ranges in these applications between 0.5% and 30%. (Glassman, 2005; Bernstein, 2008) CPO in 6.5% concentration is used as an active substance in the OTC earwax removal aid Debrox. It is indicated for occasional use as an aid to soften, loosen and remove excessive earwax.

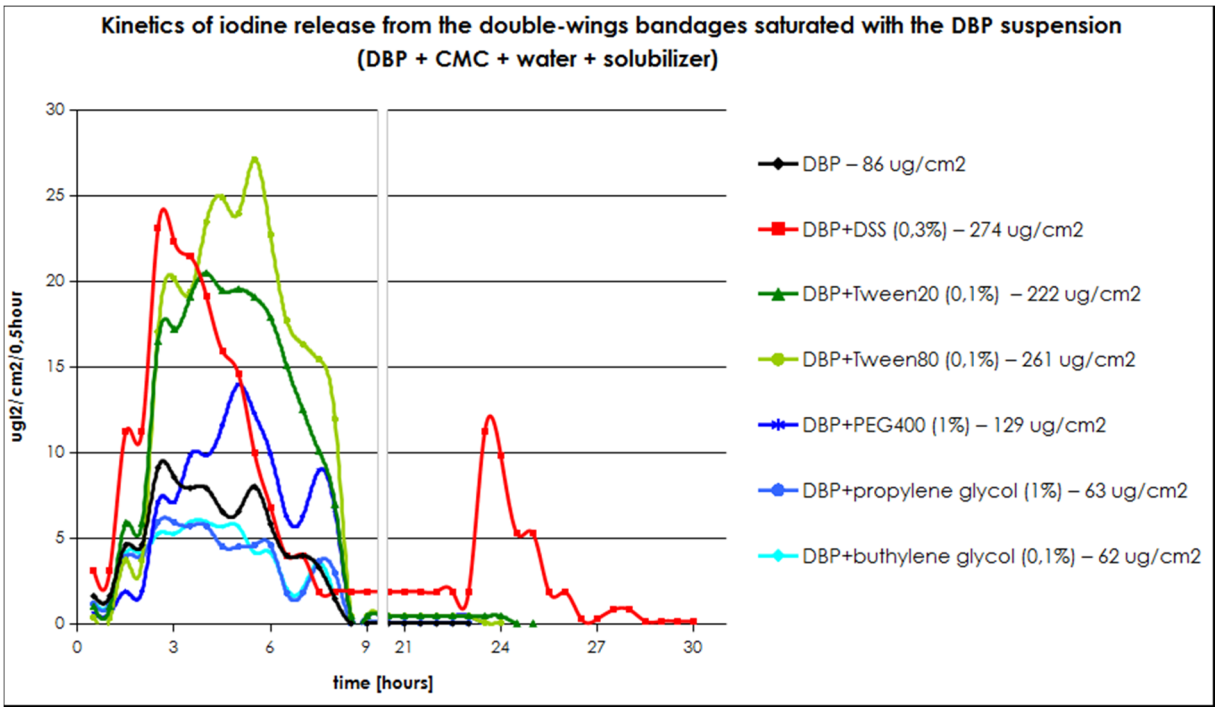
A trial experiments with the use of CPO suspended in a glycerine with succinic anhydride (a stabilizer for carbamide; based on patent US4895875 (Winston, 1990)) were performed during this research. The suspension with peroxide was applied (in the same way like DBP) on the dressing impregnated with KI. In this case the kinetics of iodine did not fulfil the expectations: the kinetics of iodine release from the wound covering was about only 22 hours and was not as steady as in the case of dibenzoyl peroxide. Possibly, finding the other stabilizer and the use of the additional iodine capacitor could decrease the initial too high concentration and prolong the time of final iodine's release.

3.1.3.1 Choosing the appropriate form of a suspension with dibenzoyl peroxide

The principle of this part of work was to find the stable formulation with dibenzoyl peroxide that would be easily applicable on the bandage impregnated with KI. The amount of the cream/gel/suspension applied on the dressing should have been big enough to cover the whole area of the dressing but should not leak from the edges simultaneously. And finally, the concentration of the peroxide should have been suitably selected to get the required concentrations of formed iodine. The short description of the development of the own-made suspensions and differences between commercially available formulations with dibenzoyl peroxide are presented below.

The first version of the suspension used for the saturation of the bandages was based on the water suspension containing DBP particles (Luperox from Arkema, USP Grade), solubilizer dioctyl sulfosuccinate (Klein, 1983) and carboxymethylcellulose (~4%) or optionally carbomer (preparation: from acidic conditions to neutral) as a gelling agent. This kind of suspension exhibited a really promising kinetics of iodine release, without high pick at the beginning of the treatment and with long time of equal distribution of iodine.

In order to increase the stability of fine particles of dibenzoyl peroxide, the research of the effective solubilizer begun. From all tested chemicals, such as Tween, DSS, PEG or propylene/buthylen glycol (Chellquist, 1992), three of them showed comparable properties of increasing the efficiency and prolonging the time of iodine release: dioctyl sulfosuccinate, Tween 20 and Tween 80. (graph 12)

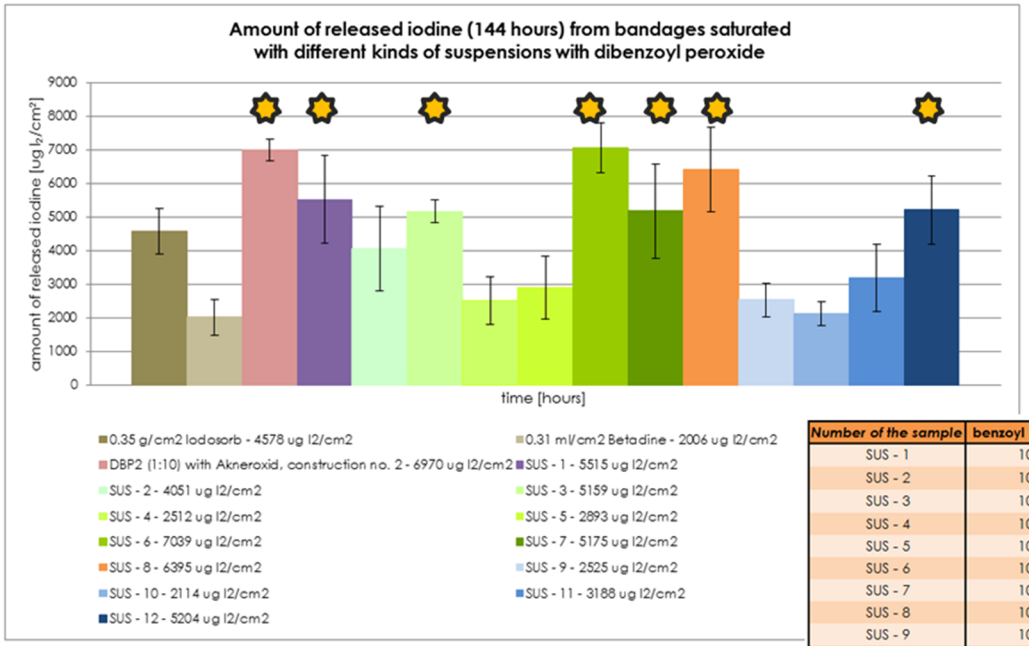


Graph 12. Kinetics of iodine release (starch gels) from double-wings bandages DBP1(1:7) saturated with the suspension containing Luperox (USP Grade), CMC and different stabilizers.

The development of the micro-analytical method for tracking the kinetics of iodine release allowed to test more own-made suspensions with DBP. The dressing BDP2 (1:10) containing 69 mg KI/cm^2, 7 mg EVA glue/cm^2, 5 mg DBP/cm^2 and prepared by the lamination technic was used as a base material. The designation DBP2 (1:10) means that there was used 20 mg of DBP per 4cm^2 of the bandage with the molar ratio DBP:KI = 1:10.

Twelve suspensions containing dibenzoyl peroxide were prepared in accordance to the patents US20110070274 (Orsoni-Segona, 2011), JPS6251666 (Nobuyuki, 1987) and US7153888 (Schwarz, 2006). The kinetics of iodine release from the bandages saturated with own-made suspensions was tested with the use of DRP electrodes (graphs 13 and 14). There were observed the following behaviours:

- Suspension no. 1 (with Tween and propylene glycol) showed the most similar kinetics of iodine release to Iodosorb;



The same construction and the same amount of dibenzoyl peroxide

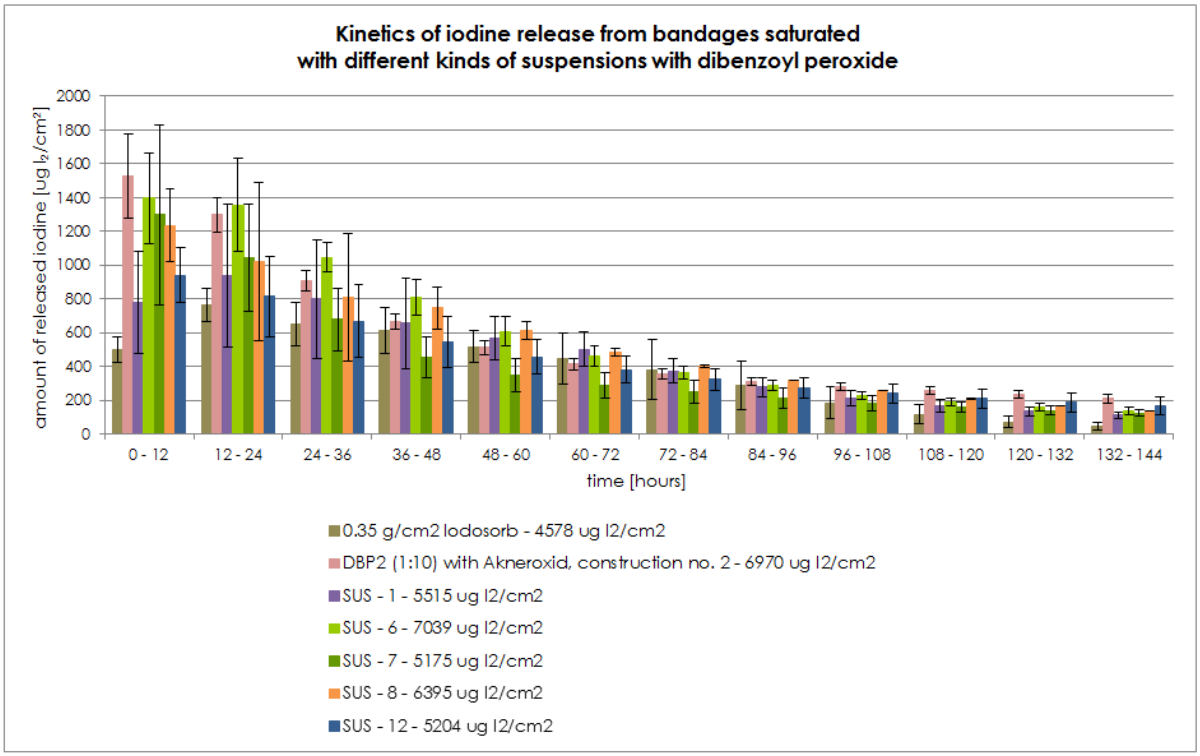
Pink column: 5% Akneroxid Gel
(the same variant as used during the *in vivo* study)

Akneroxid and suspensions that are promising to be used

Number of the sample	benzoyl peroxide	DSS	Tween 20	propylene glycol	sorbitol	xylitol	ascorbic acid (vit. C)	glycerol	DMI	Blanose I	water	*sum*
SUS - 1	10%	-	1%	3%	-	-	-	-	-	0.5%	86.5%	100%
SUS - 2	10%	10%	2%	2%	-	-	-	-	-	0.5%	75.5%	100%
SUS - 3	10%	7%	2%	2%	-	-	-	-	-	0.5%	78.5%	100%
SUS - 4	10%	4%	2%	2%	-	-	-	-	-	0.5%	81.5%	100%
SUS - 5	10%	7%	2%	-	2%	-	-	-	-	0.5%	78.5%	100%
SUS - 6	10%	7%	2%	-	-	-	-	2%	-	0.5%	78.5%	100%
SUS - 7	10%	7%	2%	-	-	2%	-	-	-	0.5%	78.5%	100%
SUS - 8	10%	4%	2%	2%	-	-	0.1%	-	-	0.5%	81.4%	100%
SUS - 9	10%	-	-	1%	-	-		-	70%	0.5%	18.50%	100%
SUS - 10	10%	4%	-	1%	-	-		-	70%	0.5%	14.50%	100%
SUS - 11	10%	-	-	1%	-	-		2%	70%	0.5%	16.50%	100%
SUS - 12	10%	4%	-	1%	-	-		2%	70%	0.5%	12.50%	100%

Graph 13. The list of prepared suspensions with dibenzoyl peroxide and the graph presenting the total amount of released iodine from the dressings saturated with mentioned suspensions.

- Suspensions no. 2, 3 and 4 contained different amounts of DSS (10, 7, 4%). Addition of 7% DSS gave the best solubility of DBP. 7% and 10% of DSS formed creams, whereas 4% formed a creamy suspension (particles of DBP fall down on the bottom of the dish after dozen of minutes);
- Addition of glycerol (SUS-11) decreased the total amount of released iodine but provided the equal release of iodine in the whole period of treatment;
- Addition of ascorbic acid (SUS-8) increased (2-times) the release of iodine from the bandage (when comparing to suspensions 4 and 12 – propylene glycol + DSS);
- Suspension no. 6 (7% DSS, 2% Tween and 2% glycerol) showed the most similar kinetics of iodine release and the same total amount of iodine as the dressing saturated with 5% Akneroxid Gel (prototype).

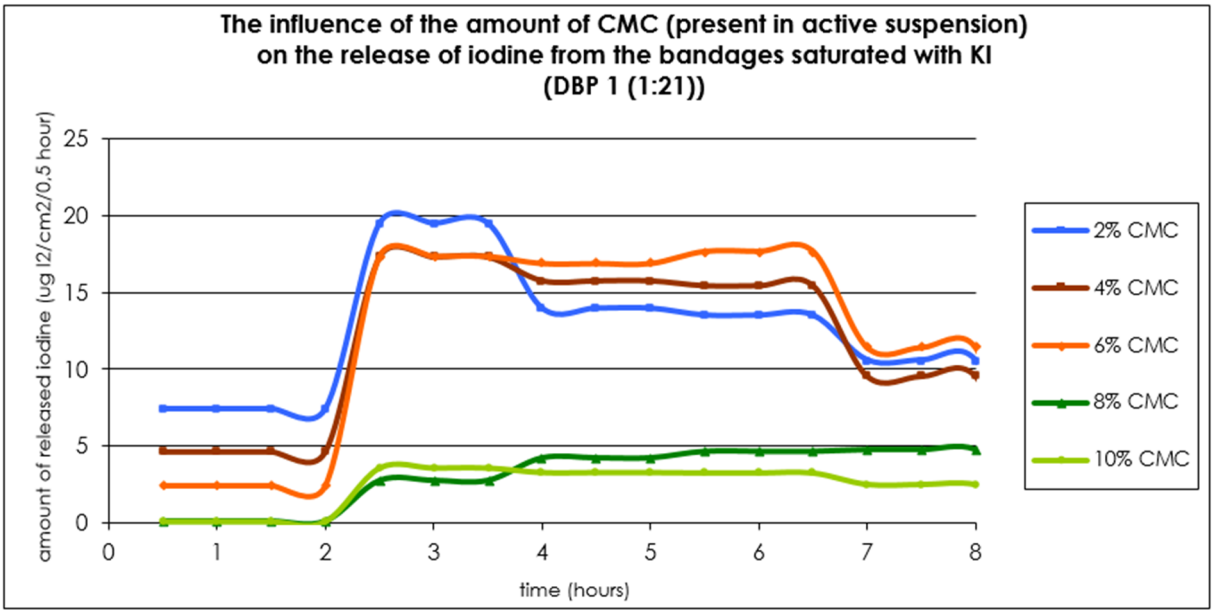


Graph 14. Kinetics of iodine release from dressings saturated with own-made suspensions in comparison to Iodosorb and the prototype with Akneroxid.

Finally, the stability of six chosen suspensions was also checked. The samples were stored for about one month in a fridge (5-10 °C). Suspensions SUS-3 and SUS-6 seemed to be the most stable. This part of research could be continued if there was the willingness of the company to develop own suspensions with dibenzoyl peroxide.

In order to aims of TAČR project the influence of polysaccharides (presented in the DBP suspension) on the kinetics of iodine release were tested. Carboxymethylcellulose

(CMC) was used for the analysis as a source of polysaccharide (graph 15). In the tested samples (especially with high ratio of DBP to KI) it was visible that the samples with 8% and more CMC released three times less iodine than samples with lower CMC content. Additionally, the period of the iodine release was at least two times shorter in case of thicker formulations.

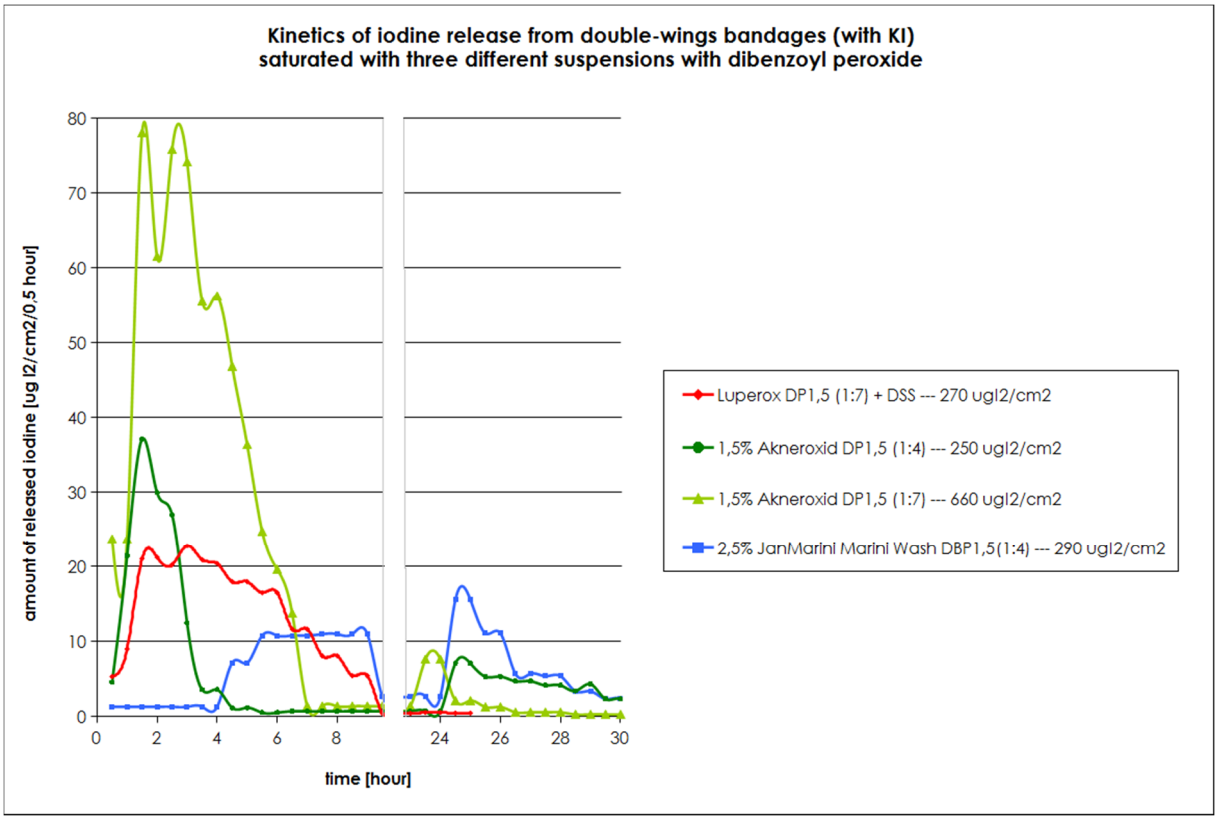


Graph 15. The influence of the amount of CMC (present in active suspension) on the release of iodine from the bandages saturated with KI (DBP 1 (1:21)).

Due to the fact that it would have been highly complicated to develop the stable gel suspension, it was indicated to use the commercial product with DBP. First of them was Akneroxid Gel (Almirall) with 10% content of DBP. It was needed to use diluted form of this product to ensure the required amount of DBP in suspension form. When looking at the total amount of iodine that released from the bandage Akneroxid was more efficient in comparison to the originally tested solution. On the other hand, the kinetics of iodine release was not as satisfied as in the case of Luperox. The reaction ran too fast and after dilution the stability of DBP in this suspension was pure (30% of loss of activity was observed after one week of storing in the fridge).

These reasons required to start looking for the other commercial product in more adequate concentration (1.5% and 2.5%). Subsequently, there were tested following formulations: 2.5% wash for treating acne from Jan Marini (USA) and also 2.5% and 5% creams from Arius (India). The last two compositions from Arius did not show the appropriate kinetics where the release of iodine was highly inhibited mainly during first 24 hours. Jan Marini Wash showed the higher efficiency and similar kinetics of iodine release to the output solution (Luperox) and more steady than for diluted Akneroxid (graph 16).

The later experiments on the *in vitro* Lubbock biofilm model revealed that 2,5% concentration of BDP did not produce iodine in the amount sufficient for biofilm eradication. Two more gels with 5% DBP were used in further research: 5% Akneroxid Gel (Almirall) and 5% Benzacne Gel (Takeda). Data concerning the mentioned formulations will be widely described in the next paragraphs. Either JanMariniWash or Akneroxid Gel and Betadine Gel are stable commercial products with three years of the shelf life.



Graph 16. The kinetics of iodine release from the double-wings bandages saturated with different suspensions containing dibenzoyl peroxide.

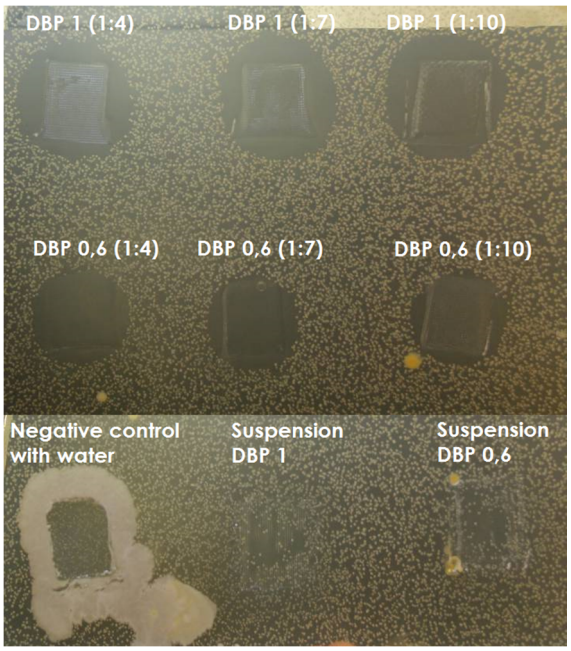
3.1.3.2 Checking the antimicrobial activity of wound coverings with DBP

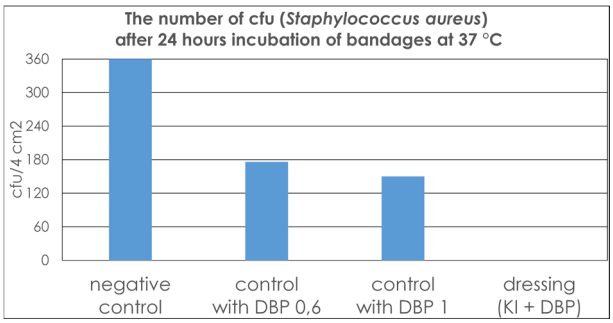
The following chapter describes the test of the antimicrobial activity of dressings with I_2G and the Akneroxid Gel by itself. The aim of this experiment was to check which of prepared coverings had the enough efficiency to inhibit the planktonic bacteria growth. The results from this test showed the minimum concentration of DBP (2,5 mg DBP per 1 cm^2) that could be used in dressings directed for the subsequently developed multibacterial biofilm model.

The antimicrobial activity of wound coverings with dibenzoyl peroxide were checked by performing the test with *Staplylococcus aureus* which was located between two agarose layers. On the dish there were put bandages with different ratios between dibenzoyl peroxide

(diluted Akneroxid) and potassium iodide (DBP1 means 10 mg of DBP per 4 cm^2 of the bandage; 1:4, 1:7 or 1:10 means the molar ratio of BDP and KI). The pH value of all tested bandages with DBP was in the range of 5.7 – 6. Additionally, the antimicrobial properties of the suspensions of benzoyl peroxide were tested.

The presence of Akneroxid in the bandage (controls) showed the ability of benzoyl peroxide (benzoyl peroxide + benzoic acid) to inhibit the growth of bacteria *Staphylococcus aureus*. Each of tested samples of Iodine Generator (DBP+KI) totally inhibited the bacteria growth. As it was expected, the higher concentration of dibenzoyl peroxide caused the bigger inhibition zone (graph 17, figure 13). Finally, it is evident that the cooperation of two (iodine with DBP) or more antimicrobial compounds plays an important role in treating biofilms.





Graph 17. The number of cfu (*Staphylococcus aureus*) after 24 hours of the incubation of samples at 37 °C.

Figure 13. The results of the antimicrobial properties of bandages with dibenzoyl peroxide.

3.1.3.3 The influence of plasma and blood on the iodine formation

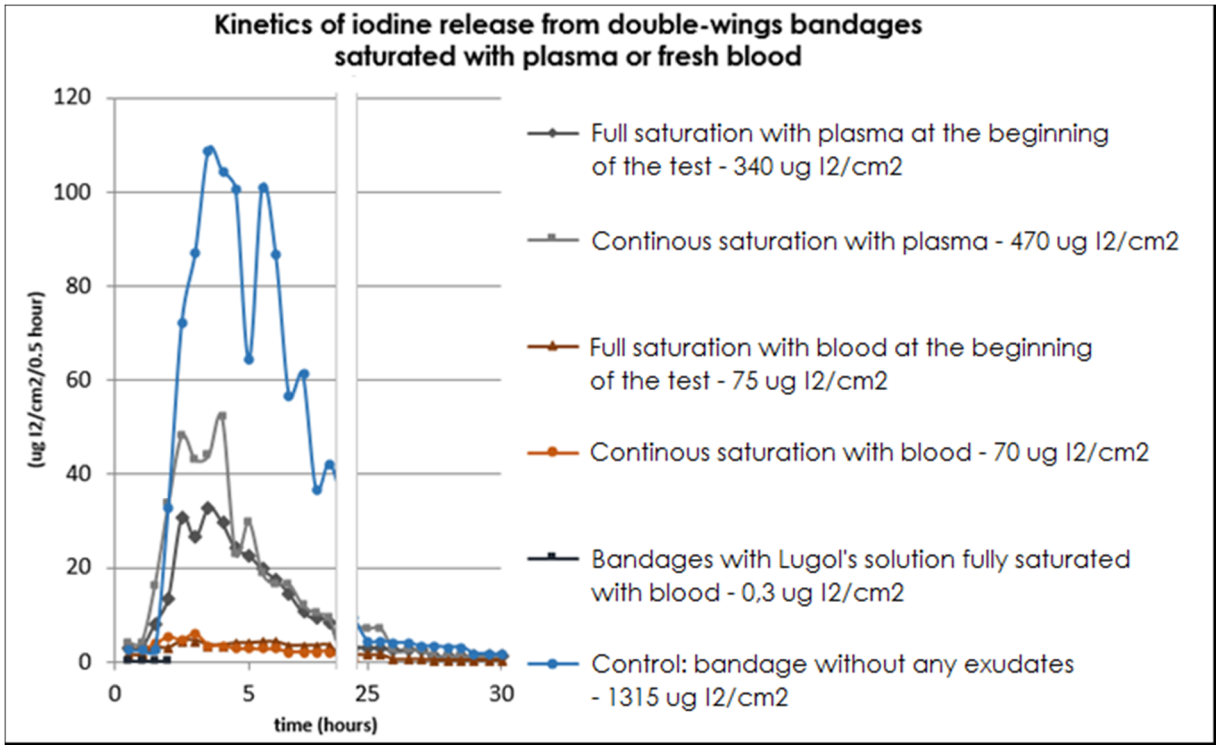
One of the next experiments was performed to check the influence of plasma and blood on the iodine release from the wound dressings. For this experiment there was used I_2G having high amount of DBP (30 mg/ 4 cm) because it was expected that the iodine production will be inhibited by the experiment's conditions.

Products containing iodine as an active ingredient for treating wounds are known as not effective in case of bleeding wounds what was proved in this work (graph 18, table 2). In the Lacey's work (1979) it was mentioned that PVP-iodine is able to be optimally effective in relatively blood-free situations. There was mentioned that this product could be effective

in the following concentrations of iodine: at least 0.1% in the presence of fat, 1% in the presence of pus and 5% in the presence of blood (1g of haemoglobin inactivates 50 mg of free iodine).

Table 2. The efficiency of the iodine release from bandages (DBP3(1:6), and the bandage with Lugol's solution) saturated with plasma and blood.

	Efficiency
I_2G	100%
I_2G + plasma (applied continuously)	36%
I_2G + plasma (applied at the beginning)	26%
I_2G + blood (applied continuously)	5%
I_2G + blood (applied at the beginning)	6%
Lugol's solution + blood (applied at the beginning)	0%



Graph 18. Kinetics of iodine release from double-wings bandages (DBP3 (1:6)) saturated with plasma or blood.

During this part of work there were performed two ways of the saturation with pig plasma or fresh cattle blood. In the first option, the dressings were fully saturated at the beginning of the application while in the second one the saturation was done continuously. The efficiency of the amount of released iodine in comparison to the dressing that was not saturated with any liquid (and which released iodine for circa 90 hours) is presented in the table no. 2. There was also tested the bandage saturated with 0.5% Lugol's solution that showed almost no ability of iodine release in the presence of blood. The iodine was completely

caught by blood's proteins. In case of chronic infected wounds covered with biofilm there should not have appeared any bleeding and even if there is some bleeding the Iodine Generator based on dibenzoyl peroxide and KI should not drastically decrease its activity.

3.1.3.4 The effect of the chosen dressings on the pH values

The low pH of the wound dressing fundamentally affects the skin irritation. The objective of the experiment described below was to compare the pH of different forms of I_2G and other wound care formulations and to find the most suitable pH range for the anti-biofilm medical device that is applied directly on the patient's skin.

A test where the bandage (25 cm^2) was put to the beaker and immersed in 35 ml of distilled water was performed. The pH of own-prepared bandages was measured 24 hours after the initiation of the reaction. Bandages* chosen from the article were incubated for 7 days but tested each day (small daily changes in the presence of water). (Uzun, 2012)

All commercially available formulations analysed by Uzun (2012) and used for wound healing has pH in the range between 5,2 and almost 7. In the process of wound biofilm inhibition, the dressing should avoid the increase of pH in the wound because higher pH in the wound causes the better conditions for pathogen bacteria growth. On the other hand, if the pH of the wound care product is much lower than 5, the irritation of the skin may appear. The gained results presented in the table no. 3 were the indication for the further research. It was deduced not to use the inorganic Iodine Generator (KI + $NaIO_3$) having pH around 4 and to concentrate on the Iodine Generator based on peroxide (pH 4,92 – 5,67 depending on the source of DBP).

Table 3. The effect of the dressings on the pH values of water.

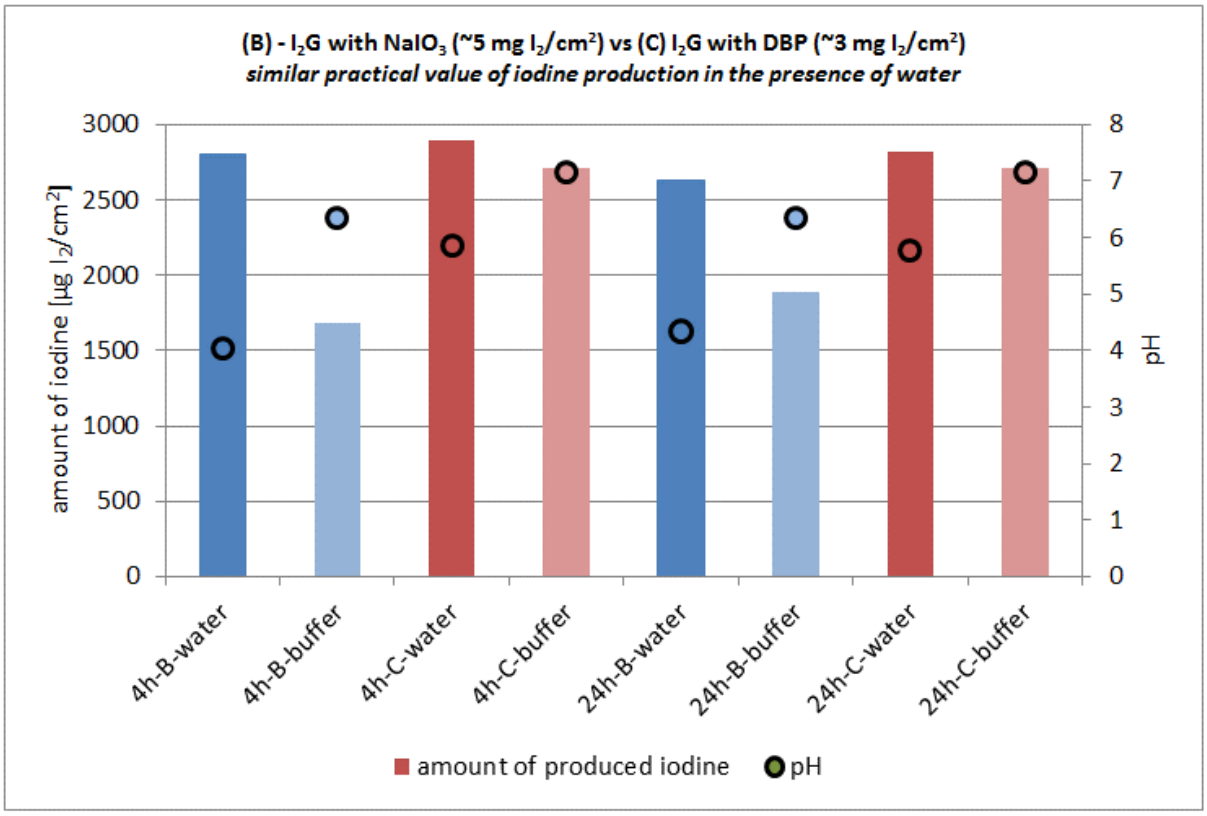
Dressing	***pH of distilled water after immersion of the dressing***
Aquacel *	5.62
Aquacel Ag *	6.08
Kaltostat *	**6.92**
Mepilex Border *	7.59
Granuflex *	**5.25**
Biatain *	6.02
Aquacel Surgical *	5.52
Bandage KI+$NaIO_3$ (920 µg/cm^2)	4.19
Bandage KI+$NaIO_3$ (9230 µg/cm^2)	**3.72**
Double-wings bandage DBP1.5 (1:2) with 2.5% Jan Marini Wash	4.92
Double-wings bandage DBP3 (1:6) with 5% Akneroxid	**5.67**

3.2 VERIFYING THE EFFECTIVENESS OF INORGANIC AND ORGANIC IODINE GENERATOR UNDER PHYSIOLOGICAL CONDITIONS

Due to the fact that high pH of body exudates can have a negative influence on the iodine production and release, the pH values of dressings containing both inorganic and organic Iodine Generator were checked and compared. The goal of this research was to prove that the iodine production in I_2G with DBP and KI should not be as much affected by the wound exudates as in case of inorganic generator. The influence of pH on the iodine production in the dressings with inorganic (B: KI + $NaIO_3$) and organic (C: KI + dibenzoyl peroxide) I_2G was checked by using a phosphate buffer (pH = 7.4). Bandage B was made of two absorbing textiles (PES, 280 g/m^2) where KI and $NaIO_3$ with the addition of acidic starters were applied and dried. Bandage C was made of two pieces of the same PES impregnated with KI (saturated and dried, construction no. 1 in a double-wings form, figure 14) between which 5% Akneroxid Gel was applied.

The bandages were put to the Erlenmayer flasks and saturated with either water or phosphate buffer. After 4 and 24 hours of storage in the incubator at 37 °C, the ethanol was added to the flasks to stop the iodine formation. The measurement was done by means of UV-Vis spectrophotometry (method: 2.2.2.1). Simultaneously, the same bandages were wetted with water or phosphate buffer and their pH values were measured after exact period of time. For the comparison, there were chosen bandages that produced the similar amount of iodine after 4 hours of incubation in the presence of water.

The pH values of bandages saturated with water were around 4 for I_2G with $NaIO_3$ and around 6 for I_2G with dibenzoyl peroxide. When phosphate buffer was used, the pH was around 6.5 and 7, respectively. So, the change of the pH of the solvent had an evident influence on the inorganic I_2G. On the other hand, formation of iodine in dressings with DBP seemed to be independent on the pH changes of the environment (ultimately: wound exudates). Moreover, the decrease of iodine release was around 40% in case of the dressing with $NaIO_3$ and only around 7% in case of the dressing with DBP (graph 19). There was also tested Betadine that showed circa 8% decrease of efficacy with the increase of pH equal to 2.8 (presenting the biggest difference between water and buffer).



Graph 19. The influence of pH on the iodine production in dressings with I_2G.

3.3 CONSTRUCTIONS OF WOUND COVERINGS WITH IODINE GENERATOR

When the principle of iodine formation was chosen, several constructions of wound coverings were prepared and used for tests. The development of constructions began with the dressing no. 1 presented in figure no. 14. It was the only construction where potassium iodide was used in a form of solution that was applied on the textile and subsequently dried at 40 °C. This application existed in a form of the double-wing bandage with lyocell or thick polyester (impregnated with KI) inside the PES and PAD's cover. The "tea-bag" sachet form was prepared by sealing the edges of the thin textiles. PAD showed sticking properties that may have helped to attach the rest of biofilm to the dressing while its removal from the wound. Just before the application the suspension with dibenzoyl peroxide was applied between the wings and then the wings were connected. This kind of construction allowed DBP presented in the suspension to react with KI (that was available from both sides of the dressing) in the most efficient way. There was also tested the influence of the way of KI application (saturation of the textile; putting the xanthan layer with KI on one or two textiles facing each other or being separated), from which the impregnation of the textiles in connection with double-wings construction seemed to be the most efficient for iodine release.

The rest of constructions having various arrangement of thick and thin textiles impregnated with KI and/or source of PVP were based on the lamination technology and are presented and described below (figures 15 – 23, (CZ 29194 U1, 2016)). Lamination thoroughly described in methods (paragraph 2.2.1.2) allowed to avoid the formation of iodide crystals after drying of the textiles and simplified the manufacturing process.

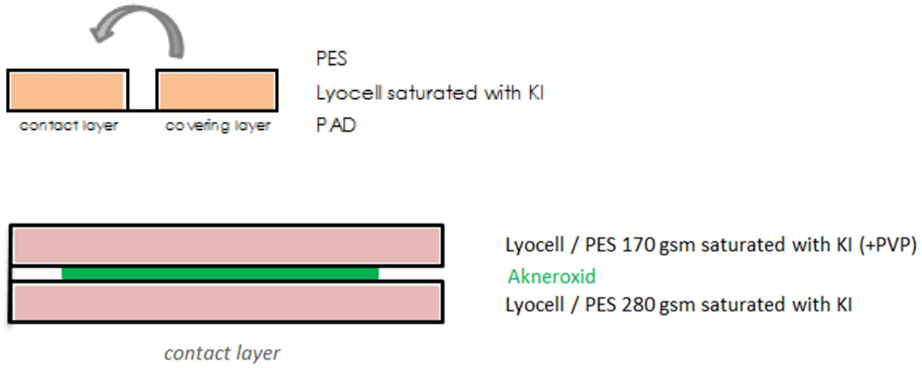


Figure 14. Double-wings bandage with the construction no. 1 in a form of sachet.

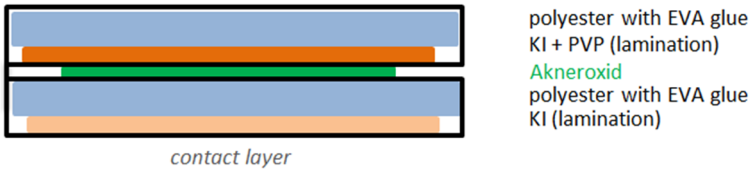


Figure 15. Bandage with the construction no. 2 in a form of a sachet.

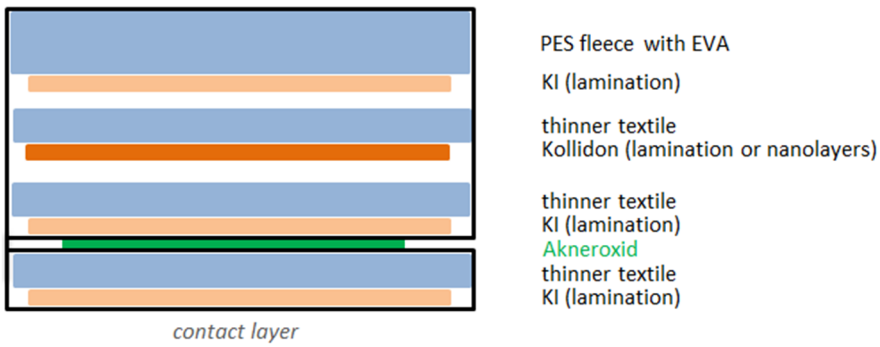


Figure 16. Bandage with the construction no. 3 in a form of a sachet.

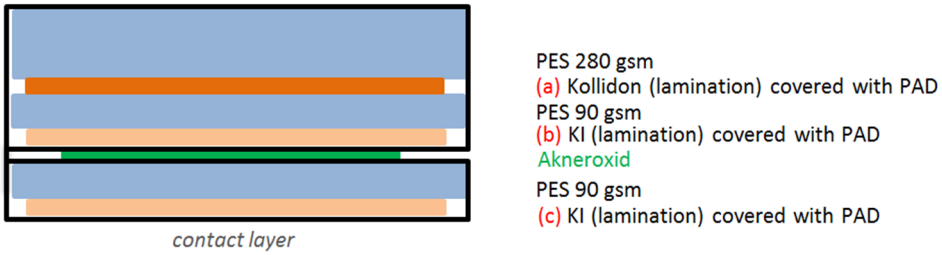


Figure 17. Bandage with the construction no. 4 in a form of a sachet.

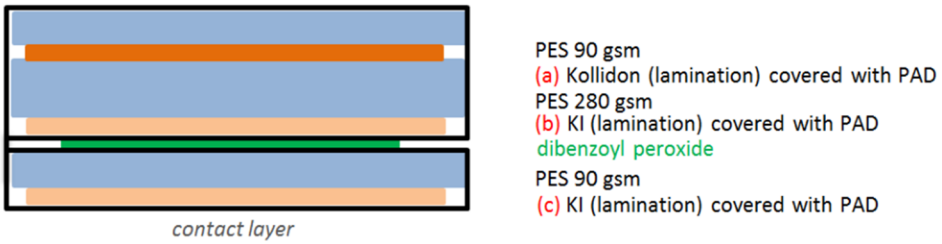


Figure 18. Bandage with the construction no. 5 in a form of a sachet.

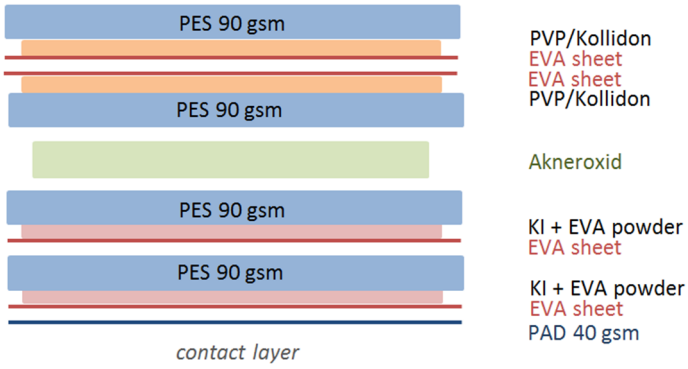


Figure 19. Bandage with the construction no. 6.

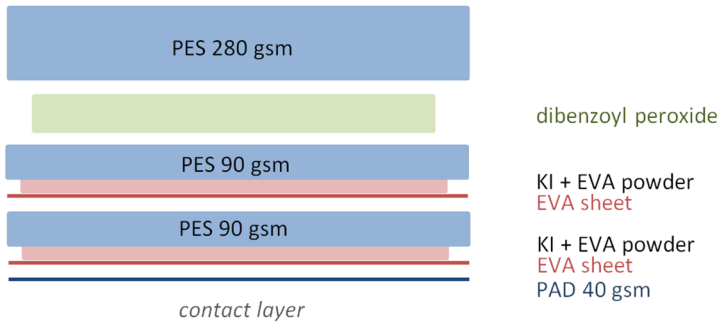


Figure 20. Bandage with the construction no. 7.

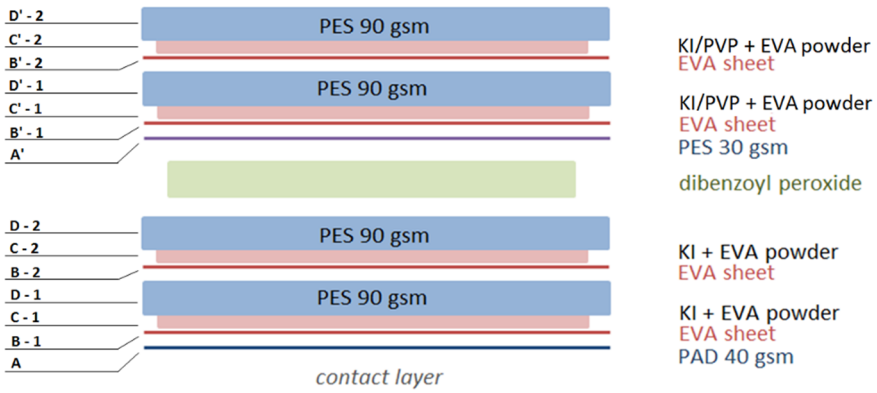


Figure 21. Bandage with the construction no. 8.

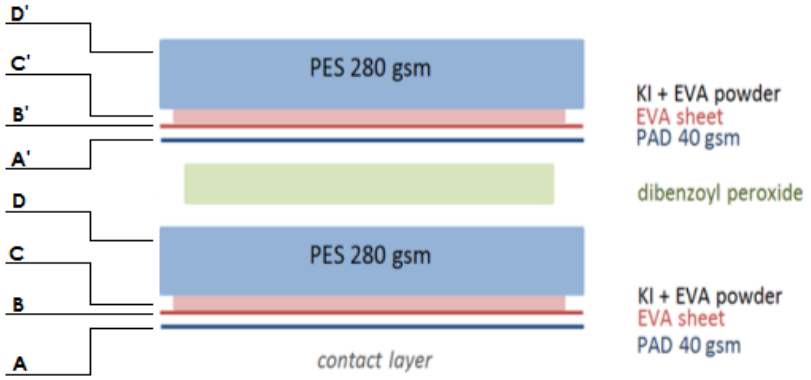


Figure 22. Bandage with the construction no. 2 (modified) – prototype for the *in vivo* study.

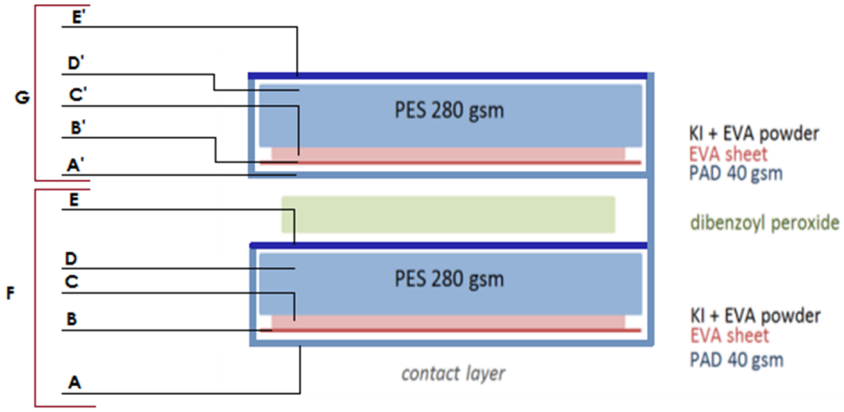


Figure 23. Bandage with the construction no. 2 (modified) in a form of a sachet.

The final alignment of layers in the wound dressing with I_2G can be described as below. The multi-layer formation of the wound covering comprises layers arranged in the following manner in the direction from the wound: the contact layer (F), which is to be in contact with the wound and the surface layer (G) being in an indirect contact with the first absorption

layer. Therefore, the lower, contact layer contains at one (lower) side preferably adhesive textile (A) destined to be contacted with the wound, then the absorbing textile (D/D') impregnated with potassium iodide and/or polyvinyl pyrrolidone with the use of a thermoplastic glue, e.g. ethylene vinyl acetate (EVA) in a form of a powder (C/C') and/or EVA sheet (B/B'), covered with a thin textile (E/E'). The edges of the contact layer (F) and the surface layer (G) may be welded together (figure 23). The upper side of the dressing can be in the same form as a lower side or can be made of different numbers of joined absorbing textiles (D or D-1, D-2, etc.). Both parts of the dressing may be welded together from three sides forming the so-called tea bag with one open edge (figure 24, on the right) or there can be two separated parts that are made of layers from A to D (figures 24 (on the left) and 22).

For more precise description, the specified information concerning two constructions is presented below. Figure 22 represents a dressing made of two separate layers where A/A' is polyamide (contact adhesive layer), B/B' is ethylene vinyl acetate in a form of sheet, C/C' is a blend of potassium iodide, ethylene vinyl acetate (EVA) in a form of powder and/or polyvinylpyrrolidone with EVA, D/D' is an absorbing textile (polyester 280 g/m^2). Figure 23 represents a dressing that differs with the previous one in the layer E/E' being a covering textile (polyester, 30 g/m^2) and in a form of welding. Here, textiles A/A' and E/E' are welded together to form a double-wings bandage or a sachet.

The final form of the developed wound dressing can be in a form of two separate layers, in a form of a double-wings bandage or in a form of a pocket/sachet (figure 24). However, from the production point of view, the first option is the most convincing. In each case the suspension containing dibenzoyl peroxide should be applied between two active layers just before the application on the patient's wound. If there is a wound with no or little exudation, it is required to saturate the wound covering with water or physiological solution.

Two separate layers *Double-wings bandage* *Pocket/Sachet*

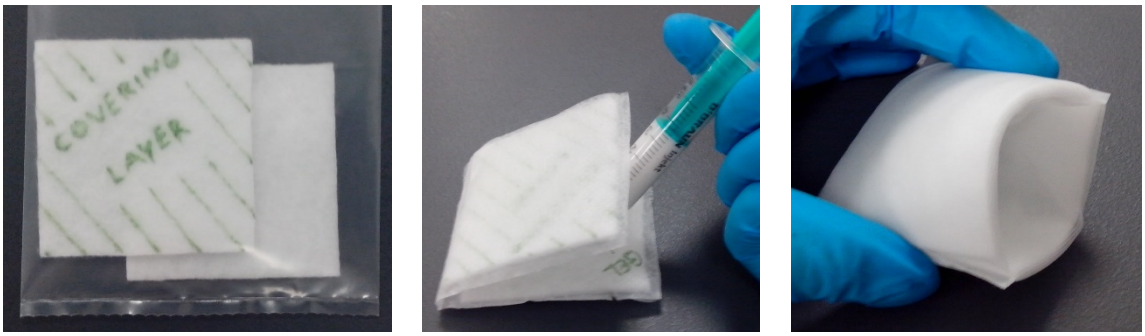


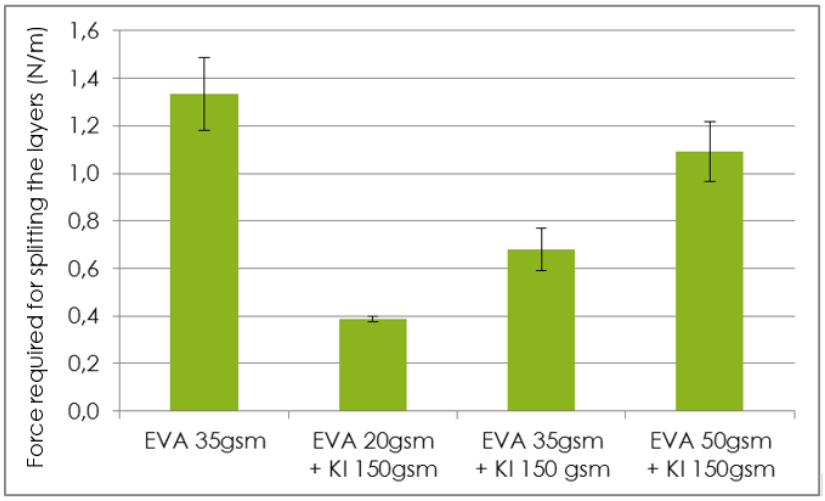
Figure 24. Final application forms of the wound coverings with Iodine Generator.

3.3.1 The amount and the form of EVA adhesive

The following paragraph describes the results from the test checking the strength of the bandages prepared by the lamination process. The purpose of these experiments was to find the sufficient amount of EVA glue to get a firm wound dressing from which no active substances were falling out while manipulating with them.

In case of a mere connection of two fabric layers laminated via an EVA (ethylene vinyl acetate) adhesive it was sufficient to use about 35 g of EVA per 1 m^2 which was derived from the proven functionality of such connections in a different product in Contipro. When EVA layer had to fix also another component, such as KI or PVP, it was likely that the weight of EVA would be increased. Strength of laminated joints, respectively the force required for delamination of the layers was determined by using a tensiometer.

Thanks to the tests performed on the „starch gel model" and the results from the first part of *in vitro* tests on the Lubbock biofilm (with four bacteria strains) it was possible to state that the optimal amount of KI in the wound covering should have been on the level of 620 g per 1 m^2. This quantity of KI provided the kinetics of iodine to be long enough (at least 48 hours) and sufficient for biofilm eradication (graph 44).



Graph 20. The force required for delamination of laminated boards for various amount of EVA adhesive and KI.

Originally, it was assumed that the total amount of KI (620 g/m^2) would have been divided in the covering into four layers that it why the first optimisation was based on 150 g/m^2 of single laminated layer. Polymer EVA was dosed in three different basis weights: 20, 35 and 50 g/m^2. In case of the incorporation of KI it was necessary to increase the amount of EVA to achieve approximately the same strength of the joint as for the EVA layer itself.

Similar strength was achieved for the weight of 50 g/m^2 EVA but subjectively (after manual peel) the weight 35 g/m^2 appeared to get a strong enough joint (graph 20).

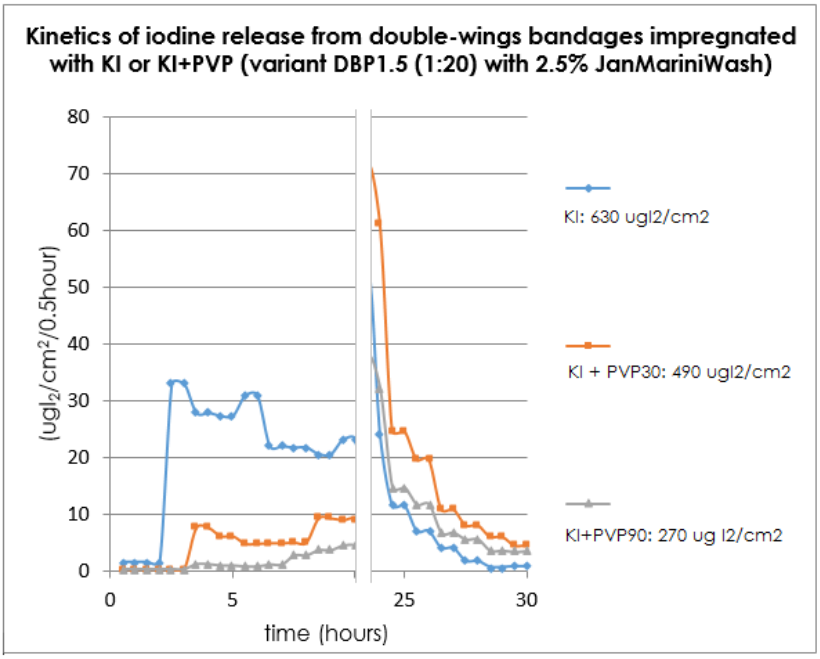
However, as it turned out that the total amount of KI can be divided only into two layers there was carried out the verification of a basis weight of KI (300 g/m^2). In this case the polymer should have been used in two forms: EVA powder (35 g/m^2, as a first cover) and the EVA powder mesh (~ 35 g/m^2, second cover). Thanks to that solution there was obtained a product which did not lose the powder even during cutting. For the preparation of such a blank the lamination should have been done with an intermediate pressure at the temperature of 120 ° C for 20s.

An analogous test was carried out for a combination EVA and PVP. The total amount of PVP in the covering was assumed on the level of 100-150 g/m^2, divided into two layers of 50 to 75 g/m^2. This amount was lower than in the case of KI for a single layer but PVP has a finer and lighter particles by volume and therefore is comparable to KI. This corresponds to the results of measuring the strength of the laminated joints. The same amount of EVA powder (35 g/m^2 powder + EVA mesh) as in the case of KI had to be used for forming the sufficiently strong laminated joints with PVP.

3.3.2 Incorporation of polyvinylpyrrolidone - prolonging the kinetics of iodine release

The objective of this part of research was to check the properties of PVP that should work as a capacitor of iodine and decrease the high concentration of iodine in the initial phase of kinetics and prolong the time of iodine release simultaneously. That is why the bandages with Iodine Generator were optimized by the incorporation of polyvinylpyrrolidone (PVP K30; PVP K90; Kollidone 25).

During the first attempt, PVP was mixed together with KI and then the suspension was applied on the absorbing layer that was subsequently dried. The results from the starch gels showed that the kinetics of iodine release when PVP K30 had been used was inhibited during the first day but showed the higher amount of released iodine in the following days. Total value of iodine which released from the dressing was 20% lower than in the bandage with the absence of PVP. On the other hand, PVP K90 inhibited the release of iodine during the whole period of the experiment so finally was not used in further development. (graph 21)



Graph 21. The kinetics of iodine release from the dressings impregnated with KI and PVP ("starch gels").

Addition of Kollidon (cross-linked PVP) or PVP K30 (40 or 60 mg/4 cm^2) decreased the efficacy of iodine release in the first phase of the kinetics but subsequently increased the amount and prolonged the time of iodine release in comparison to the dressing without the addition of PVP. The total amount of released iodine was respectively 10 % or even 30% lower when compared with the dressings with the absence of PVP. In case of PVP K30 there was similar inhibition of iodine release in the first phase of the release but no significant prolongation of the iodine release (also around 10% loss of the total amount of released iodine). The addition of 20 mg PVP per 4 cm^2 almost did not influence the kinetics of iodine release. In the further part of work with chosen covering (p.3.4.1) the influence of PVP on the kinetics of iodine release was checked again (measurement on glass sensors) and compared to the results from *in vitro* test on Lubbock biofilm model.

Exposure of PVP powder to high temperature (150 °C) can cause the darkening in colour and decreased water solubility. However, PVP appeared to be quite stable when heated repeatedly at 110 - 130 °C for relatively short intervals (ISP, 2013). The bandages used in this research went the process of lamination under 120 °C. It did not cause the darkening of PVP (if was laminated alone). However, together with the incorporation of PVP to the dressing, there appeared the problem with yellowing of the textile. In case of the bandage no. 2 it was visible that the contact layer impregnated only with KI maintained with no colour change (without yellowing). Whereas the second layer with KI mixed with PVP was yellowing. That is why it was indicated to separate the layer with PVP from the layers containing KI.

In the next variants the used textiles were thinner (90 g/m^2) than in the previous construction to maintain the similar thickness of the bandage (constructions 4-8, figures: 17-21).

During this work there was also performed a pilot experiment with nanolayers containing PVP (PVP K30 + Kollidon; 29:71). The results did not differ from that gained from the bandages impregnated with Kollidon in a form of powder. Again, the kinetics was inhibited in the first phase of the application (first 10 hours on the "starch gel" model) and there was visible the prolongation of the iodine release for several hours.

3.4 COMPARISON OF THE KINETICS OF IODINE RELEASE FROM CHOSEN VARIANTS OF WOUND COVERINGS WITH IODINE GENERATOR

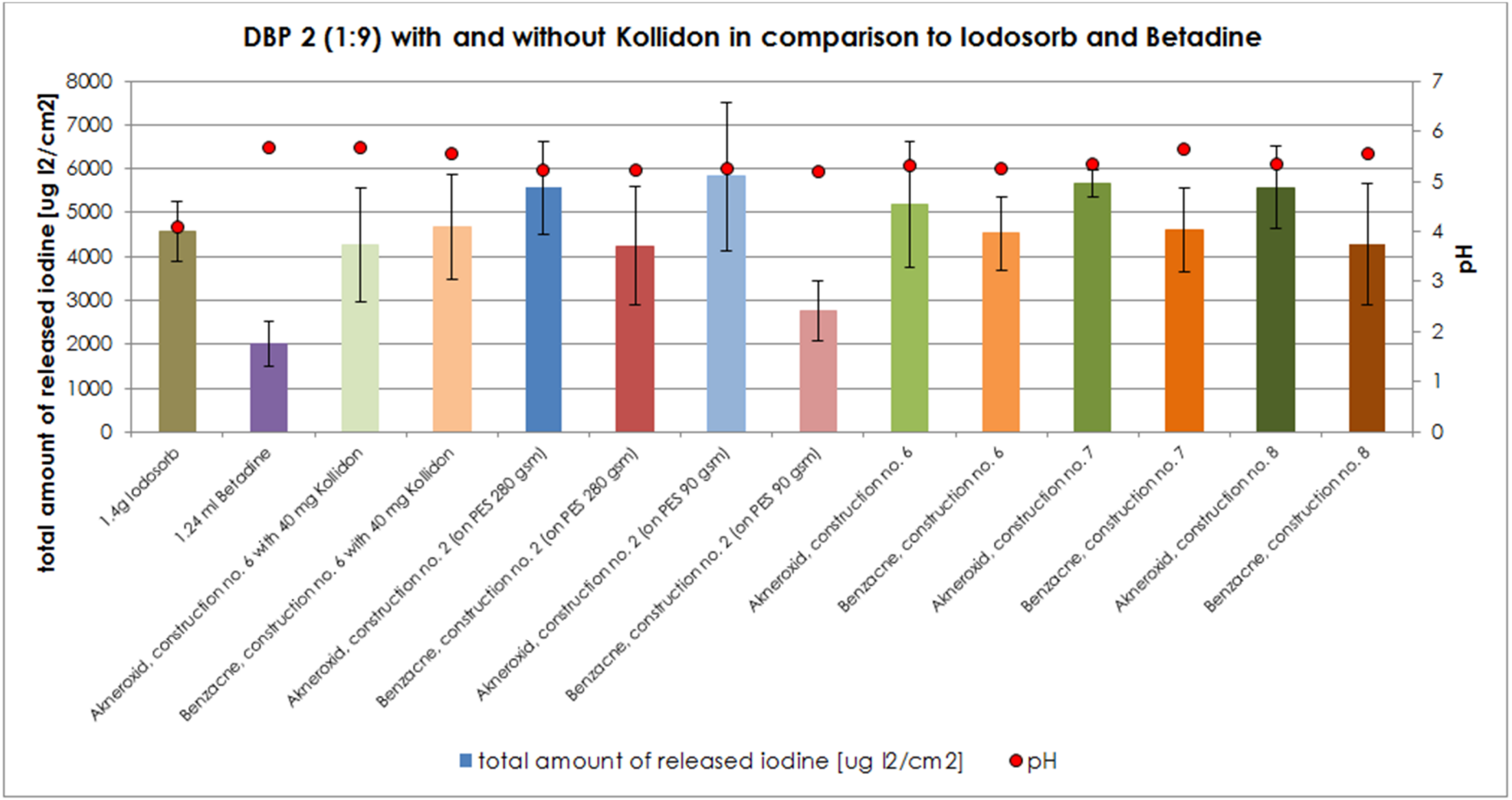
The experiments made with the use of starch gel model (mentioned above) and the results from the first model of Lubbock biofilm (see: paragraph 3.5.1) gave the direction for receiving the prototype of the wound dressing with I_2G. The next method of tracking the long-term kinetics of iodine release was based on the electrochemical method where the platinum sensors formed on a corundum ceramic base were used. The experiments with the BVT sensors demonstrated that there is no significant difference in the kinetics of iodine release from wound dressings having the constructions number 2, 3 and 4 (figures 15-17). These sensors did not allow to check the properties of PVP/Kollidon impregnated on the dressing and to compare the rest of constructions due to the fact of the fast corrosion of electrodes on account of long-lasting iodine action.

Due to this fact, the new kind of sensors was taken into consideration. The electrodes with interdigitated platinum arrays formed on the glass substrate by lithographic techniques were subsequently used and met with the expectations of being durable in contact with iodine. The last part of experiments of tracking the kinetics of iodine release were performed with a use of DRP glass sensors from DropSens. The following paragraphs presents the results of testing the kinetics of iodine release from three main variants of laminated wound dressings from which the last one (described in the paragraph 3.4.3) became the prototype that was subsequently directed for the *in vivo* study on the porcine model (see: paragraph 3.7).

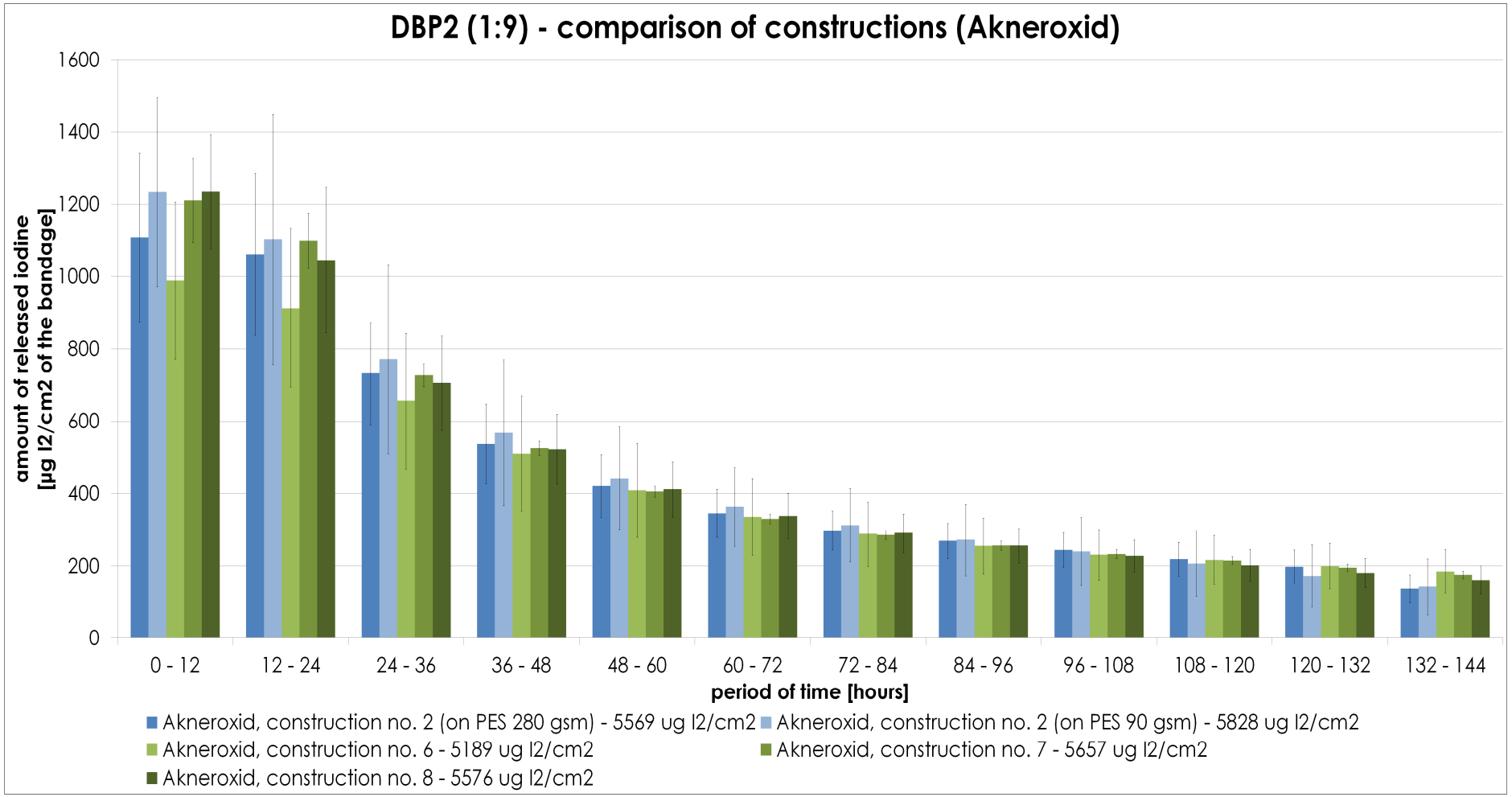
3.4.1 DBP2(1:9) with and without Kollidon (KI: 62 mg/cm^2, EVA: 7 mg/cm^2, DBP: 5 mg/cm^2, Kollidon: 10/15 mg/cm^2)

This variant of wound covering was chosen due to the results from the first model of the *in vitro* Lubbock biofilm (p. 3.5.1). Here, there were tested several constructions of laminated bandages (with or without PVP) saturated with 5% Akneroxid Gel or 5% Benzacne Gel. The pH of bandages after 6 days of the experiment was monitored, too. (figure 22). All dressings with I_2G had the pH value slightly under 5, identically to Betadine that met with the objectives of this project. On the other hand, Iodosorb (cadexomer-iodine) caused the decrease of pH value to 4.

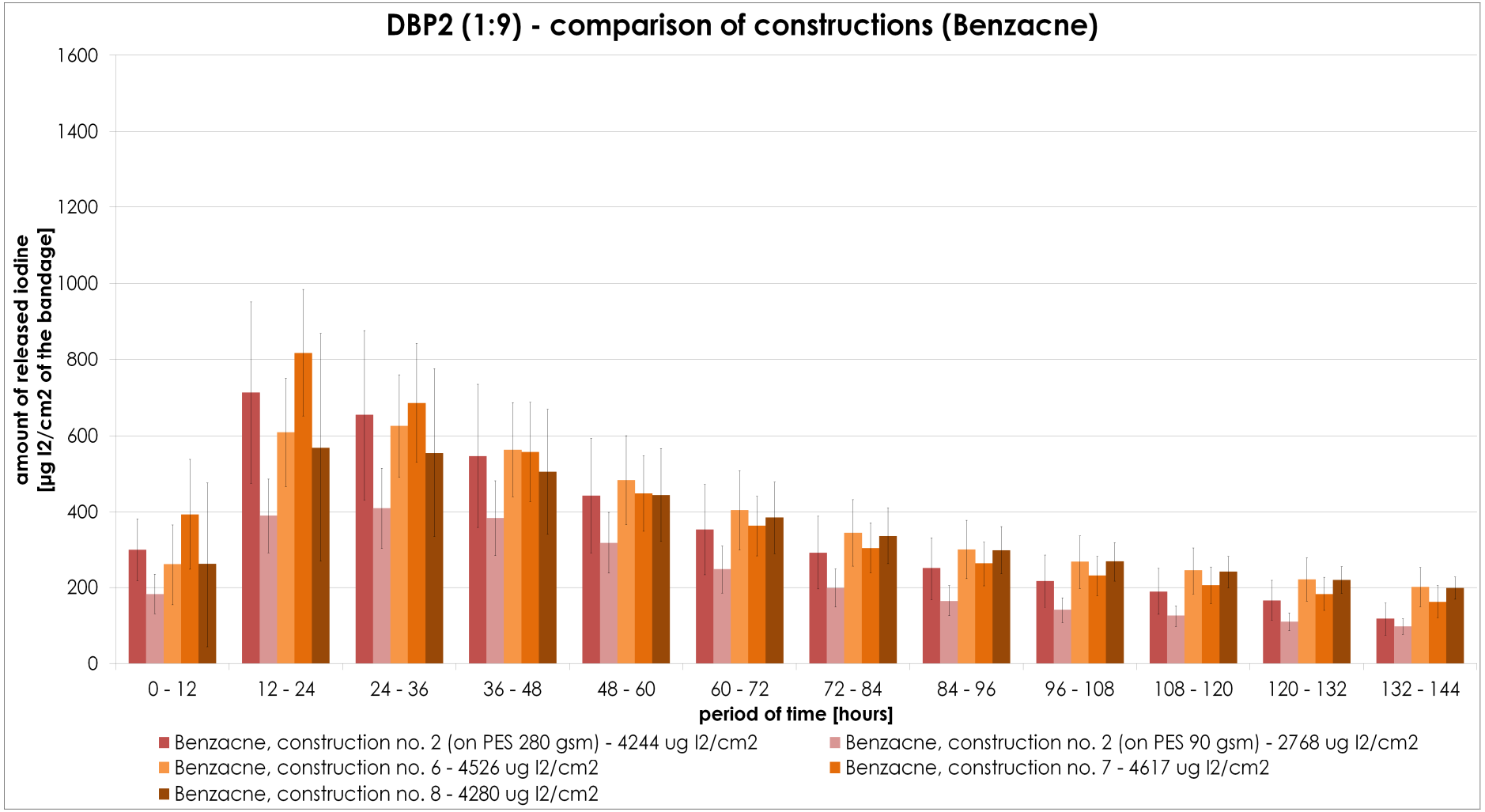
In the figures 23 and 24 there are presented the kinetics of iodine release from dressings saturated with Akneroxid or Benzacne, where only small differences between five constructions are visible. Only in case of the construction no. 2 with thin polyester (90 g/m^2) and with the application of Benzacne the release of iodine was inhibited that therefore excluded this variant from the next tests. Figure 25 presents the differences between formulations containing different sources of DBP. In case of Akneroxid the amount of released iodine in the first 48 hours was evidently higher in comparison to Benzacne (with the kinetics similar to Iodosorb) that showed the stable way of iodine release but with the lower total quantities of released iodine. The same variant of the bandage with the construction no 6 was tested on the *in vitro* model of biofilm. It was proved that it is required to have a high enough peak of iodine concentration mainly during first 12 hours of treatment and the steady decrease of its value. The bandage with Akneroxid almost totally eradicated the polymicrobial biofilm whereas the bandage with Benzacne (with the kinetics similar to Iodosorb) was less effective (graph 51).



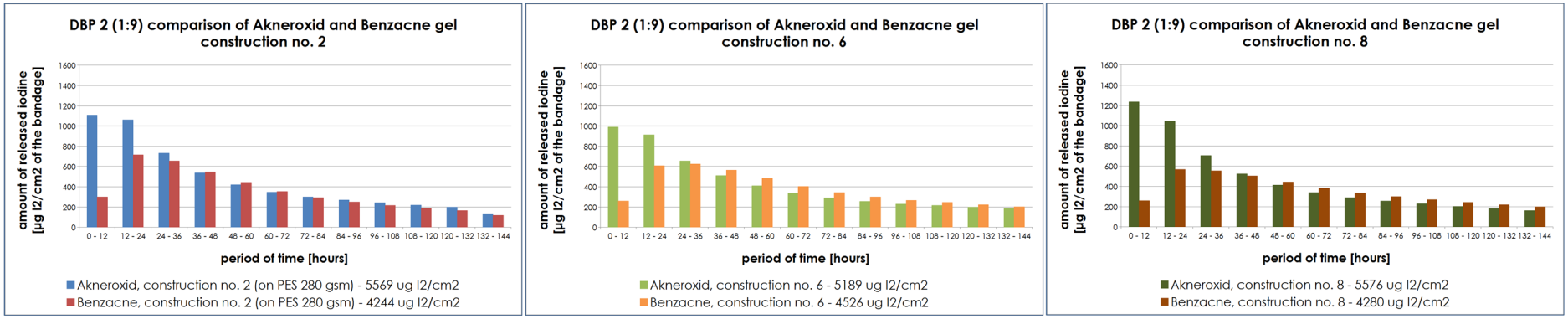
Graph 22. Total release of iodine from the dressings with Iodine Generator DBP2 (1:9) after 6 days of experiment and the pH values of bandages (measured on their contact layers).



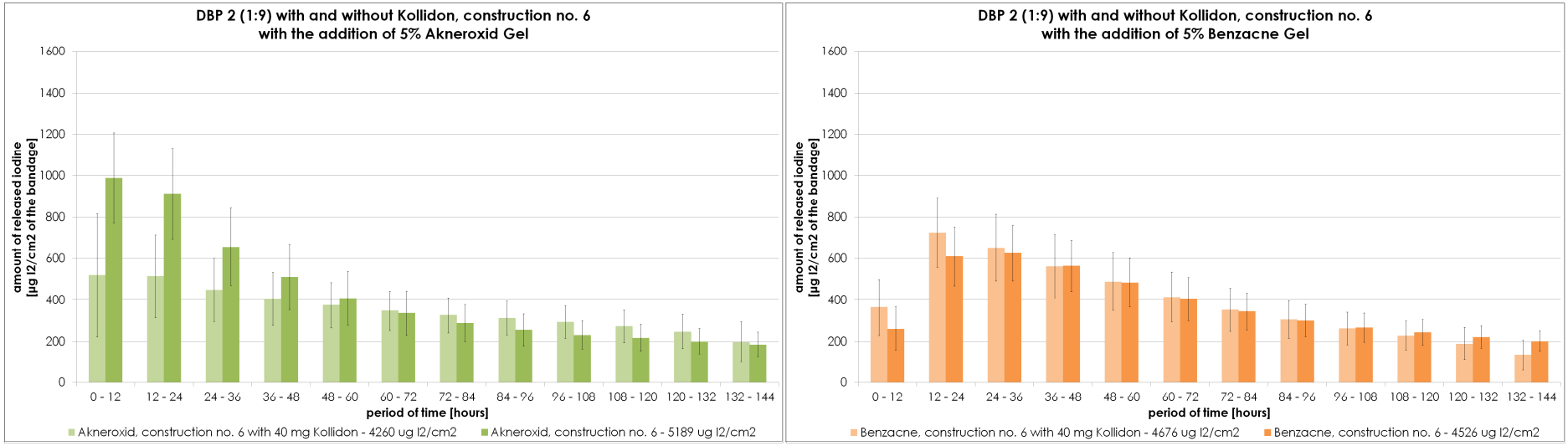
Graph 23. Kinetics of iodine release from the dressings with I_2G – DBP2 (1:9) saturated with 5% Akneroxid Gel and having 5 different constructions.



Graph 24. Kinetics of iodine release from the dressings with I2G – DBP2 (1:9) saturated with 5% Benzacne Gel and having 5 different constructions.

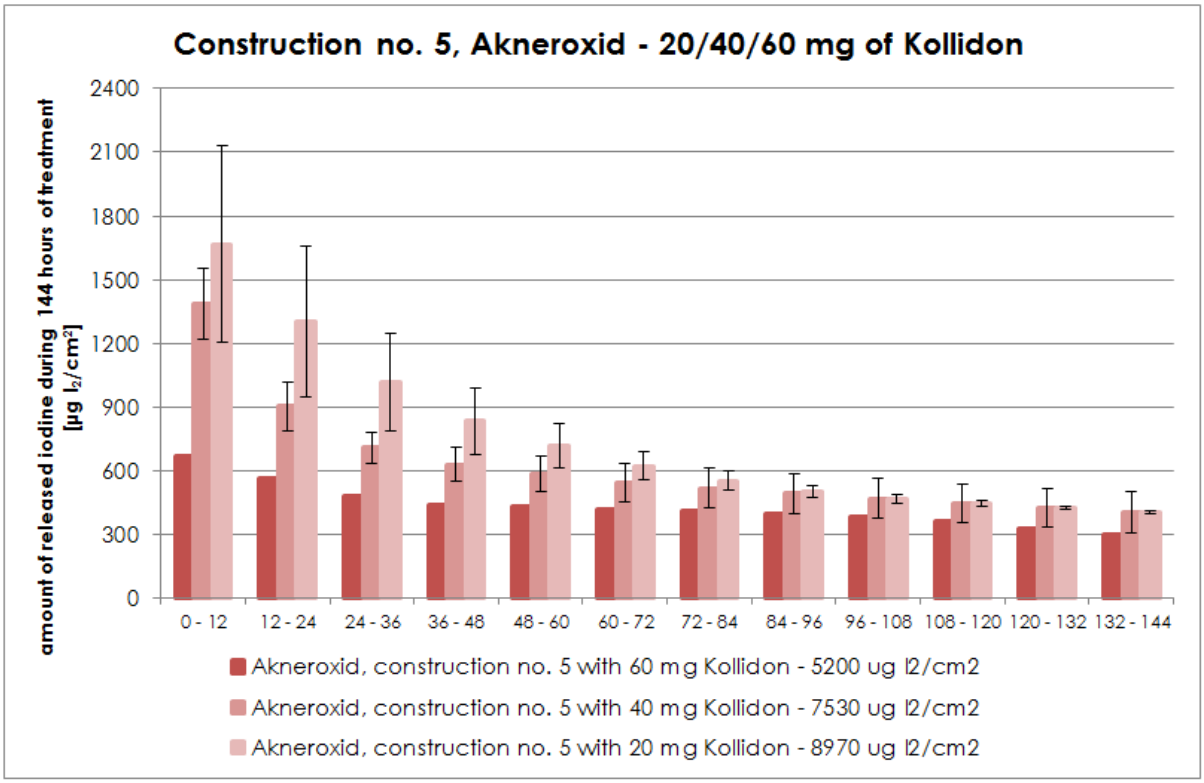


Graph 25. Comparison of the kinetics of iodine release from three constructions of the bandages DBP2 (1:9) saturated with 5% Akneroxid Gel or 5% Benzacne Gel.



Graph 26. Kinetics of iodine release from the dressings with I_2G – DBP2 (1:9), construction no. 6, saturated with gels containing DBP - influence of the addition of Kollidon (10 mg/cm^2)

The last coverings from this variant were incorporated with PVP or cross-linked PVP (Kollidon) used as a capacitor of iodine (see: paragraph 3.3.2). When Akneroxid was used as a DBP source, the inhibition of iodine release in the first hours of the release was clearly visible. At the last 24 hours of the experiment the amount of iodine was slightly higher for the bandages with PVP, but the increase of the efficacy was expected on 3rd and 4th day, not on 5th and 6th. Moreover, the addition of PVP also caused the decrease of the total amount of released iodine (figure 26). Moreover, there was checked the influence of the amount of Kollidon on the kinetics of iodine release from the covering. There were used following variants: 5, 10 and 15 mg of Kollidon per 1 cm^2 of the bandage. It was observed again that the more Kollidon, the lower amount of released iodine, mainly during first 60 hours of treatment. The increase of 5 mg of Kollidon caused a 15% loss in the amount of released iodine (figure 27). The inhibition of iodine release during first two days in dressing with PVP caused the deterioration of anti-biofilm properties (table 5). Due to the fact that PVP increased the efficacy of iodine release after nearly three days (instead of one day) there was no sense of incorporating it to the final dressing. No addition of PVP will make the process of manufacturing easier and will not unnecessarily raise the price of the wound covering with Iodine Generator.

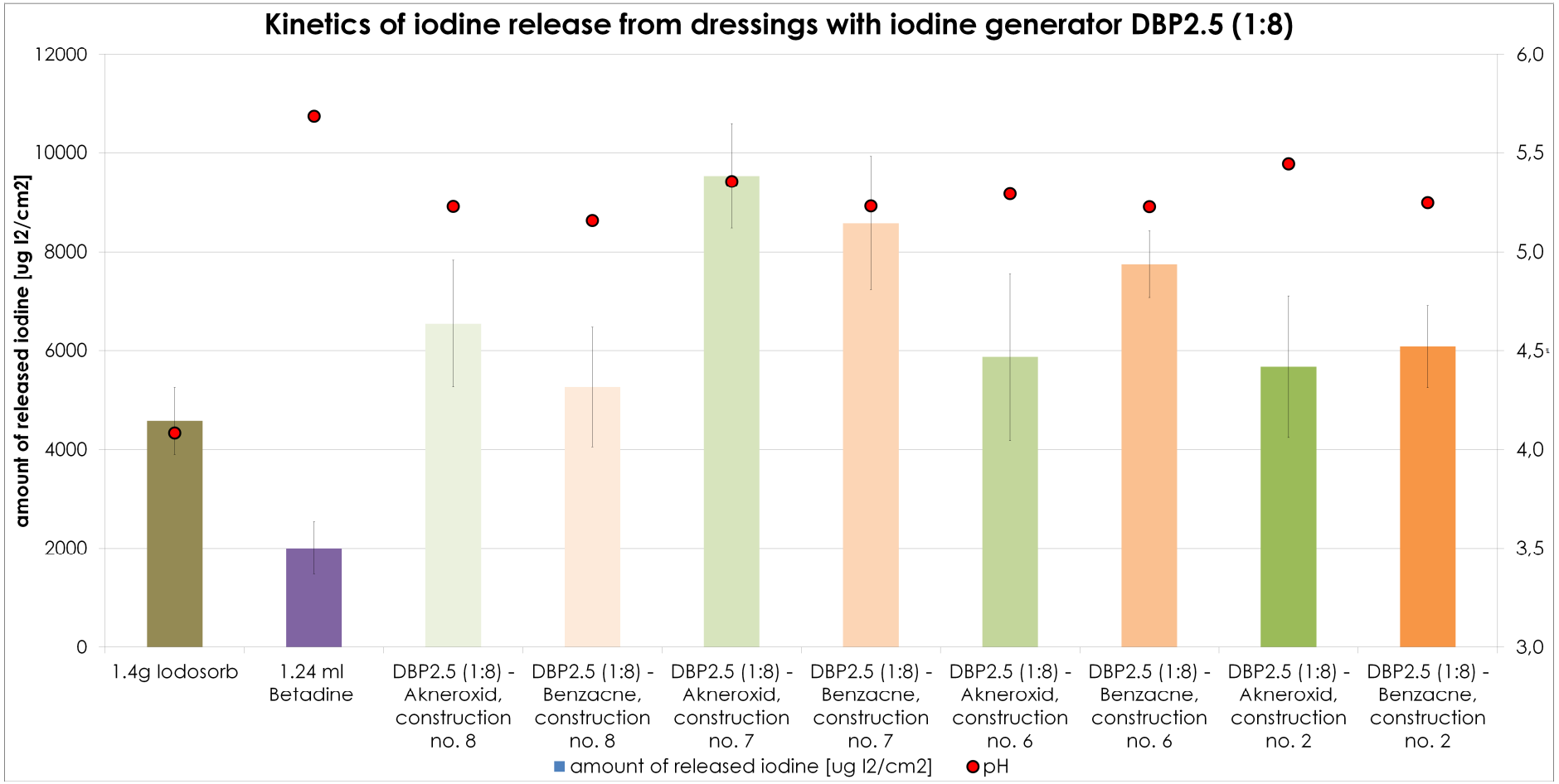


Graph 27. Kinetics of iodine release from the dressings with I_2G – DBP2 (1:9) saturated with 5% Akneroxid Gel. Dressings with construction no. 5; contain 5, 10, 15 mg/cm^2.

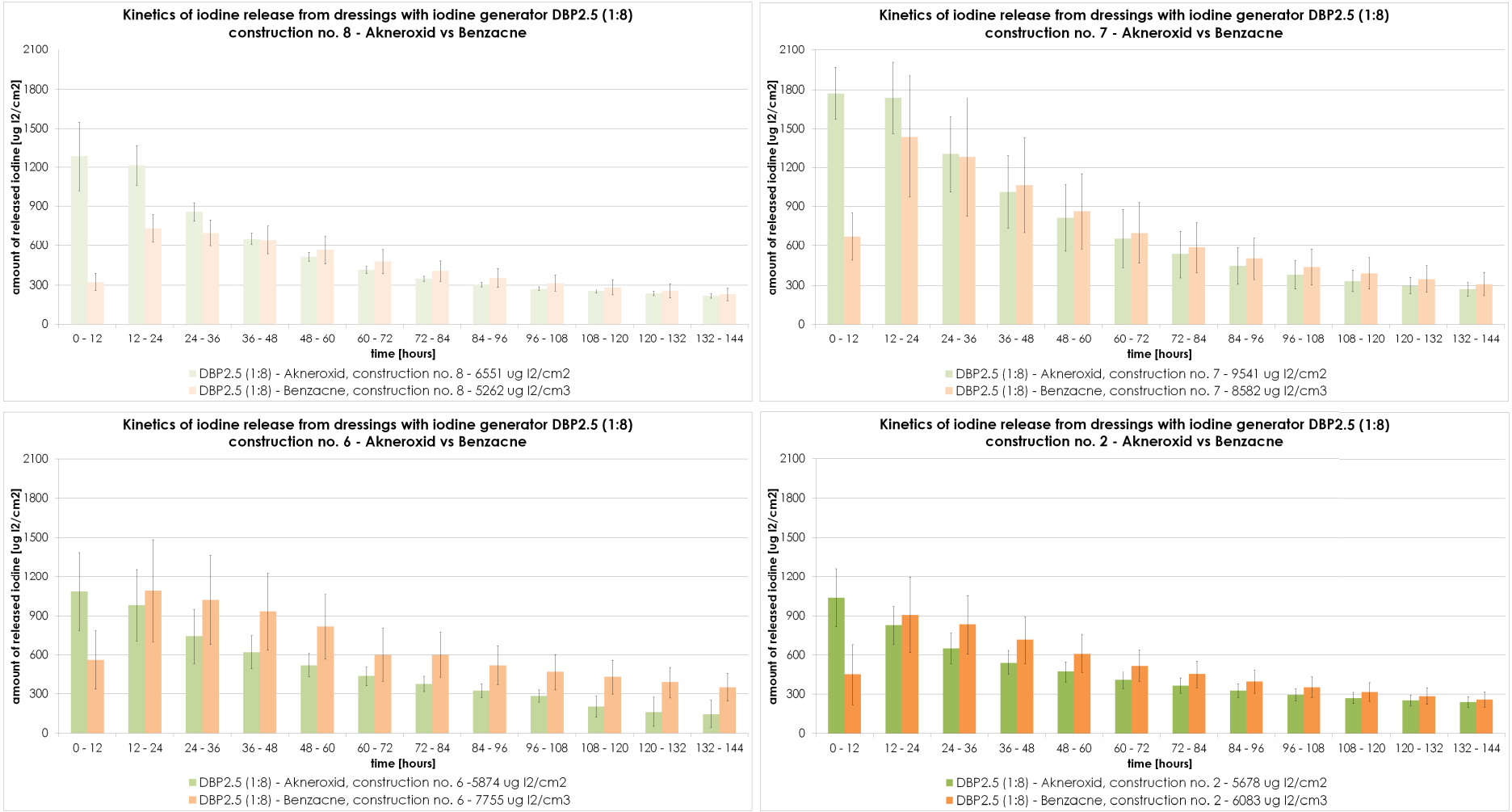
3.4.2 DBP2.5 (1:8) (KI: 69 mg/cm^2, EVA: 7 mg/cm^2, DBP: 6.25 mg/cm^2)

The similar observations as in the previous variant were done for the DBP2.5 (1:8) containing more KI and DBP. This variant of the dressing was prepared and tested to have the alternative for the weaker dressing that was not fully efficient in biofilm eradication (it turned out when the second *in vitro* model with biofilm containing 5 bacteria strains was used).

The pH values for dressings with Benzacne were slightly lower (0.2 pH) than in dressings with Akneroxid (graph 28). Mainly during first 12 hours the release of iodine from the dressings with Benzacne was three or four-times lower in comparison to Akneroxid, what meant problems with biofilm eradication. There was not visible the difference in the kinetics of iodine release for four tested constructions (2, 6, 7 and 8), in exception of construction no. 7 that released the highest amounts that may be too high for the clinical use, causing the skin irritation. (graph 29).



Graph 28. Total release of iodine from the dressings with I_2G DBP2.5 (1:8) after 6 days of experiment and the pH values of bandages (measured on their contact layers).



Graph 29. Comparison of the kinetics of iodine release from four constructions of the bandages DBP2.5 (1:8) saturated with 5% Akneroxid Gel or 5% Benzacne Gel.

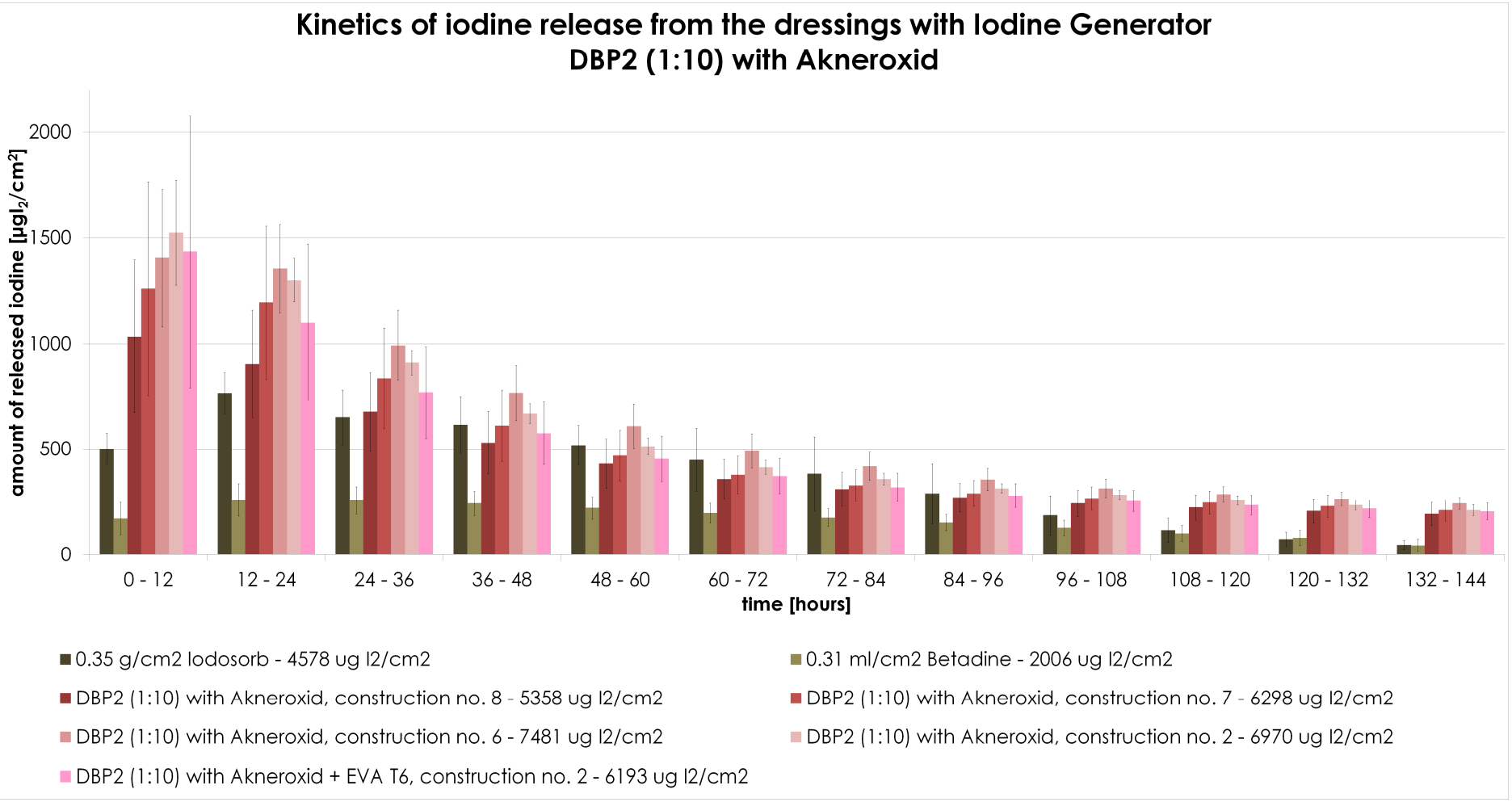
3.4.3 DBP2 (1:10) (KI: 69 mg/cm^2, EVA: 7 mg/cm^2, DBP: 5 mg/cm^2)

Finally, the third variant of the I_2G with 69 mg of potassium iodide per cm^2 and 5 mg of dibenzoyl peroxide per 1 cm^2, being sufficiently effective in biofilm eradication (table 5, graphs: 54 - 56), was also analysed with the use of DRP sensors.

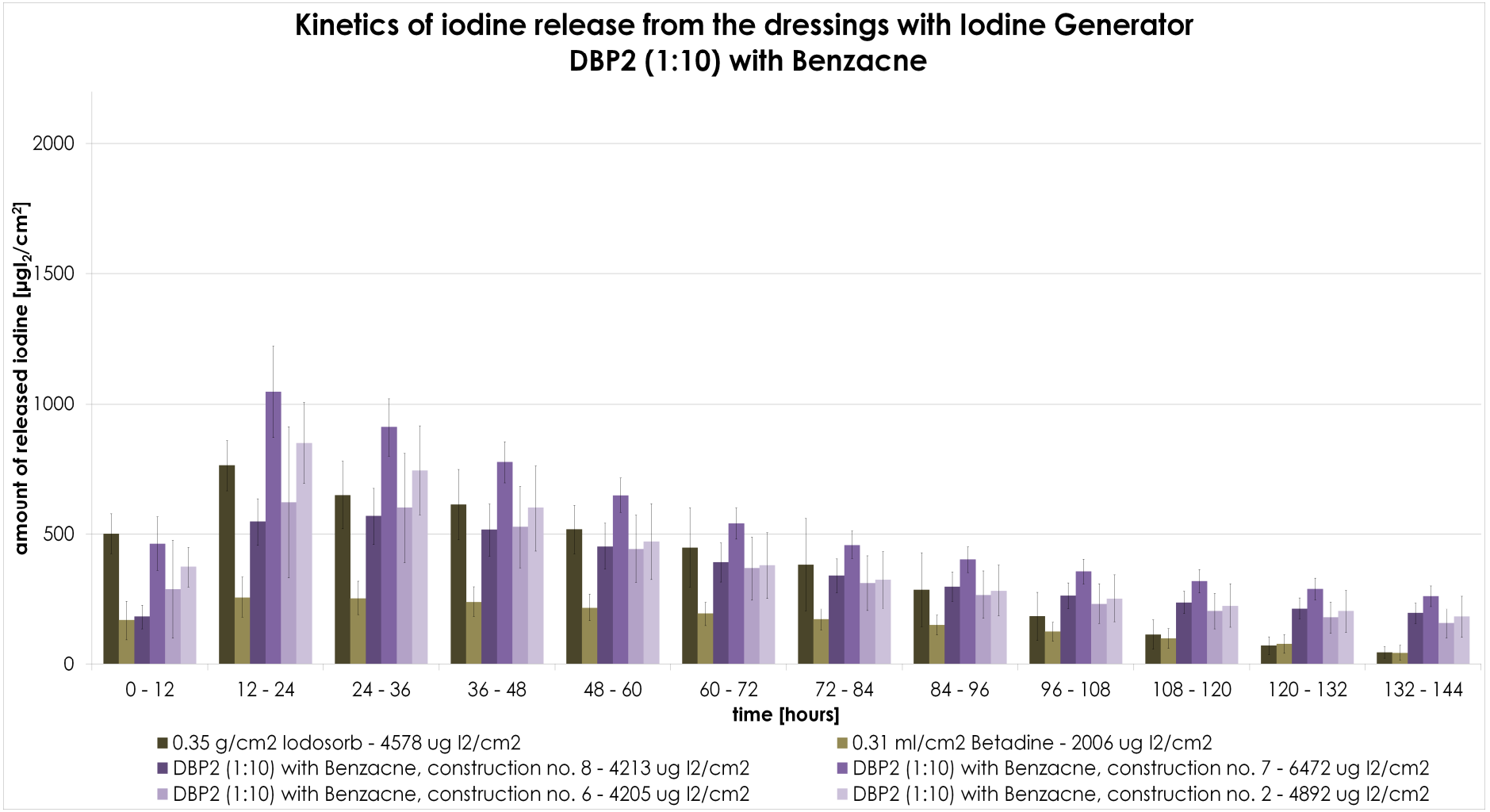
Figures 30 and 31 show the kinetics of iodine release from four different constructions of bandages saturated with Akneroxid or Benzacne but in comparison to Betadine (PVP-I_2) and Iodosorb (cadexomer-iodine). Iodosorb and the dressings with Benzacne presented the similar kinetics of iodine release. However, it was shown during the *in vitro* tests on biofilm that the Iodine Generator is more effective in biofilm eradication.

The next two mentioned graphs (graph 32 and graph 33) proved the differences in the kinetics when different amounts of KI or DBP were used. For instance, the use of the same amount of DBP in the dressing but with the higher amount of KI (62 and 69 mg/cm^2) only slightly increased the amount of released iodine in all periods of time but ensured that the whole mass of BDP reacted (graph 32). This growth allowed the chosen wound covering (DBP2 (1:10)) to be totally effective in *in vitro* biofilm eradication (table 5). When the same amount of KI in the coverings and different amounts of DBP (5 mg/cm^2 in DBP2 (1:10) and 6.25 mg/cm^2 in DBP2,5 (1:8)) were used, the slight increase of iodine release in case of the dressing containing more DBP was observed (graph 33).

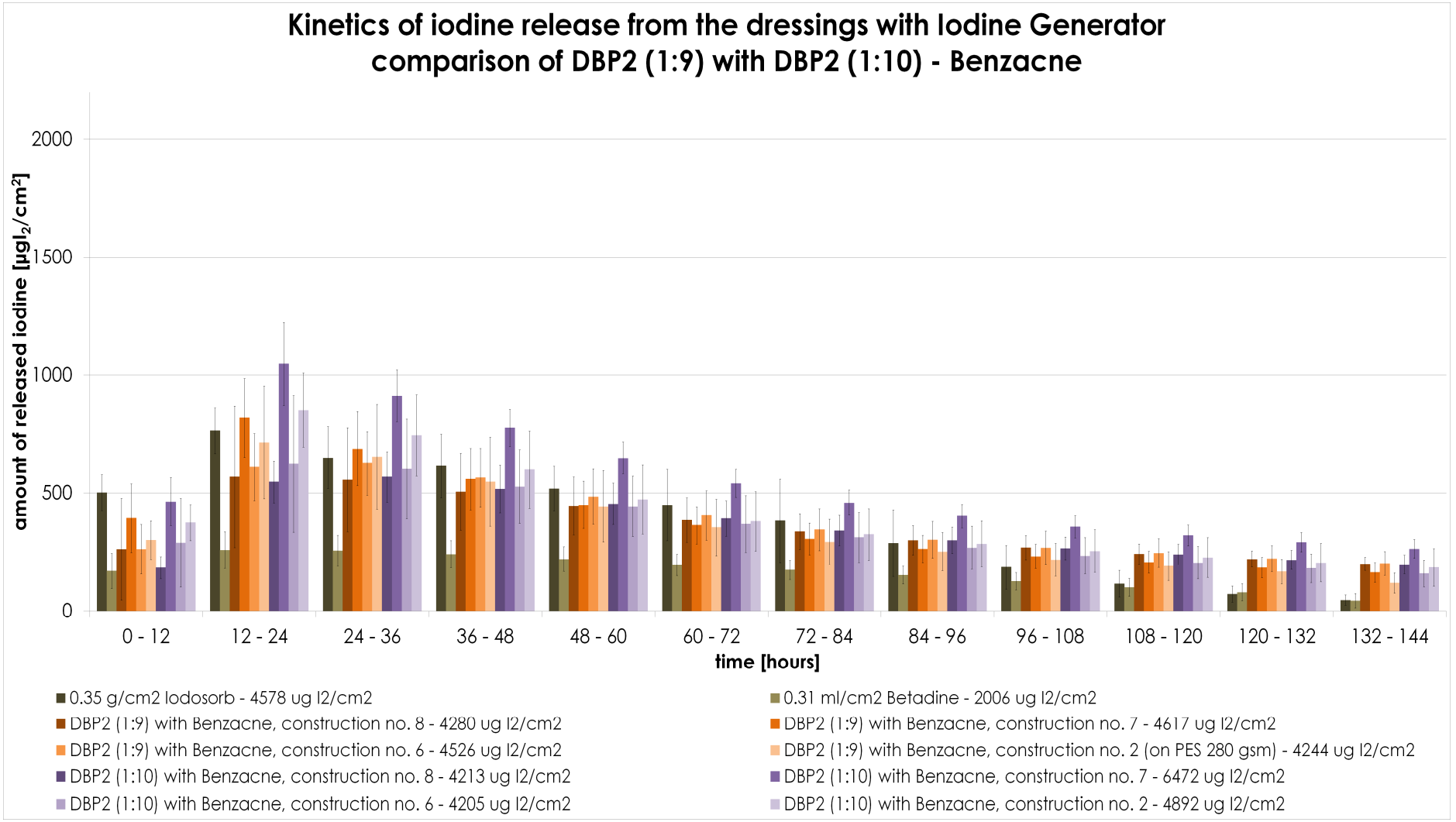
It is important to mention that all samples tested with the use of electrodes were saturated with buffered peptone water. It gave the opportunity to observe their absorbing properties that definitely influenced on the prototype choice. Constructions 6 and 8 had really bad absorbing properties, both of them hardly absorbed the mentioned solution. On the other hand, construction no. 2 with the use of 280 g/m^2 polyester seemed to be a really good absorbent for wound exudates because easily absorbed buffered water. Moreover, construction no. 2 possesses the easiest form to manufacture. Both layers are identical and there is no need of using the second step of lamination process.



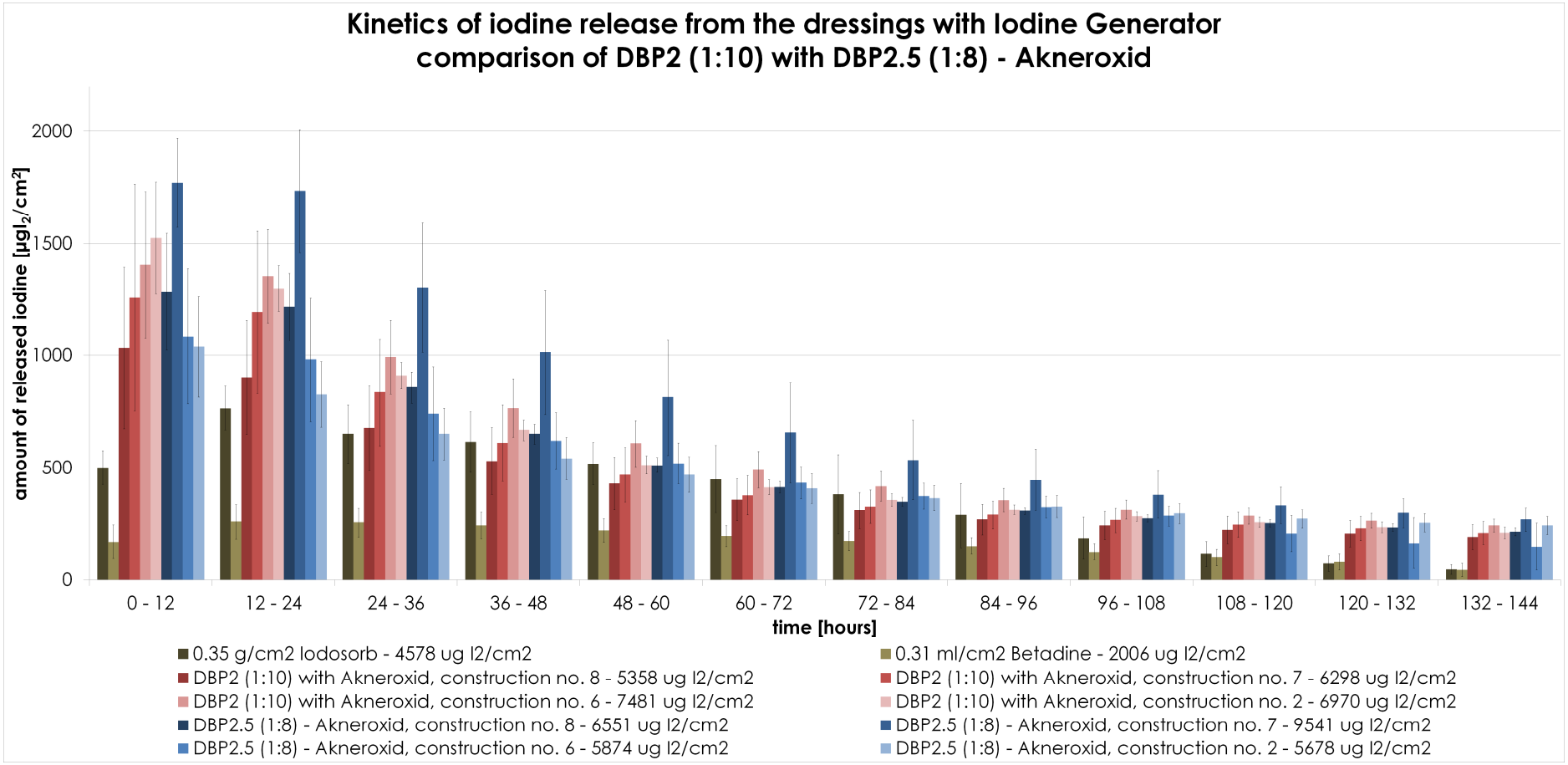
Graph 30. Kinetics of iodine release from Iodosorb, Betadine and dressings with I_2G: DBP2 (1:10) saturated with 5% Akneroxid Gel and having 4 different constructions.



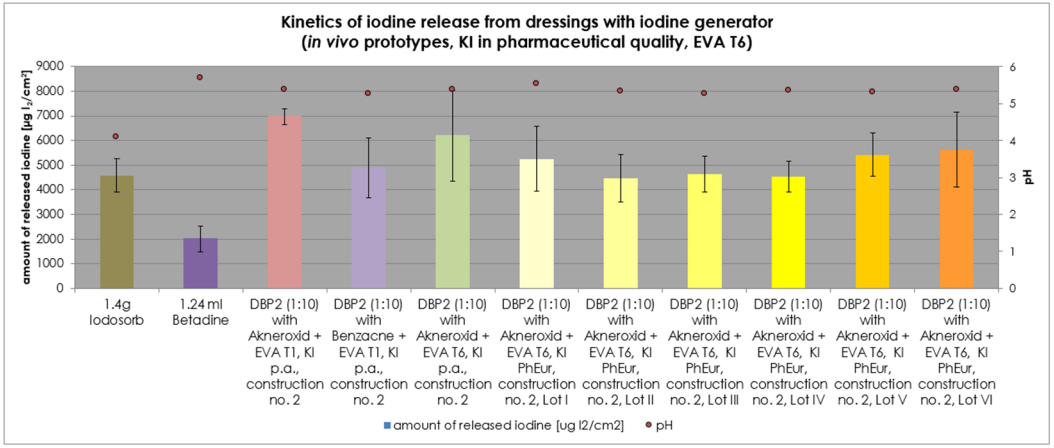
Graph 31. Kinetics of iodine release from Iodosorb, Betadine and dressings with I_2G: DBP2 (1:10) saturated with 5% Benzacne Gel and having 4 different constructions.



Graph 32. Comparison of the kinetics of iodine release from dressings: DBP 2 (1:9) and DBP 2 (1:10).



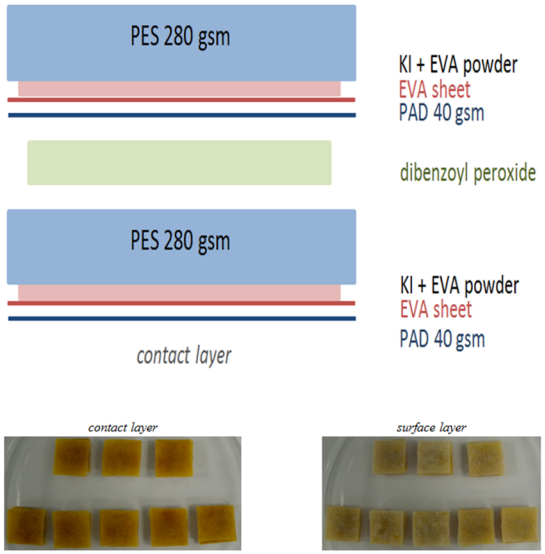
Graph 33. Comparison of the kinetics of iodine release from dressings: DBP 2 (1:10) and DBP 2.5 (1:8).

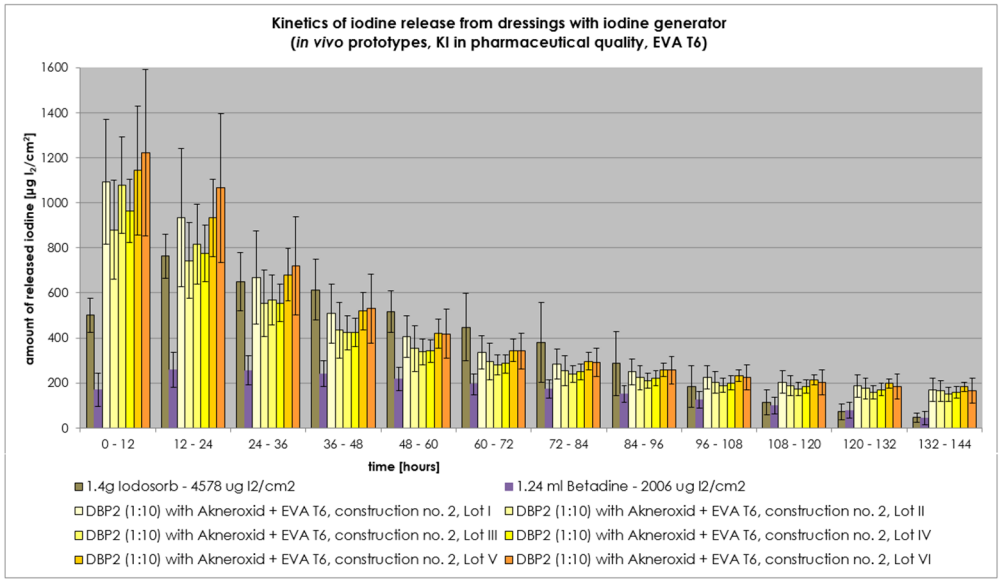


Potassium iodide: **690 g/m²**

Ethylene vinyl acetate (glue, T6): **70 g/m²**

5% Akneroxid Gel: **1000 g/m²**
(dibenzoyl peroxide: **50 g/m²)**





Graph 34. Data concerning the chosen prototype: DBP2 (1:10) saturated with 5% Akneroxid Gel; photos represent the look of dressings after 6 days of treatment.

Finally, construction no. 2 was chosen as a prototype for the *in vivo* study. Therefore there were prepared six batches of the dressings that were directed for the kinetics tests (graph 34). Potassium iodide used in finally prepared dressings was changed from a quality for analysis to a pharmaceutical quality (Ph.Eur. from Penta Chemicals, CZ). In parallel, the efficacy of the biofilm eradication of five batches of the same kind of dressings was checked, too (graphs 56). The chosen prototype of the Iodine Generator released iodine with a kinetics that allowed to eradicate *in vitro* biofilm during first day of treatment and then prevent from its reappearance

3.5 BIOFILM INHIBITION AND ERADICATION – PERFORMED EXPERIMENTS

The following chapter presents data from the *in vitro* study of the wound dressings with Iodine Generator performed on the modified Lubbock biofilm model. First part of the experiments was done with the blend of four bacteria strains (*P. aeruginosa, S. aureus, E. faecalis* and *B. subtilis*) and the second part with the subsequently modified model with five bacteria strains such as *P. aeruginosa, S. aureus, E. faecalis, E. coli* and *S. agalactiae*.

The list of the dressings, both saturated with the solution of KI and laminated with KI powder, that were tested in this work is presented in the tables below (tables 4 and 5). In the tables there are also presented the generalized information concerning the effectivity of wound coverings with iodine on the biofilm inhibition and eradication. A symbolic designation of viable bacteria colonies after treatment with wound dressings was adopted as follows:

+++ (almost) no eradication of biofilm
++ slight eradication of biofilm (10-16 log CFU growth after treatment)
+ medium eradication of biofilm (3-10 log CFU growth after treatment)
+/- strong eradication of biofilm (1-3 log CFU growth after treatment)
.- total eradication of biofilm (max. 1 log CFU growth after treatment).

Table 4. The list of dressings with Iodine Generator tested on the first *in vitro* Lubbock biofilm model (with four bacterial strains).

Sample (2cm x 5 cm) 10 cm^2	KI (mg)	5% Akneroxid (ml)	PVP/Kollidon (mg)	A symbolic designation of viable bacteria		Number of test
				after 24 hours	after 48 hours	
Negative control	-			+++	+++	1 - 15
Betadine (PVP-iodine) – solution (3,1 ml)	-			-	+/-	12
Betadine (PVP-iodine) – ointment (3,1 ml)	-			-	+	11, 14, 15
Iodosorb – gel (3,5 ml)	-			+/-	+/-	e.g. 3, 5, 6
Inadine	-			+++	+++	14
Saturation: DBP 6 (1:3) + PVP K30	617	1.5 (10%)*	180 (90 + 90)	-	-	3
Saturation: DBP 3 (1:6)	617	0.75 (10%)*	-	-	-	8
Saturation: DBP 3 (1:6) + PVP K30	617	1.5	180 (90 + 90)	-	-	4
Saturation: DBP 3 (1:6)	617	1.5	-	-	-	4, 6-10
Saturation: DBP 3 (1:5)	515	1.5	-	+/-	-	10
Saturation: DBP 3 (1:4)	412	1.5	-	+/-	+	9
Saturation: DBP 3 (1:3)	309	1.5	-	+	+/-	6
Saturation: DBP 2.5 (1:7)	600	1.25	-	+	-	12
Lamination: DBP 2.5 (1:7)	600	1.25	-	+/-	-	12
Lamination: DBP 2.5 (1:7) + Kollidon	600	1.25	150 (covering layer)	-	-	15
Lamination: DBP 2.5 (1:6)	512	1.25	-	-	-	15
Lamination: DBP 2.5 (1:6) + Kollidon	512	1.25	150 (covering layer)	-	-	15
Saturation: DBP 2 (1:25)	1713	2 (2.5%) JM*	-	-	+/-	4
Saturation: DBP 2 (1:9)	617	1	-	+/-	-	7
Lamination: DBP 2 (1:9)	617	1	-	-	+/-	14
Lamination: DBP 2 (1:9) + Kollidon	617	1	150 (covering layer)	+/-	-	14
Saturation: DBP 2 (1:8)	550	1	-	-	-	10
Lamination: DBP 2 (1:8)	550	1	-	-	-	13
Lamination: DBP 2 (1:8) + PVP K30	550	1	200 (covering layer)	+/-	-	13
Saturation: DBP 2 (1:7)	480	1	-	+	-	10
Lamination: DBP 2 (1:7)	480	1	-	+/-	+/-	13
Lamination: DBP 2 (1:7) + PVP K30	480	1	200 (covering layer)	+/-	-	13
Saturation: DBP 2 (1:6)	412	1	-	+/-	+	9
Saturation: DBP 1.5 (1:20)	1028	1.5 (2.5%) JM*	-	+	+	3
Saturation: DBP 1 (1:18)	247	0.5	-	+	+	11
Saturation: DBP 1 (1:14)	193	0.5	-	++	++	11

(10%) - there was used 10% Akneroxid Gel, JM* - there was used 2.5% Jan Marini Wash*

Table 5. The list of laminated dressings with Iodine Generator tested on the modified *in vitro* Lubbock biofilm model (with five bacterial strains).

Sample (2cm x 5 cm) 10 cm^2	Number of construction	KI [mg]	5% Akneroxid [ml]	5% Benzacne [ml]	Kollidon [mg/]	A symbolic designation of viable bacteria			Number of test
						after 24 h	after 48h	after 72h	
Negative control			-			+++	+++	+++	1-14
0.9% Iodosorb (3.5 ml)			-			+/-	+/-	+	1-14
(1% I2) Betadine ointment (3.1 ml)			-			+/-	+	++	1, 3
(1% I2) Betadine solution (3.1 ml)			-			+/-	+	++	2, 4-14
(0,1% I2) Betadine solution (3.1ml)			-			+++	+++	+++	12
DBP2 (1:8) - *Akneroxid*	2	550	1	-	-	-	+	no data	2
DBP2 (1:9) - *Akneroxid*	2	620	1	-	-	+/-	-	no data	1
DBP2 (1:9) - *Akneroxid*	2	620	1	-	-	-	-	+	5
DBP2 (1:9) - *Akneroxid*	2	620	1	-	-	+	+/-	+	8
DBP2 (1:9) - *Benzacne*	2	620	-	1	-	+++	++	+	6
DBP2 (1:9) - *Akneroxid*	6	620	1	-	-	+	+/-	-	8
DBP2 (1:9) - *Benzacne*	6	620	-	1	-	++	+	+	8
DBP2 (1:9) - *Akneroxid*	7	620	1	-	-	+	+	+	9
DBP2 (1:9) + Kollidon (5) *Akneroxid*	4	620	1	-	50	+	+	+	4
DBP2 (1:9) + Kollidon (10) *Akneroxid*	6	620	1	-	100	+	+/-	+	6
DBP2 (1:9) + Kollidon (10) *Benzacne*	6	620	-	1	100	+/-	+	+	6
DBP2 (1:9) + Kollidon (15) *Akneroxid*	2	620	1	-	150	-	+/-	no data	2
DBP2 (1:9) + Kollidon (15) *Akneroxid*	3	620	1	-	150	++	++	++	3
DBP2 (1:9) + Kollidon (15) *Akneroxid*	4	620	1	-	150	+	-	+	4
DBP2 (1:10) - *Akneroxid*	2	690	1	-	-	-	-	+/-	5
DBP2 (1:10) - *Akneroxid*	2	690	1	-	-	-	+/-	-	10
DBP2 (1:10) - *Akneroxid*	**2**	**690**	**1**	**-**	**-**	**-**	**-**	**-**	**11 - 14**
DBP2 (1:10) - *Benzacne*	2	690	-	1	-	+	-	+	12
DBP2 (1:10) - *Akneroxid*	6	690	1	-	-	-	-	-	11
DBP2 (1:10) - *Akneroxid*	7	690	1	-	-	-	-	-	10, 11
DBP2 (1:10) - *Akneroxid*	8	690	1	-	-	+	-	+/-	11
DBP2 (1:10) + Kollidon (10) *Akneroxid*	5	690	1	-	100	+	+/-	+/-	5
DBP2 (1:10) + Kollidon (10) *Akneroxid*	6	690	1	-	100	++	+	+	7
DBP2 (1:10) + Kollidon (10) *Benzacne*	6	690	-	1.25	100	-	-	-	10
DBP2.5 (1:6) + Kollidon (15) *Akneroxid*	2	512	1.25	-	150	+/-	+/-	no data	2
DBP2.5 (1:7) - *Akneroxid*	2	600	1.25	-	-	+/-	+	+/-	7
DBP2.5 (1:7) + Kollidon (10) *Akneroxid*	6	600	1.25	-	100	+	+/-	-	7
DBP2.5 (1:8) - *Akneroxid*	7	690	1.25	-	-	+/-	-	-	9
DBP2,5 (1:8) - *Akneroxid*	2	690	1,25	-	-	-	-	-	14
DBP2.5 (1:8) - *Benzacne*	7	690	-	1.25	-	+	+/-	-	9
DBP2.5 (1:8) - *Benzacne*	2	690	-	1.25	-	+/-	+/-	+/-	12
DBP3 (1:6) - *Akneroxid*	2	620	1.5	-	-	-	-	no data	1

3.5.1 First model of the *in vitro* Lubbock biofilm (4 bacterial strains, 48 hours)

3.5.1.1 Commercially available products containing iodine

The first experiments performed in this part of work were done to choose the appropriate concentrations of the commercially available products with iodine to play a role of a positive control in the *in vitro* Lubbock biofilm model. Iodosorb from Smith&Nephew (cadexomer-iodine gel) was used as a positive control in the further experiments. Iodosorb was mainly used in a concentration of 0.9% in the following doses: 2 ml (~18 mg I_2/cm^2), 3.5 ml (~32 mg I_2/cm^2) and 4 ml (~36 mg I_2/cm^2) per 10 cm^2. Due to the fact that these were the biological experiments, it was hard to get the repeatable results for the same concentration of Iodosorb, but finally 3,5 ml of this formulation was subsequently used as a positive control.

In this first model of Lubbock biofilm there were used four bacteria strains: *P. aeruginosa, S. aureus, E. faecalis* and *B. subtilis*. However, in most of experiments there was no statistically significant difference in *Bacillus subtilis* growth and eradication between the dressings and the control samples. So, there was no sense to take *B. subtilis* into consideration while comparing data. It seemed that *B. subtilis* only played a role of a contaminant in the biofilm.

The initial test concerned the use of the same concentrations of iodine in commercial products. Alfadin (PVP-iodine) and Iodosorb were diluted with buffered peptone water. Both of them, having 0.1% concentration of iodine (what is clinically relevant for Alfadin), showed almost no biofilm eradication. Only Alfadin caused a statistically evident 1log CFU inhibition ($p<0.05$) in case of *S. aureus* and Iodosorb - in case of *E. faecalis* after 48 hours of treatment. The other commercial product containing iodine that was subsequently tested was Betadine (PVP-iodine) in a form of solution and ointment (1% of iodine). In case of this product there was a statistically proved inhibitory effect on the biofilm growth when compared to the negative control. However, when used in a diluted form (0.1% iodine, clinically used for a long-time treatment), there was not visible any significant difference to the negative control (graph 42). Betadine in a form of solution was more effective in biofilm inhibition (total eradication after 24 hours and 3 log CFU regrowth of *P. aeruginosa* after 48 hours) than Betadine in a form of ointment. The gel formulation showed at least 4 log CFU inhibition after 24 hours of treatment but after 48 hours the load of bacteria increased by 3 log CFU for *P. aeruginosa* and 0.5-3 log CFU for *E. faecalis* (graphs 41 and 45). Due to the fact that the base of the ointment formulation was macrogol, this compound was also tested

on the *in vitro* model. It was proved by getting no statistically significant difference between the results from macrogol alone and the negative control sample that the base of Betadine had no influence on its antimicrobial properties (graph 41).

Also Inadine (a sheet of the viscose impregnated with 10% PVP-iodine) was tested and did not show any biofilm eradication properties after 24 hours of the treatment, but there was 1log CFU of *S. aureus* inhibition and almost 2 log CFU inhibition of *E. faecalis*. (graph 44) It was proved that this formulation is not able to fight with the wound biofilm.

Presented results concerning the kinetics of iodine release from the commercially available compositions coincide with data obtained from both *in vitro* models of biofilm. In case of PVP-iodine the high amount of iodine released during first few hours and then decreased quite drastically (graph 33) what was observed in case of tests on biofilm where the bacteria begun to regrowth in the second and especially third day of treatment (graph 56). When Iodosorb was used, the kinetics of iodine release was comparable to Iodine Generator (sample directed for *in vivo* study on minipigs) that means that the kinetics was steady and the amount of iodine reduced slowly over time (graph 33). These properties occurred simultaneously during performing the *in vitro* study with biofilm: all bacteria strains were eradicated during first day and in most of repetitions no bacterial growth was observed during next two days of the study.

3.5.1.2 "Saturated" bandages with Iodine Generator

Bandages impregnated with the solution of potassium iodide were wetted with the gels containing dibenzoyl peroxide just before the application on the biofilm's model. 2.5% Jan Marini Wash and 10% Akneroxid were used only in few experiments. Initially, it was suggested to get the dressing with the possibly low concentration of DBP (mainly due to lower costs of dressings and the assumption of having the iodine concentration based on the clinically relevant concentrations of PVP-iodine). But Jan Marini Wash, which contains 2.5% of DBP, required the presence of high amounts of potassium iodide (> 1.5 g/10 cm^2) in the bandage to be effective in biofilm eradication (graph 35). Moreover, the application of more than 1.5 ml of the gel between the layers of the double-wings bandage was impossible due to the fact of its leaking outside the bandage's edges. It made these variants useless for the *in vivo* application. On the other hand, the application of Akneroxid containing 10% of DBP seemed to be strong enough to eradicate biofilm. In case of the dressing presented in the graph no. 35 (DBP6 (1:3) that is 617 mg KI, 1.5 ml of 10% Akneroxid) the concentration of produced iodine was firmly too high while in case of the dressing DBP3 (1:6) containing 617 mg KI

and 1.5 ml of 5% Akneroxid (graph 38), the iodine concentration seemed to give the highest possible concentration of iodine that can be used. It is worth mentioning that the amount of 0,75 ml of the Akneroxid Gel (10% DBP) was not sufficient for the equal distribution of that formulation on the whole area of the dressing.

The rest of the experiments were done with the use of 5% Akneroxid Gel. That is why it was also checked if Akneroxid itself influences on the biofilm inhibition. (graph 36). It was showed that 1.5 ml of Akneroxid gel (the same amount as in DBP3) caused the statistically significant inhibition 0.5 log CFU of *S. aureus* (24 hours) and *E. faecalis* (48 hours) in comparison to the negative control ($p<0.05$). Simultaneously, it was proved that there is a statistically significant difference in biofilm eradication between Akneroxid itself and the whole bandage (DBP 3 (1:6)) which completely reduced the population of biofilm bacteria for up to 48 hours. Moreover there was no significant difference between the dressing with Iodine Generator and the positive control - Iodosorb (4 ml).

Subsequently, there were tested dressings with the reduced amounts of potassium iodide. Bandage DBP 3 (1:5) containing 515 mg KI/10 cm^2 (graph 40) completely eradicated biofilm after 48 hours of treatment, but bandages with 412 mg (graph 39, DBP 3 (1:4)) and 309 mg KI/10 cm^2 (graph 37, DBP3 (1:3)) only inhibited the growth of bacteria (not statistically significant).

Due to the fact that it is required to have no excess of DBP in the dressing after the treatment, it was indicated to use at least 3 times more KI than results from the molecular basis (when looking at the chosen names of the dressings with Iodine Generator, the ratio of DBP to KI is presented in the brackets). That is why the bandages DBP3 (3 means 30 mg/4 cm^2) with lower than mentioned amounts of KI were not taken into consideration in further tests.

Therefore, lower quantities of dibenzoyl peroxide were used to find possibly the weakest dressing that was able to eradicate all bacteria populations during 48 hours of treatment. For these tests the amount of 5% Akneroxid was reduced to 1.25 ml and 1 ml. Bandage containing 1 ml of the gel (that is ~ 5 mg DBP/cm^2 of the dressing) was the most suitable option from the application's point of view (equal distribution on the whole area of the dressing). There were performed the experiments with 1 ml of 5% Akneroxid and with the dressings containing the following amounts of KI:

- 617 mg/ 10 cm^2 (DBP2 (1:9), graph 44),
- 550 mg/ 10 cm^2 (DBP2 (1:8), graph 40),
- 480 mg/ 10 cm^2 (DBP2 (1:7), graph 40),

- 412 mg/ 10 cm^2 (DBP2 (1:6), graph 39).

All of these dressing, except of DBP2 (1:6) were significantly effective in biofilm eradication up to 48 hours of treatment. Dressing DBP2 (1:7) had still 4 log CFU of viable *E. faecalis* 24 hours after the application. Therefore, dressing DBP2 (1:8) could be recognized as the prototype of "saturated" dressing directed for tests on the *in vivo* model.

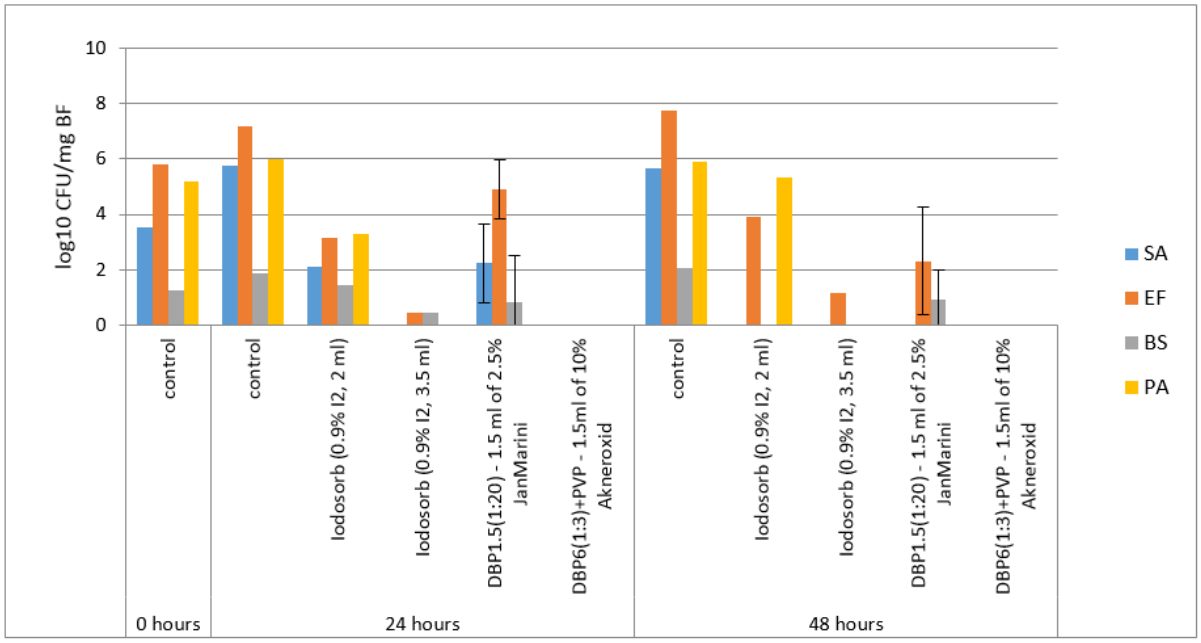
3.5.1.3 Laminated bandages with Iodine Generator

The second tested group was the group of laminated bandages. From the first experiment with their use, when comparing with "saturated" bandages, there was shown no significant difference between Betadine (1%, solution) and tested bandages (DBP2.5 (1:7)). The only exception was the slight growth of *E. faecalis* and *P. aeruginosa* after 24 hours of treatment with the saturated bandage. But, finally all bandages eradicated biofilm after 48 hours of treatment (graph 42). The total eradication of biofilm was repeated again in the other experiment (graph 45) when the samples containing smaller amount of KI (100 mg less) were tested. All of them showed a 0.5 log CFU (24 hours) and 3 log CFU (48 hours) of *P. aeruginosa* difference in comparison to Betadine (1%, ointment).

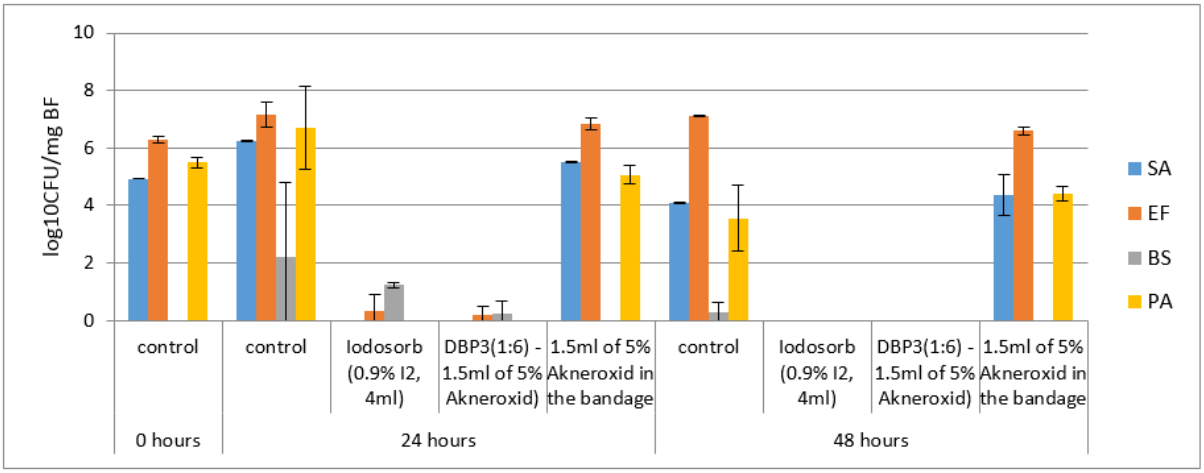
In the graphs no 43 and 44 there are presented results from the treatment of biofilm with the use of the dressings containing 1 ml of 5% Akneroxid (DBP 2). There were compared samples without and with the addition of PVP/Kollidon. There was not visible a significant difference between the chosen samples. The slight inhibition (but not statistically significant) of the efficacy of biofilm eradication was seen in case of the dressings containing PVP.

Finally, the research on the first Lubbock biofilm model moved to the conclusion that the dressing DBP2(1:9) seemed to be sufficiently effective dressing in the viable cell eradication.

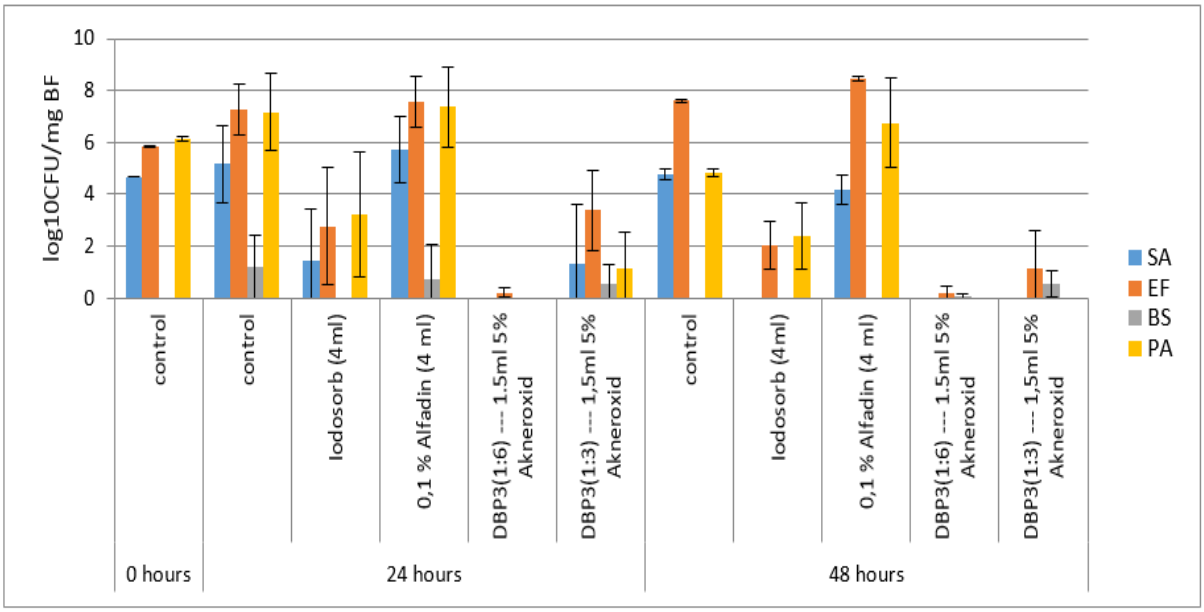
3.5.1.4 Graphical presentation of the results



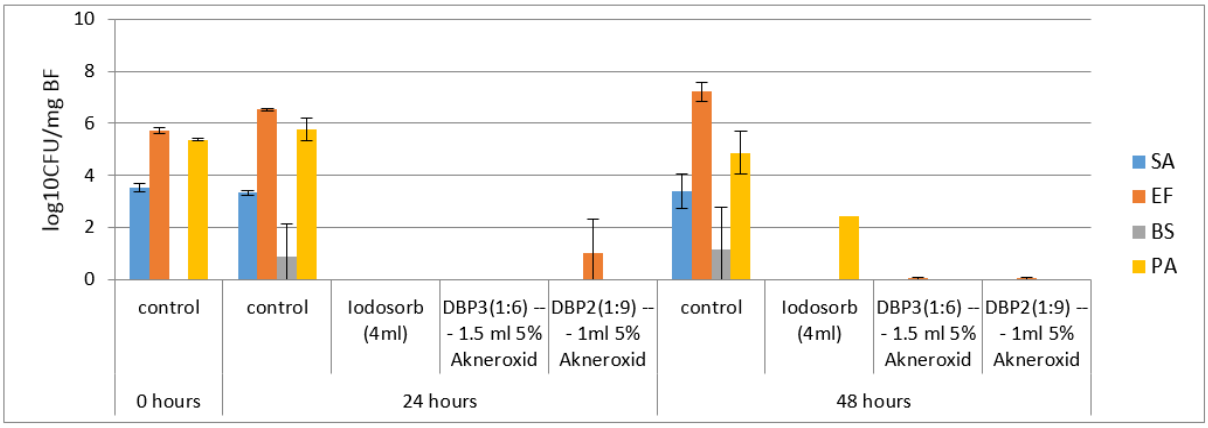
Graph 35. The bacterial population counts 24 and 48 hours after the application of the bandages DBP6(1:3) with PVP K30 (10% Akneroxid), DBP1.5(1:20) with JanMarini Wash (2.5% dibenzoyl peroxide) and Iodosorb as a positive control. Test BF-3.



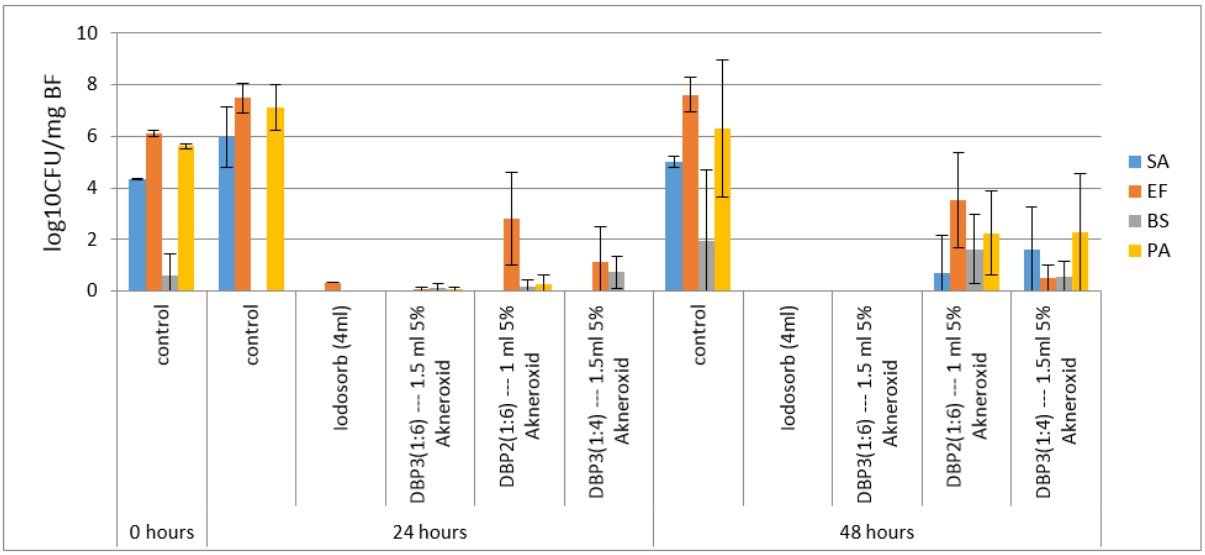
Graph 36. The bacterial population counts 24 and 48 hours after the application of the bandages DBP3(1:6) and Akneroxid (in the same amount as in DBP3). Test BF-5.



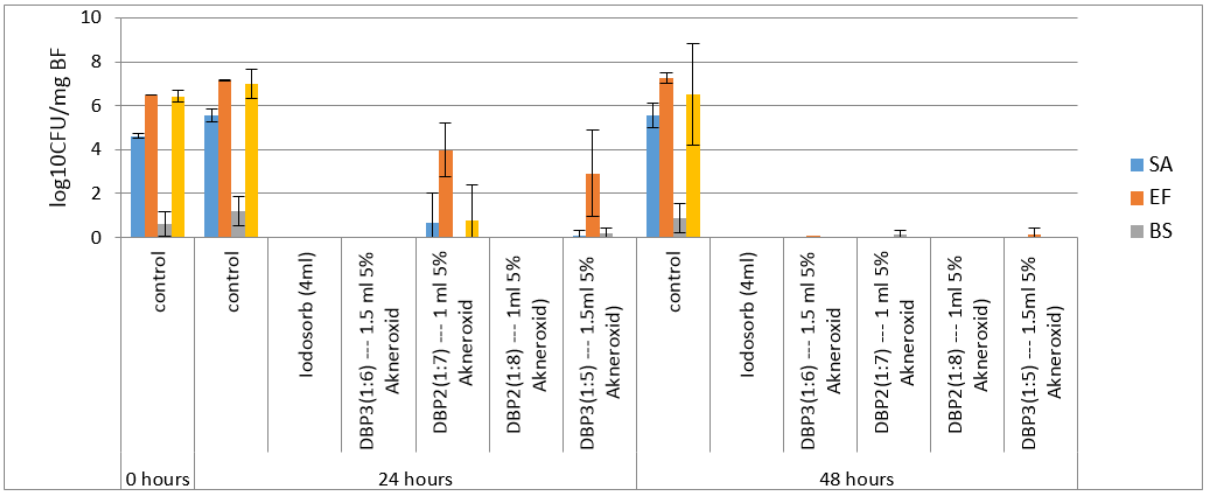
Graph 37. The bacterial population counts 24 and 48 hours after the application of the bandages DBP3(1:6), DBP3(1:3) and Alfadin (0.1% iodine). Test BF-6.



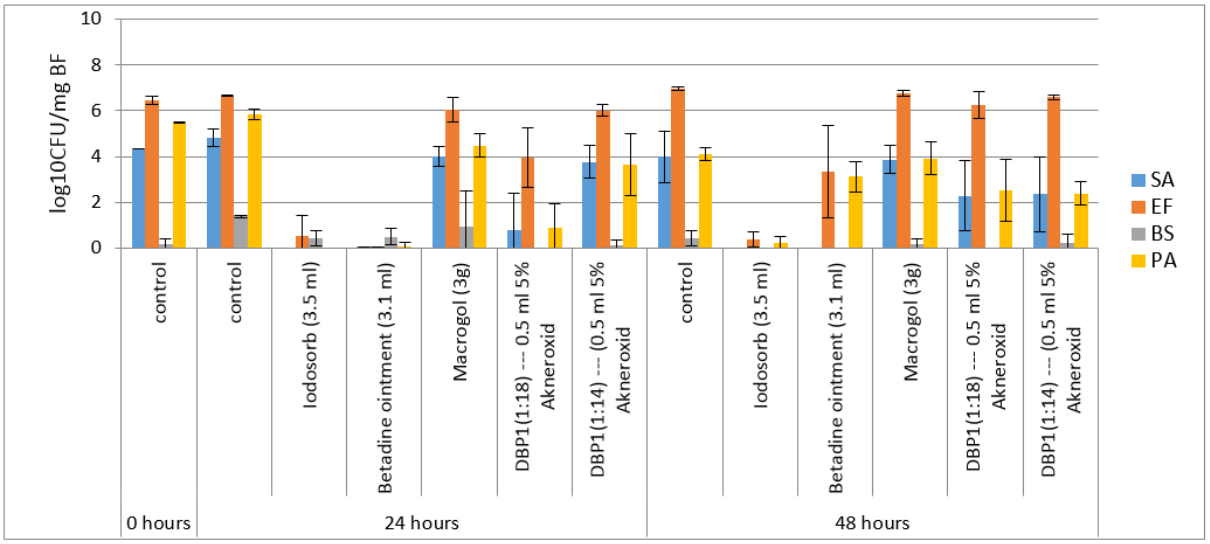
Graph 38. The bacterial population counts 24 and 48 hours after the application of the bandages DBP3(1:6) and DBP2(1:9). Test BF-7.



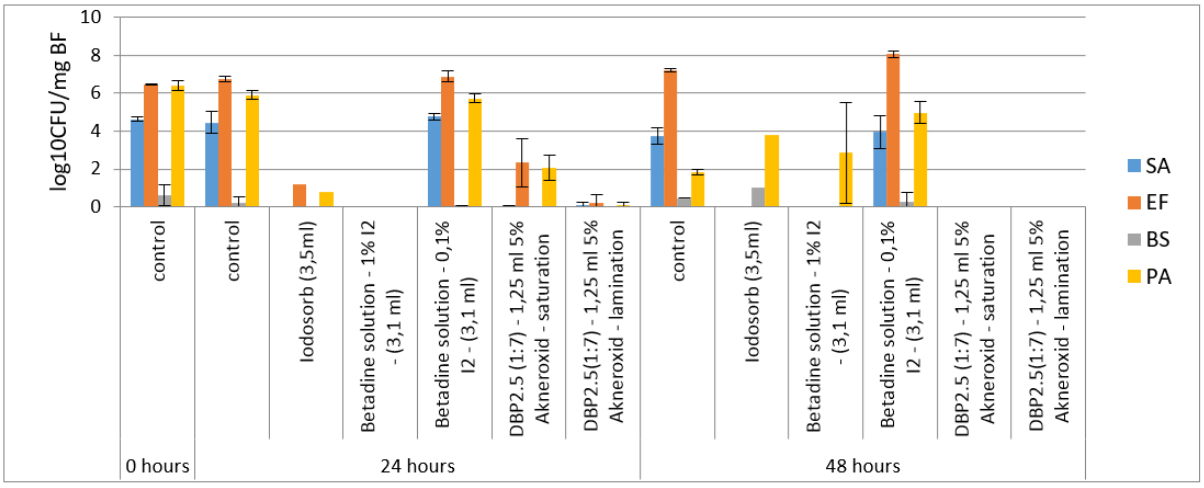
Graph 39. The bacterial population counts 24 and 48 hours after the application of the bandages DBP3(1:6), DBP3(1:4), DBP2(1:6). Test BF-9.



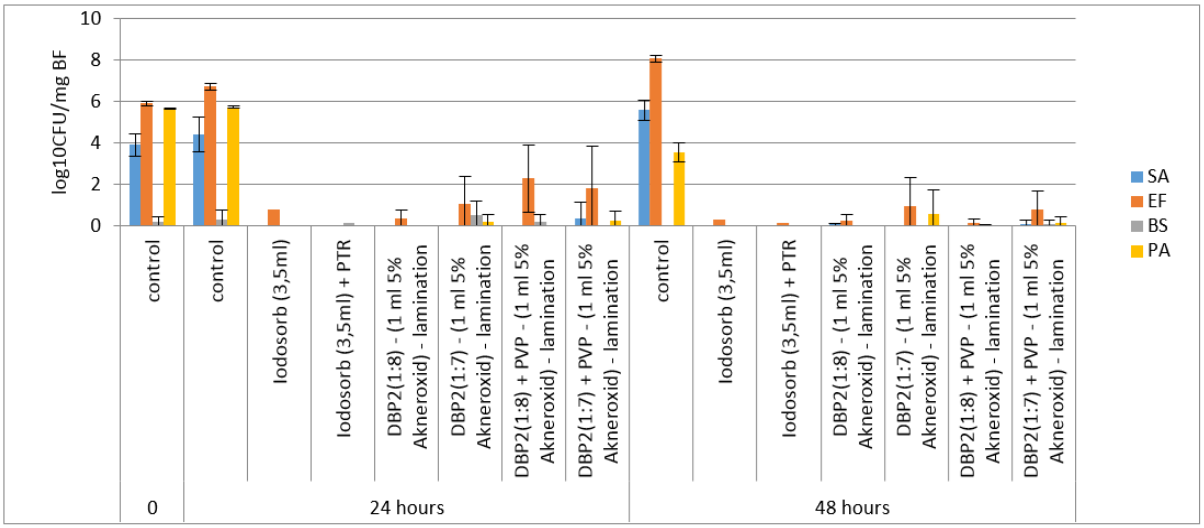
Graph 40. The bacterial population counts 24 and 48 hours after the application of the bandages DBP3(1:6), DBP3(1:5), DBP2(1:8) and DBP2(1:7). Test BF-10.



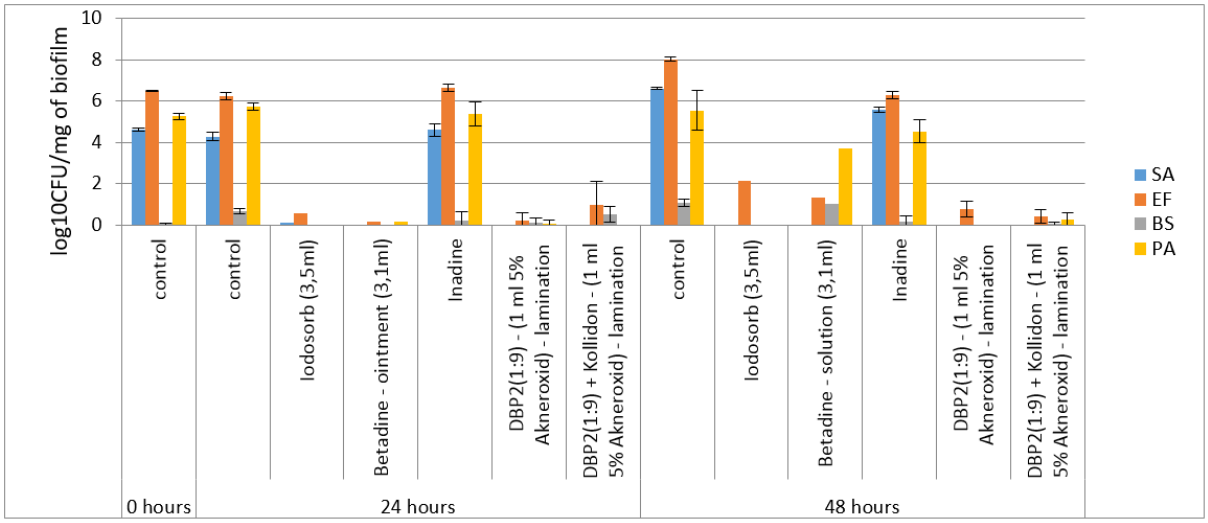
Graph 41. The bacterial population counts 24 and 48 hours after the application of the bandages DBP1(1:18), DBP 1(1:14) and bandages saturated with Betadine ointment (1% iodine) and macrogol (similar amount to this in Betadine). Test BF-11.



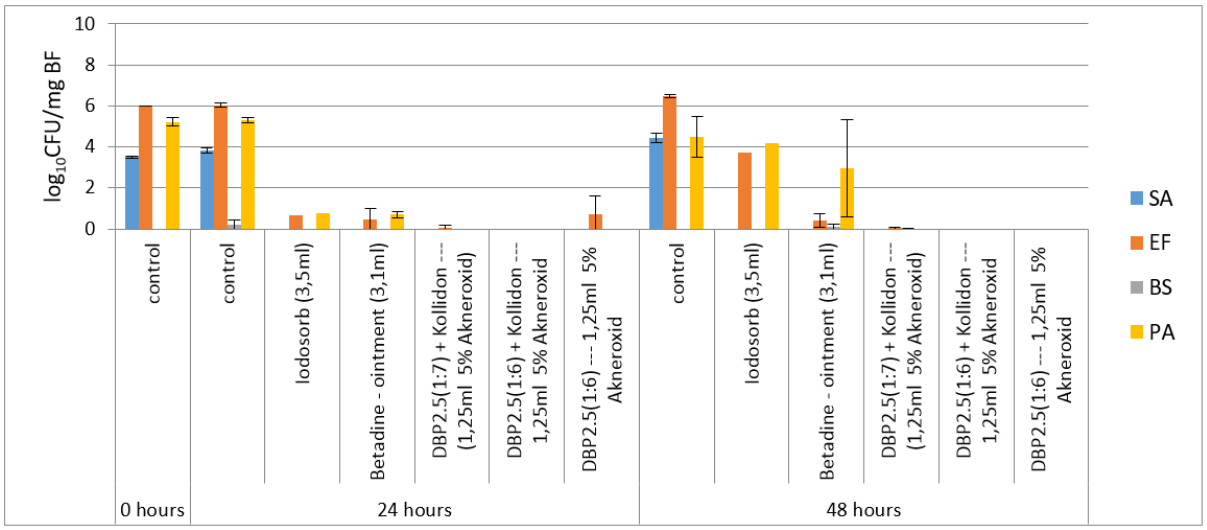
Graph 42. The bacterial population counts 24 and 48 hours after the application of the bandages DBP2.5 (1:7), laminated DBP 2.5(1:7) and bandages saturated with Betadine (0.1% and 1% iodine). Test BF-12.



Graph 43. The bacterial population counts 24 and 48 hours after the application of the laminated bandages DBP2 (1:7), DBP2 (1:7) with PVP K30, DBP2 (1:8) and DBP2 (1:8) with PVP K30. Test BF-13.



Graph 44. The bacterial population counts 24 and 48 hours after the application of the laminated bandages DBP2 (1:9) with Kollidon, DBP2 (1:9) and Inadine (PVP-iodine impregnated on the textile). Test BF-14.



Graph 45. The bacterial population counts 24 and 48 hours after the application of the laminated bandages DBP2.5(1:7) with Kollidon, DBP2.5(1:6) with Kollidon, DBP2.5(1:6) and the bandage saturated with Betadine ointmnent (1% iodine). Test BF-15.

3.5.2 Second model of the *in vitro* Lubbock biofilm (5 bacterial strains, 72 hours)

In the further experiments the *in vitro* model was modified and the time of the proper part of the experiment was prolonged to 72 hours. It is the time after which the bandage should be changed during the treatment of patient wound. In this second model there were always used at least two controls: negative control (textile saturated with buffered peptone water) and a positive control (Betadine and/or Iodosorb). After 72 hours of incubation the biofilm was weaker and not as firm as after 24 or 48 hours (for negative control). However, it was more stable than the biofilm from the previous model. During first five experiments *S. aureus* showed a really weak growth that was caused by too long time of bacteria passages.

In most of performed experiments Iodosorb eradicated biofilm after 24 hours of incubation but there appeared the regrowth of some bacteria strain(s) even after 48 hours. But during the experiment no. 9 (graph 52) Iodosorb was extremely effective. It totally eradicated biofilm; there was no regrowth of bacteria even after 72 hours of treatment.

Betadine in a form of ointment almost eradicated biofilm after 24 hours (in exception of *E. faecalis*) but then the evident regrowth of bacteria was observed: even 5 log CFU for *E. faecalis* and *P. aeruginosa* (graph 47). Usually, the efficacy of Betadine (1% of iodine) in biofilm inhibition was not effective, mainly in the second and third day of the experiment when the regrowth of bacteria was observed. Moreover, there was tested a 10-times diluted Betadine (clinically relevant) that had almost no biofilm inhibition properties. Diluted Betadine did not statistically differ from a negative control that was in a form of a textile saturated with buffered peptone water (graph 55).

3.5.2.1 Iodine Generator – required amount of KI

The use of the first *in vitro* model gave the conclusion that the dressing DBP2 (1:9) releases the smallest possible amount of iodine that is able to be effective in total biofilm eradication (48 hours). However, it has changed in case of the modified biofilm model with five bacteria strains (graph 49). Three variants of the wound dressings with Iodine Generator (containing the same amount of dibenzoyl peroxide) were compared to find the possibly weakest product for biofilm eradication. First of them was dressing DBP2 (1:8) impregnated with 550 mg of KI which only inhibited the biofilm growth. After 48 hours of treatment the 3 log CFU regrowth of each of three bacteria strains was visible (graph 46). Second of them was dressing DBP2 (1:9) impregnated with 620 mg KI (per 10 cm^2) which inhibited the bacteria growth during first 24 hours but was not effective in the two following days (1 log CFU

of *E. faecalis* after second day and additionally 1 log CFU more regrowth of *E. faecalis* and 2 log CFU of *E. coli* after third day of treatment). It turned out that the bandage DBP2 (1:10) with 690 mg KI was effective enough in the whole period of time with 1 log CFU regrowth of *E. coli* after 72 hours of the study (graph 49). In other experiments with this variant the regrowth of any bacteria strains (after 72 h) was not higher than 1 log CFU/mg of initial biofilm (graphs: 53 - 56).

3.5.2.2 Various constructions of wound coverings

The purpose of this experiment was to double-check if there exist any crucial difference in biofilm eradication between various contructions of wound dressings. During this part of work there were tested six constructions that were chosen on the basis of the electrichemical methods.

It was observed from the determination of the amount of released iodine (DRP sensors) during defined time that there is no evident difference between tested constructions of wound coverings. The *in vitro* study of the dressing DBP2 (1:9) with Kollidon (15 mg/cm^2) showed that the samples with constructions no. 2 (graph 46) and no. 4 (graph 48) were more effective in bacterial biofilm inhibition than the sample having the construction no. 3 (graph 47). The last one was even weaker than Betadine after first 24 hours of incubation what means that too small amount of KI was in close contact with DBP.

Constructions no. 2, 6 and 7 were compared when variant DBP2 (1:9) with Akneroxid was tested. Dressing with construction no. 6 inhibited the growth of all bacteria strains up to 72 hours. Construction no. 2 gave a slightly weaker inhibition of biofilm that time (main difference: 4 log CFU of *E. faecalis* after 72 hours, graph 51). The dressing having the seventh construction gave even weaker results in biofilm inhibition (graph 52). On the other hand, when the dressing DBP2 (1:10) with Akneroxid was applied on the biofilm model it was found that there was no statistical difference between bandages having the constructions no. 2, 6, 7 and 8 (graphs: 53, 54).

3.5.2.3 Wound coverings with PVP (Kollidon)

As previously mentioned (paragraphs: 3.3.2 and 3.4.2) there were made the attempts to achieve a long-term kinetics of iodine release with the decreased accumulation of iodine formation in the first phase of iodine production. The dressings with the addition of polyvinyl

pyrrolidone were also tested on the modified Lubbock biofilm model to double check the results from the measurements of the kinetics of iodine release.

Two dressings with the same amount of KI but having the difference in the presence of cross-linked PVP were compared (graph 49). There was a 2 log CFU difference for *S. aureus* and 4 log CFU for *E. faecalis* when comparing both dressings after 24 hours of incubation what proved again that Kollidon causes the inhibition of the iodine release from the wound dressings. After longer incubation there was no statistical difference between the efficacy of compared Iodine Generators. Similarly, the dressings DBP2 (1:10) with and without the addition of Kollidon were compared. The dressing without Kollidon (graph 54) almost totally eradicated biofilm (24 h) and was maintaining its efficiency during the whole experiment (till 72 h) whereas the dressing with Kollidon (graph 50) only inhibited the bacteria growth on the level corresponding to Betadine.

In the test no. 4 (graph 48) the coverings (DBP2 (1:9), construction no. 4) containing different amounts of Kollidon (5 and 15 mg/cm^2) were tested. The bandages differed between each other in the growth of *E. faecalis* and *E. coli* after 48 hours of treatment (the bandages with 15 mg of Kollidon eradicated biofilm) but after next 24 hours there was no statistical difference between them (regrowth of bacteria).

The presented data made the confirmation that PVP in the developed dressings is not playing the expected role that was a slight inhibition of iodine release during first day and an increase of the amount of iodine release in the second and third day of treatment. In this situation the incorporation of PVP to the Iodine Generator became useless.

3.5.2.4 Comparison of DBP formulations

The objective of this paragraph was to test the wound dressings containing eventually chosen formulations containing DBP (5% Akneroxid Gel and 5% Benzacne Gel) and to choose one of these forms to be used for the preparation of the final prototype of Iodine Generator that could be subsequently directed to the *in vivo* study.

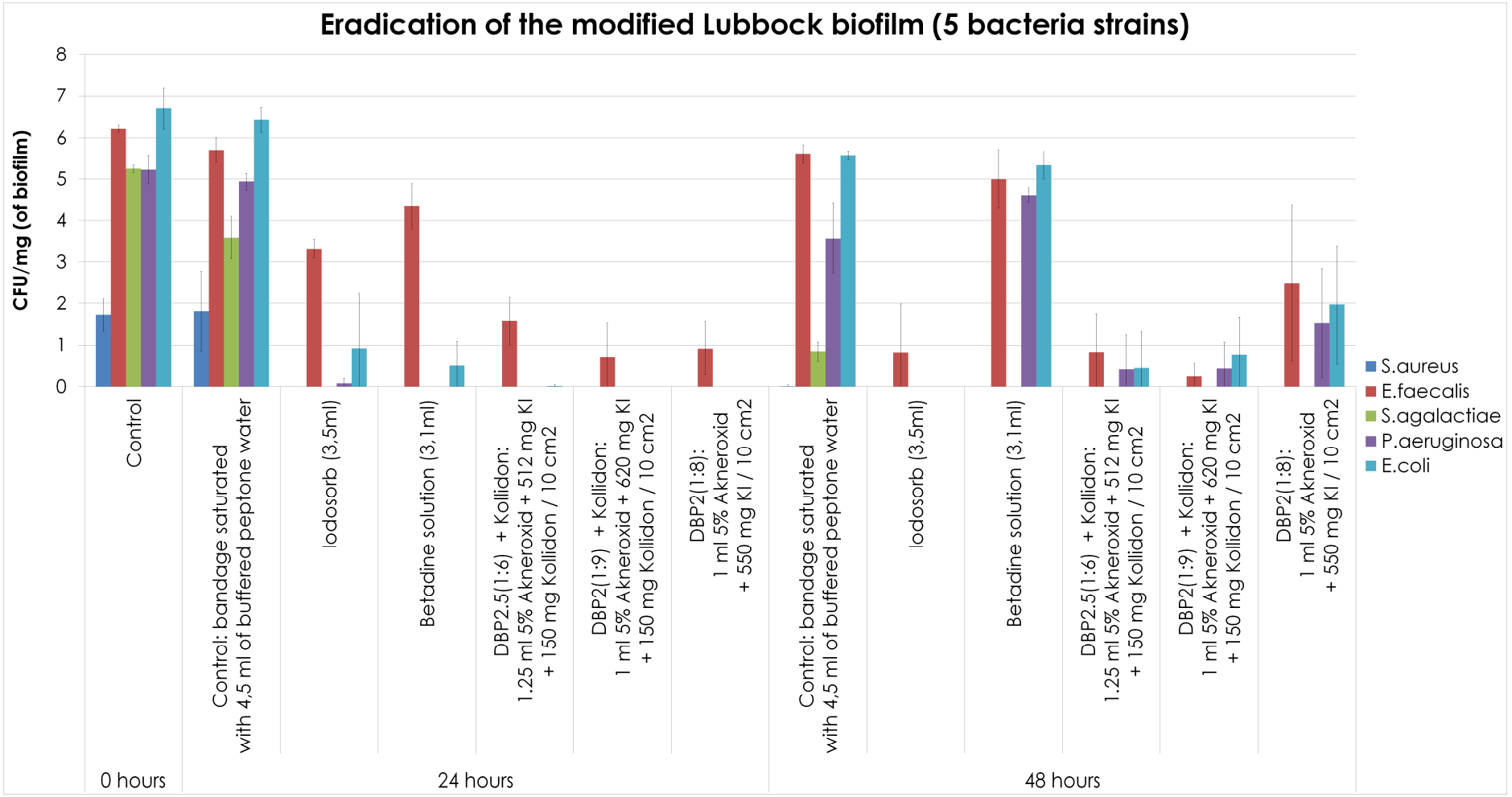
In the test no. 8 (graph 51) there were compared Akneroxid and Benzacne as a DBP sources. Akneroxid showed the higher efficacy in biofilm eradication in comparison to Benzacne. The use of the dressing with construction no. 6 saturated with Akneroxid gave the really good results. It eradicated most of bacteria (in exception of *E. faecalis*) up to 48 hours and all bacteria up to 72 hours. The eradication of *E. faecalis* and *P. aeruginosa* up to 72 hours by this bandage was statistically significant in comparison to Betadine. The same covering

but with Benzacne inhibited *E. faecalis* and *S. aureus* with 2 log CFU and almost totally eradicated the rest three bacteria strains.

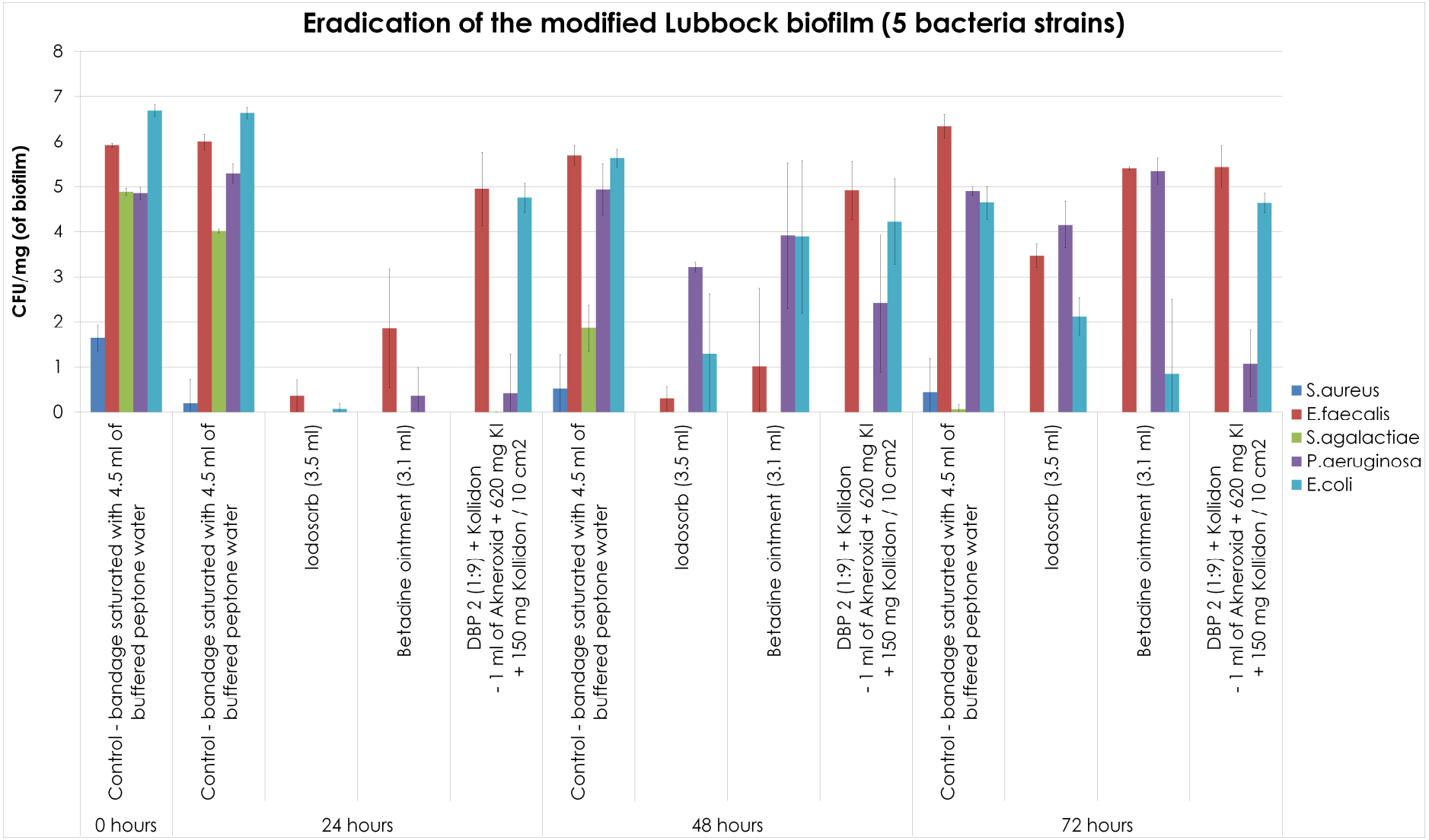
Test no. 12 was based on the comparison of two variants of bandages saturated with Benzacne – DBP 2 (1:10) and DBP 2.5 (1:8). They showed the statistically identical biofilm inhibition as Betadine. Moreover, there was no statistical difference between these two tested samples. It directed to the decision of not applying the higher amounts of DBP to the dressing (graph 55). Moreover, when comparing the dressing DBP2 (1:10) soaked with Benzacne to the dressing soaked with Akneroxid (graph 56) it is evident that the second one is faster and more efficient in biofilm eradication than the first one (Benzacne contains small particles of DBP in the gel base; it requires longer time to be dissolved).

The last two experiments were based on testing the finally chosen prototype in five repetitions. The prototype is a dressing with Iodine Generator that contains 69 mg KI/cm^2 and 5 mg DBP/cm^2 having the second (modified) construction. All analysed batches gave the same result that is the eradication of biofilm during first 24 hours of treatment and maintaining these properties till the end of the experiment that is 72 hours (graph 56).

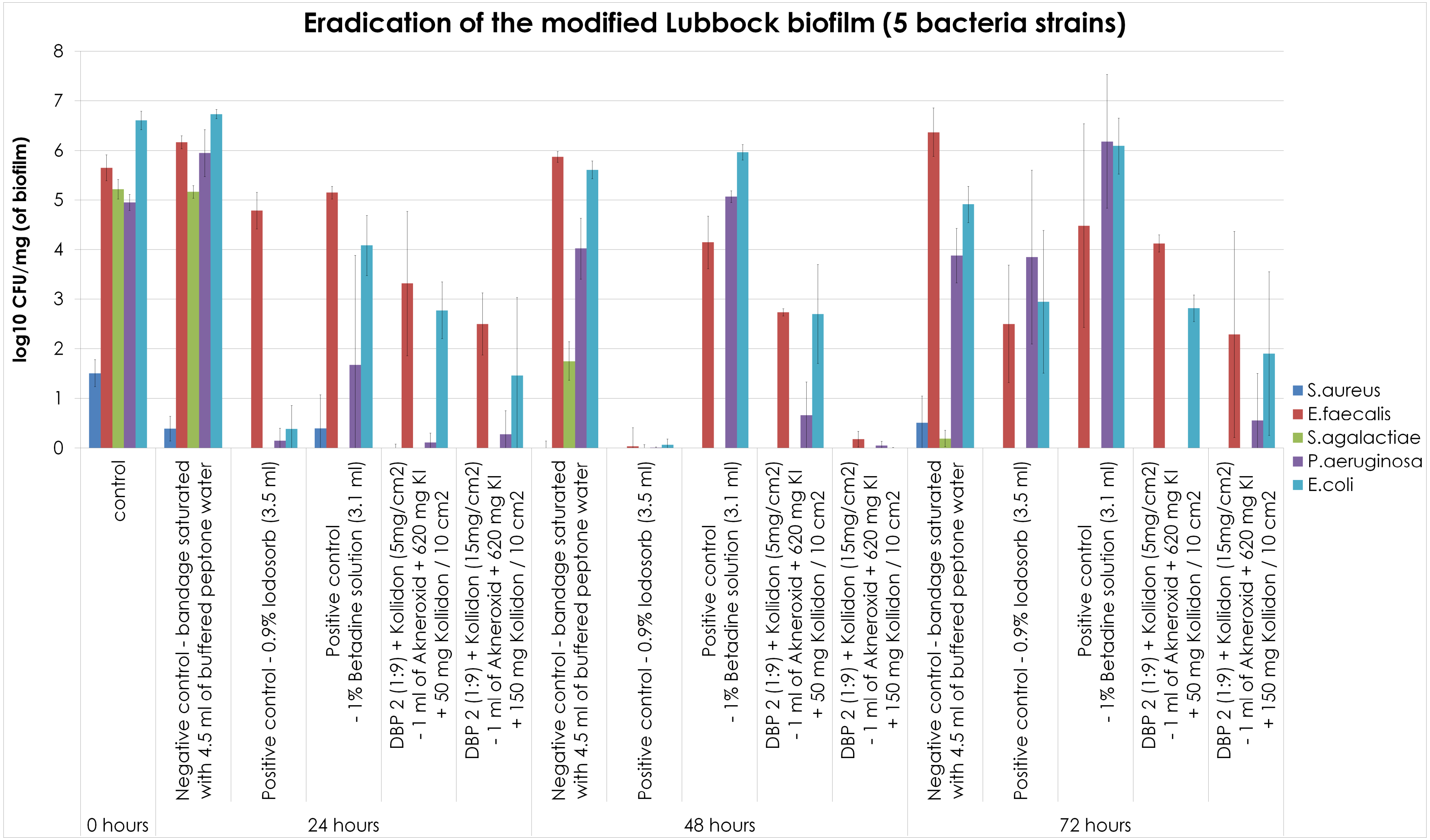
3.5.2.5 Graphical presentation of the results



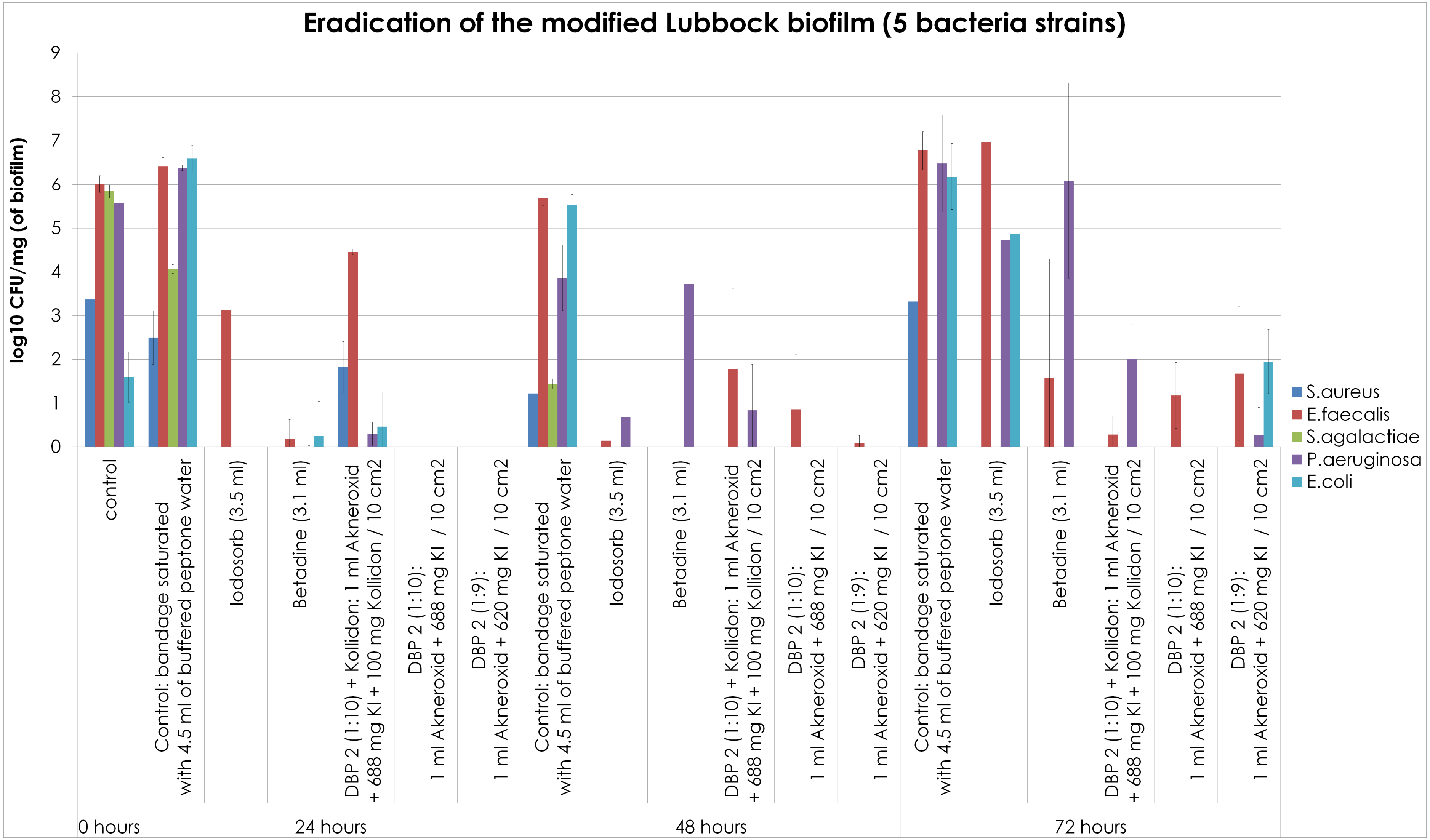
Graph 46. The bacterial population counts 24 and 48 hours after the application of the bandages with Iodine Generator (construction no. 2). Test BF-2 (modified).



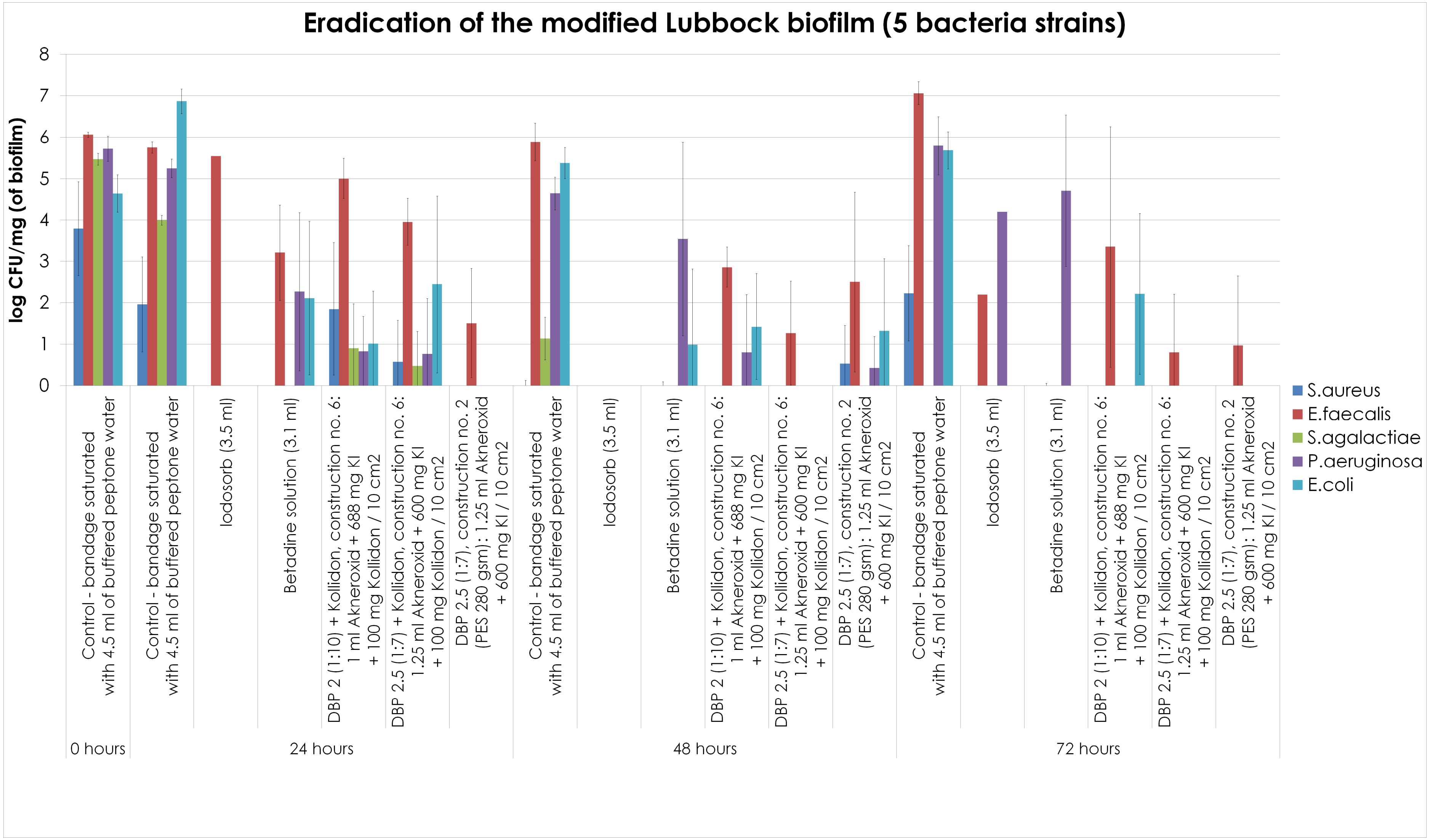
Graph 47. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator (construction no. 3). Test BF-3 (modified).



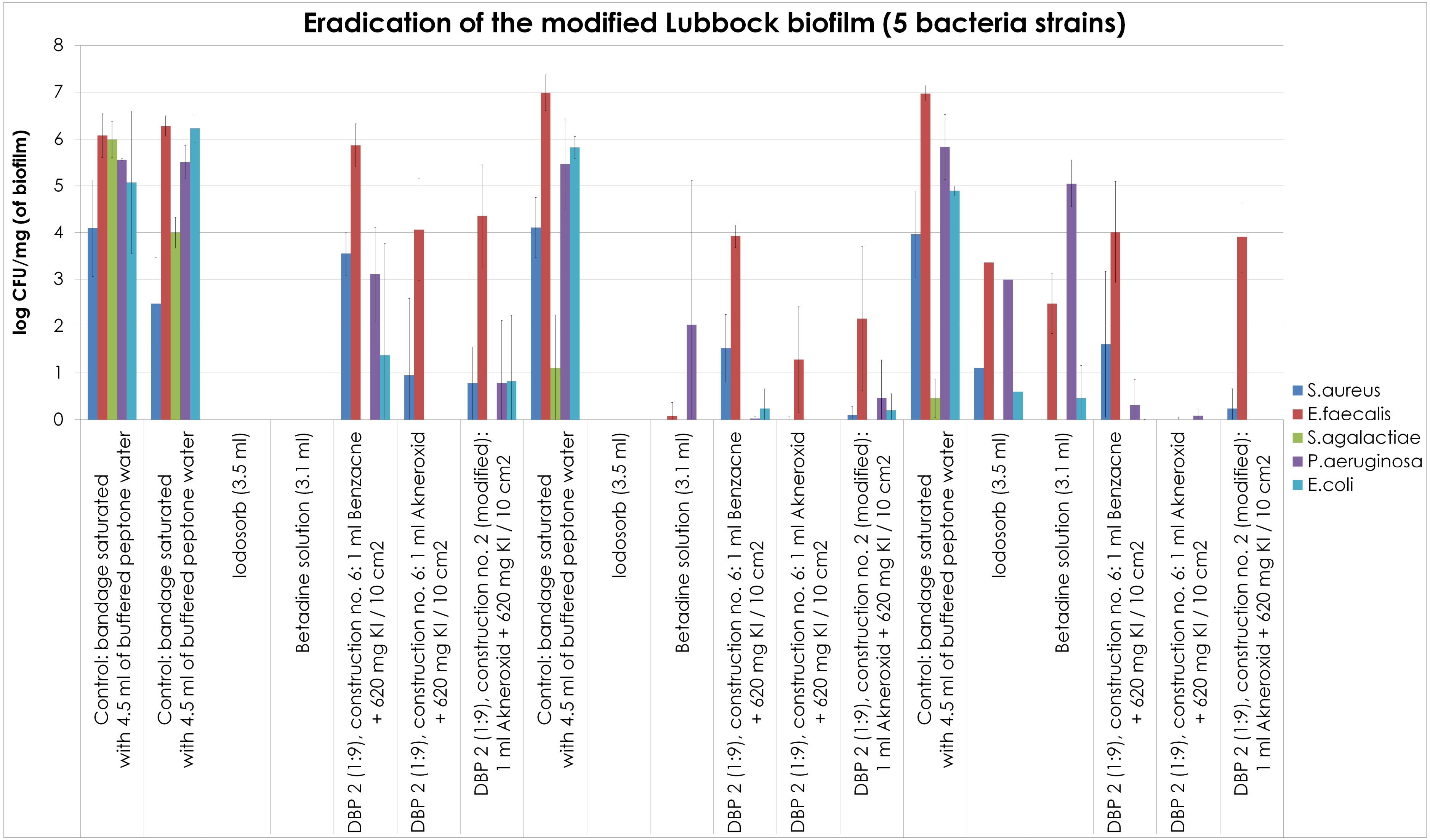
Graph 48. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator (construction no. 4). Test BF-4 (modified).



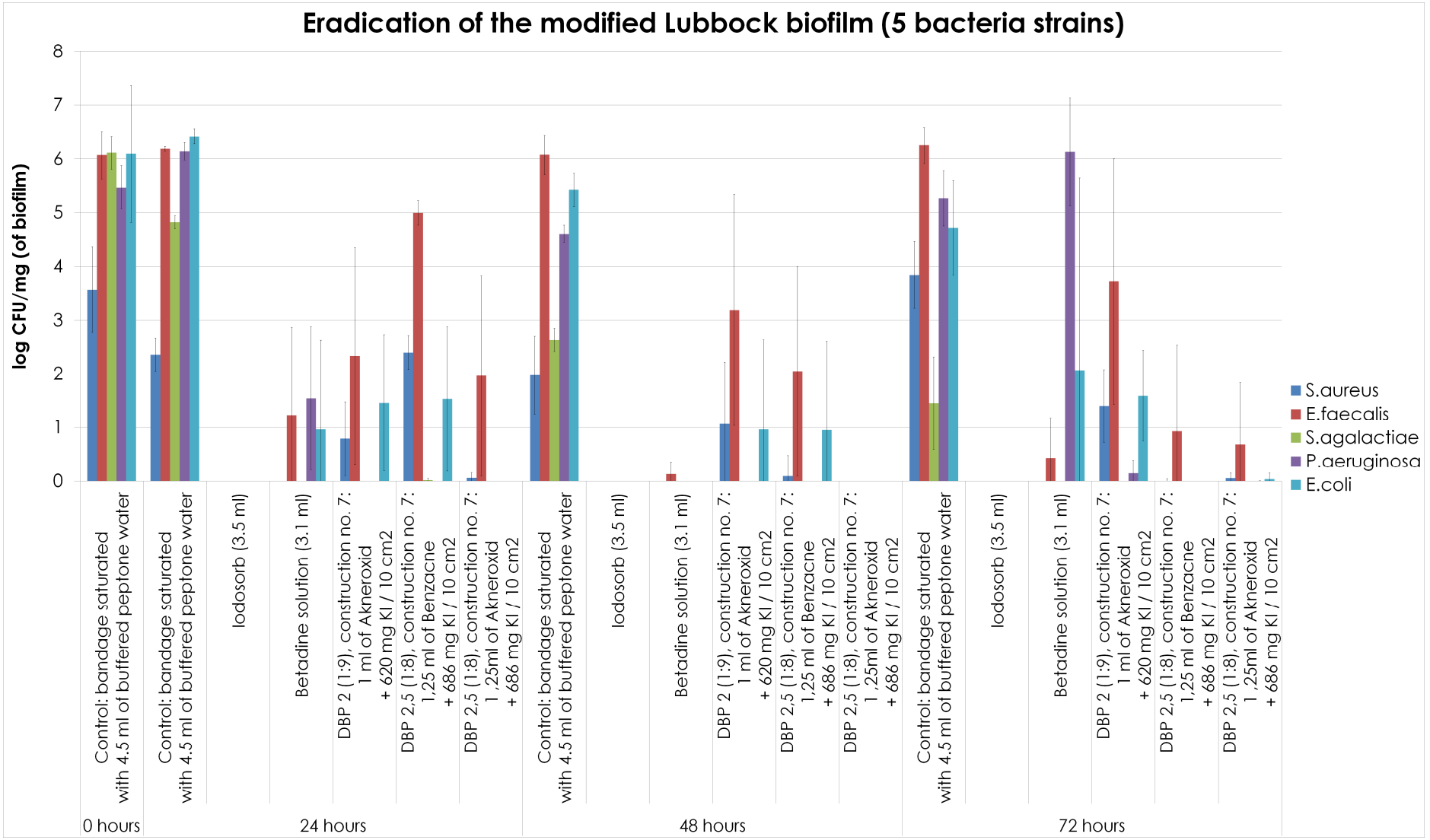
Graph 49. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-5 (modified).



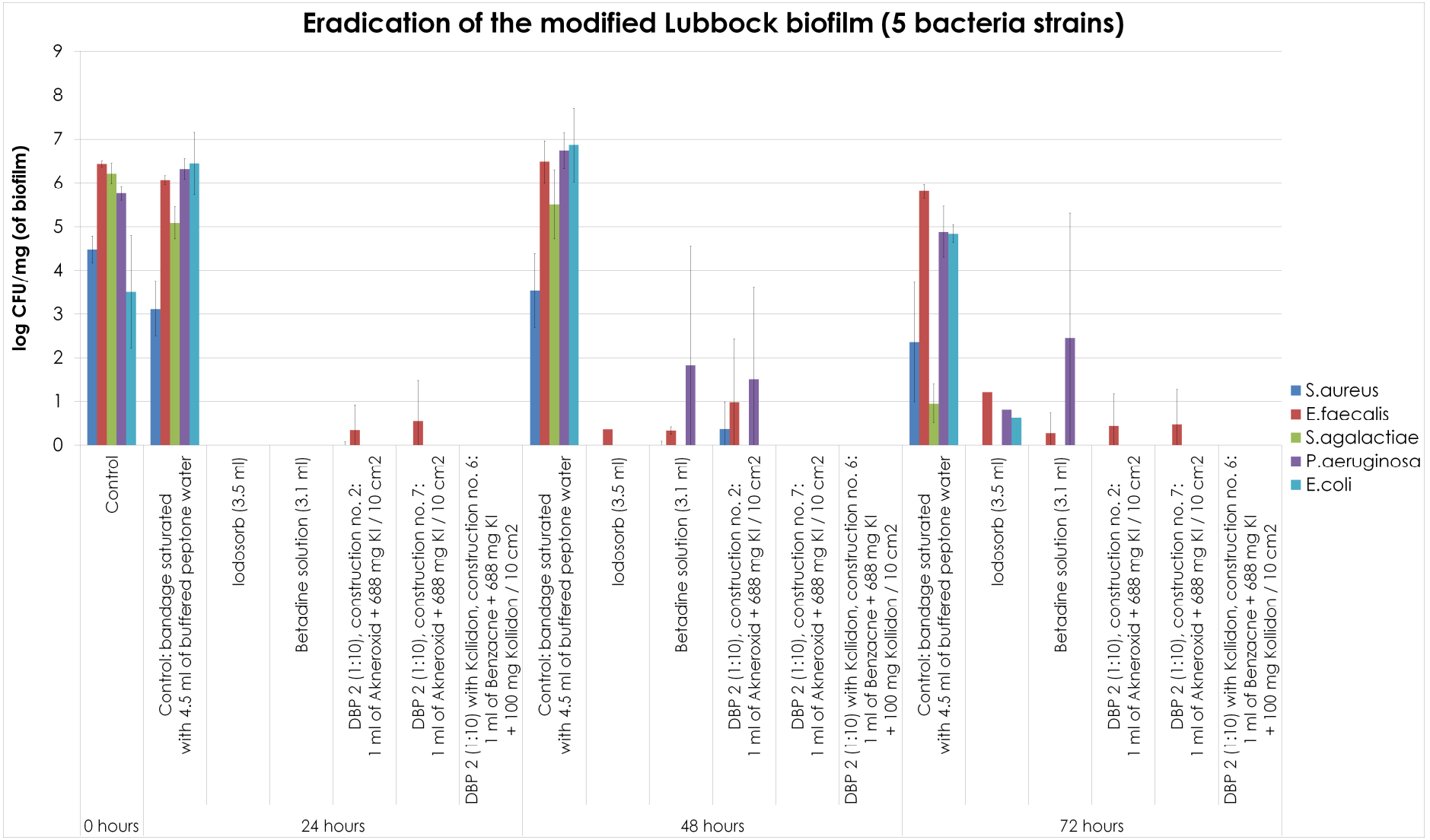
Graph 50. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-7 (modified).



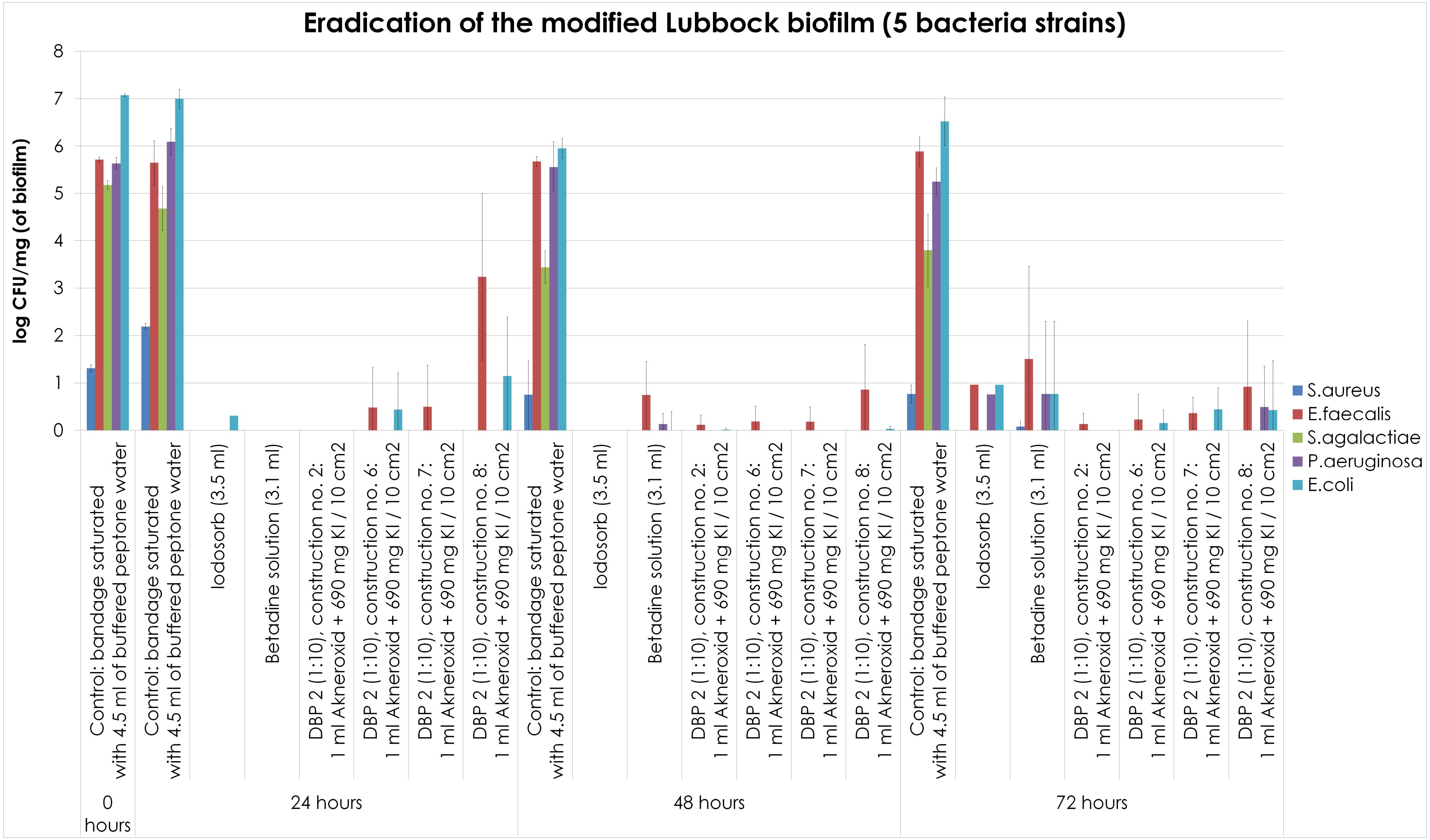
Graph 51. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-8 (modified).



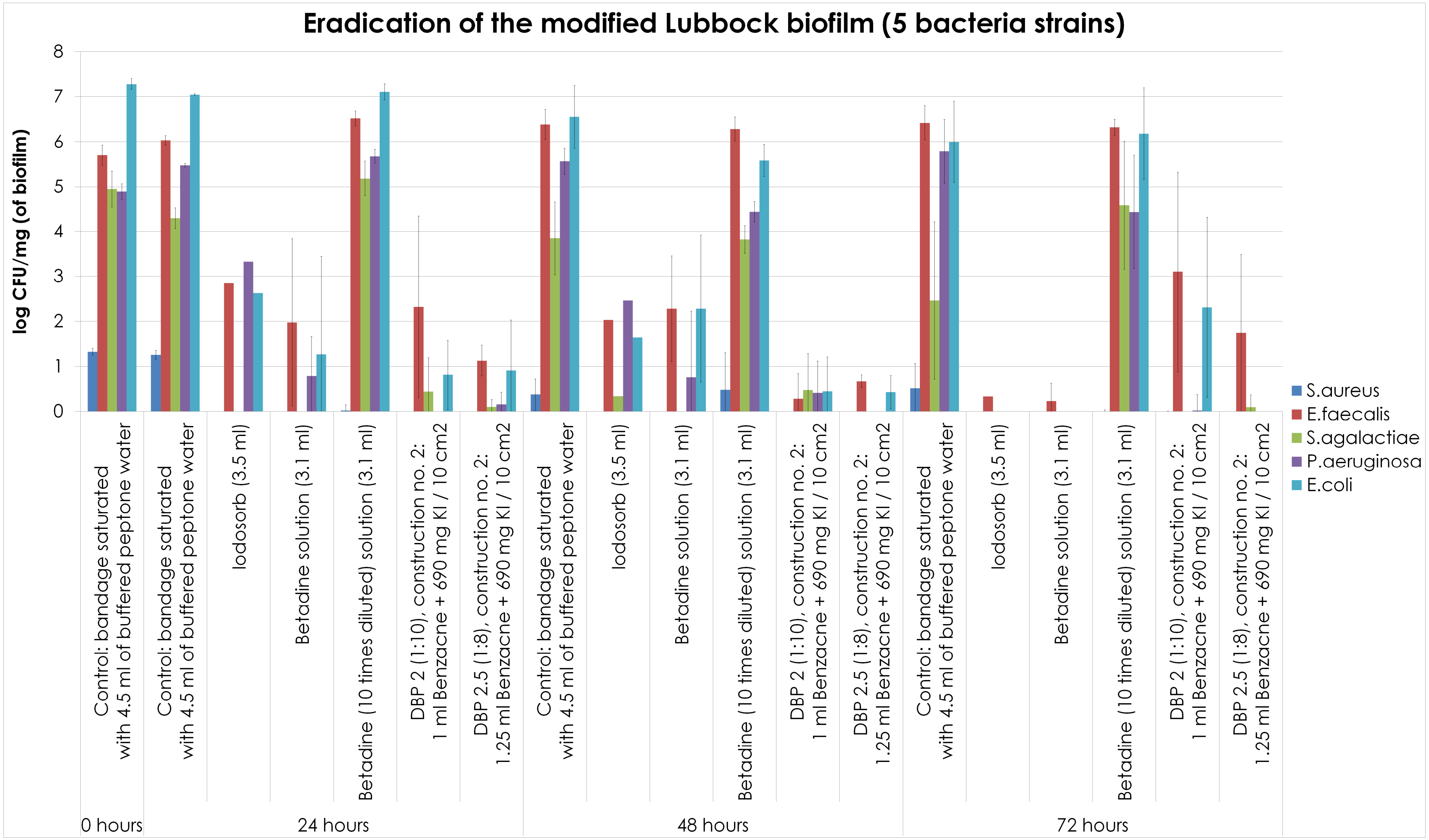
Graph 52. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-9 (modified).



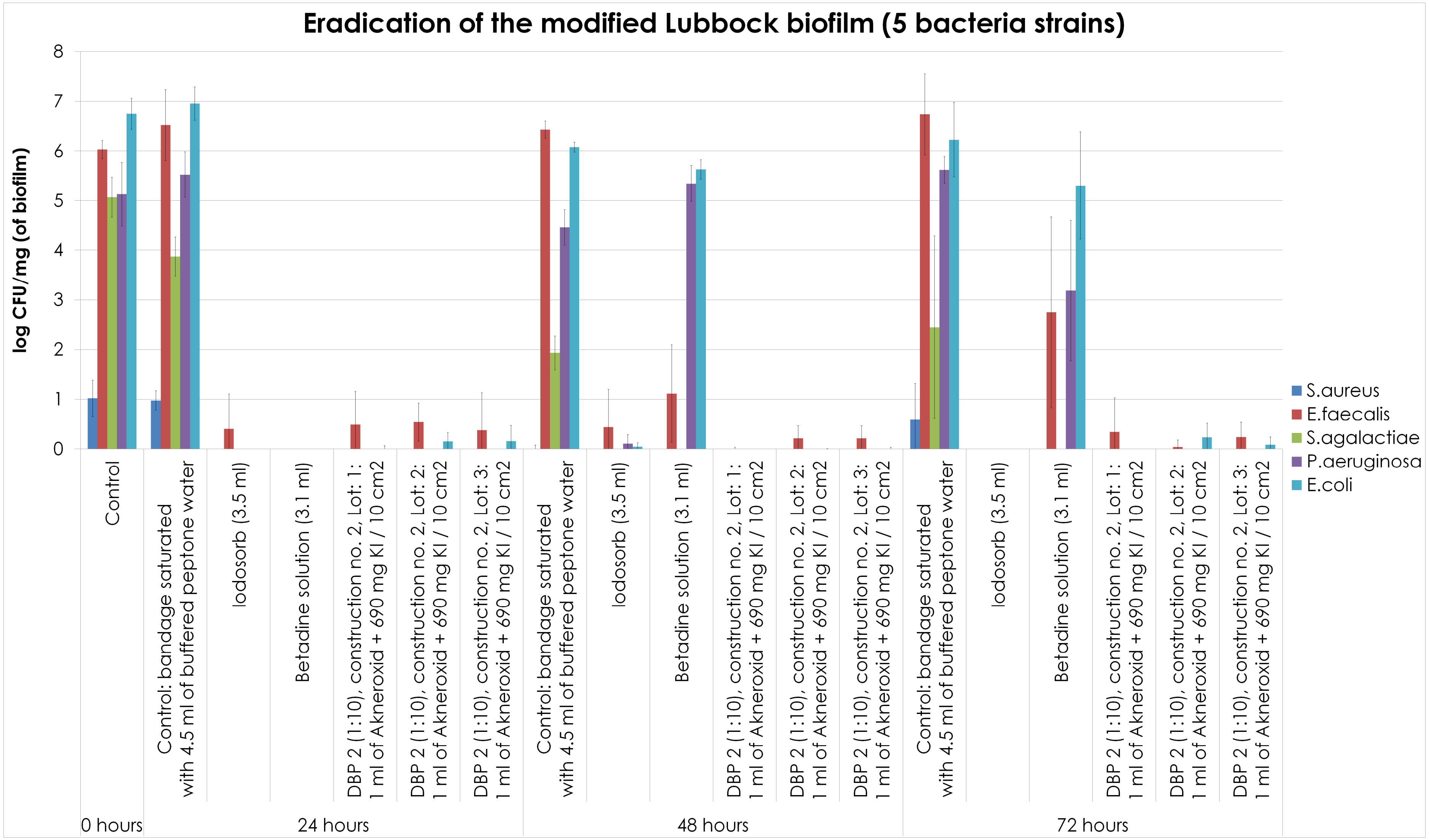
Graph 53. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-10 (modified).



Graph 54. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-11 (modified).



Graph 55. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-12 (modified).



Graph 56. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-13 (modified).

3.6 CYTOTOXICITY OF DRESSINGS WITH IODINE GENERATOR

The aim of the below described research was to check the cytotoxicity of the prototype of the dressing with Iodine Generator. At the same time it was expected that the living cells would be damaged due to the fact that the dressing was intended to be used on the chronically infected wounds covered with biofilm and its action had to be strong enough to destroy pathogenic microorganisms.

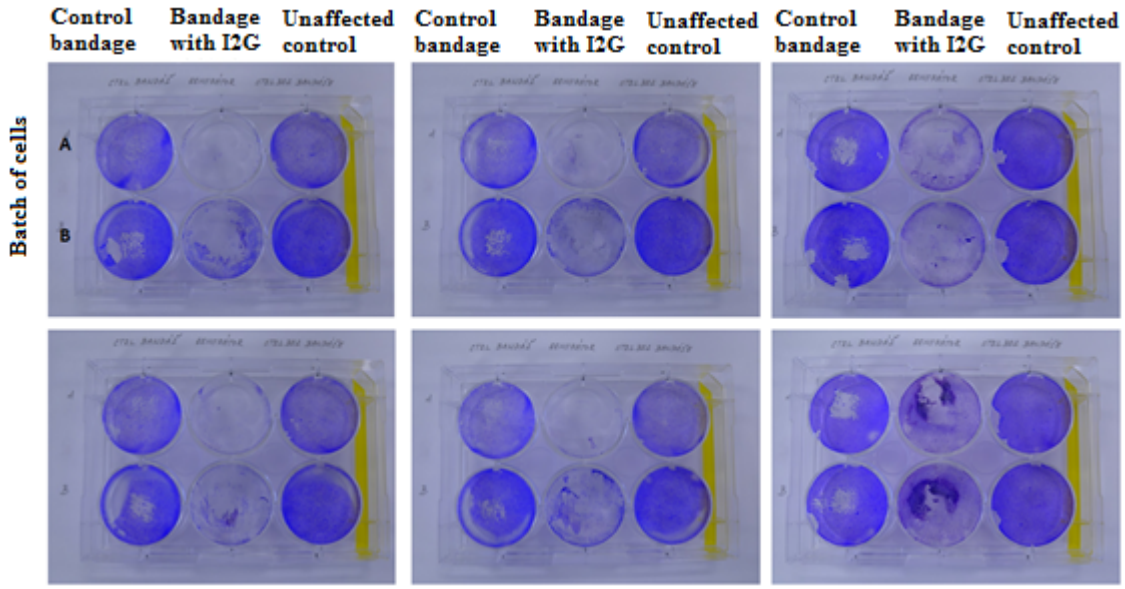


Figure 25. Presentation of the results from testing the cytotoxicity of the wound dressings with Iodine Generator on the 3T3 fibroblasts.

Two batches of Swiss mouse fibroblasts (A and B) were seeded into 6-well panels in the concentration 300.000 cells/well. They were cultured for 48 hours and after reaching confluency 1 cm^2 of bandages with Iodine Generator (DBP2 (1:10) that is 69 mg/cm^2 of KI Ph.Eur. from Penta, 7 mg/cm^2 of EVA T6 and 0.1g of 5% Akneroxid Gel), control bandages (textiles without KI and Akneroxid) and control wells without affecting were tested. After 2 hours of cultivation with bandages, the cells were fixed and coloured with crystal violet.

In each repetition, there was visible a small mechanical damage in case of control bandages and a huge damage of cells in case of the dressing with Iodine Generator. Covering with Iodine Generator caused the death of tested fibroblasts and can therefore be considered as cytotoxic (due to the release of a huge amount of iodine). This effect was expectable due to the concentration of released iodine and design of the dressings addressable to the highly antibacterial effect on the microbial wound biofilm. (figure 25)

3.7 FIRST RESPONSES FROM THE *IN VIVO* STUDY – CHRONIC INFECTED WOUNDS

After several double-checks of the kinetics of the iodine release and the responses from the *in vitro* study on the modified Lubbock biofilm model, the prototype of the wound dressing with Iodine Generator was chosen. The purpose of the next step of research that was *in vivo* study on the porcine model was to qualify if the chosen prototype of the anti-biofilm dressing with I_2G would be efficient enough to eradicate biofilm occurring in the chronically infected wounds.

Before the tests on the *in vivo* model, the bandages impregnated with KI were sent for the gamma radiation sterilization (25 kGy). The sterilization process did not influence on the stability of KI in the bandage (in opposite to the sterilization with ethylene oxide that caused a significant loss of KI). Cultivation of bandages after sterilization in TSB medium (aerobic cultivation) and TG medium (anaerobic cultivation) gave the negative results what proved the propriety of the sterilization method.

When it came to the *in vivo* study, one batch of chosen prototype of the wound dressing with Iodine Generator (DBP2 (1:10); KI: 69 mg/cm^2, EVA: 7 mg/cm^2, DBP: 5 mg/cm^2) was prepared and subsequently tested on the porcine model of chronic skin wound infected with polybacterial biofilm (the same biofilm as in the modified *in vitro* Lubbock biofilm model). The similarity of the healing process in the pig as compared to humans indicates that this model is useful for research on the pathogenesis of wound infections and for preclinical testing of treatments of biofilm-infected human wounds (Klein, 2014).

Irritation of the skin of minipigs during *in vivo* study was not observed (the skin did not change the colour to red). Photos of wounds (surrounded by a healthy skin) after 7 and 21 days of treatment with Iodine Generator, Betadine (10-fold diluted, clinically relevant) and negative control covered with gauze saturated with physiologic solution are presented below (figure 26). However, it would be better to check the irritating properties of bandages with the use of histological examination of the healthy area of the skin surrounding the wound on which the active part of the dressing was applied (figure 27). It should be proved that there are no inflammatory cells and necrosis.

The first results from the *in vivo* study (that is the presence of bacteria colonies on the surface of the wounds) revealed that the dressing with Iodine Generator is more effective in biofilm eradication than 10% Betadine (clinically relevant concentration). In the graph no. 57

there is only presented data for *Staphylococcus aureus* and *Pseudomonas aeruginosa* where there is respectively 60% and almost 50% of the absence of cultivated bacteria when dressing with I_2G was applied. It showed a six-time better efficacy of I_2G in comparison to Betadine. These results are really promising for this medical device but the more specified data will be described in the final report for TACR project.

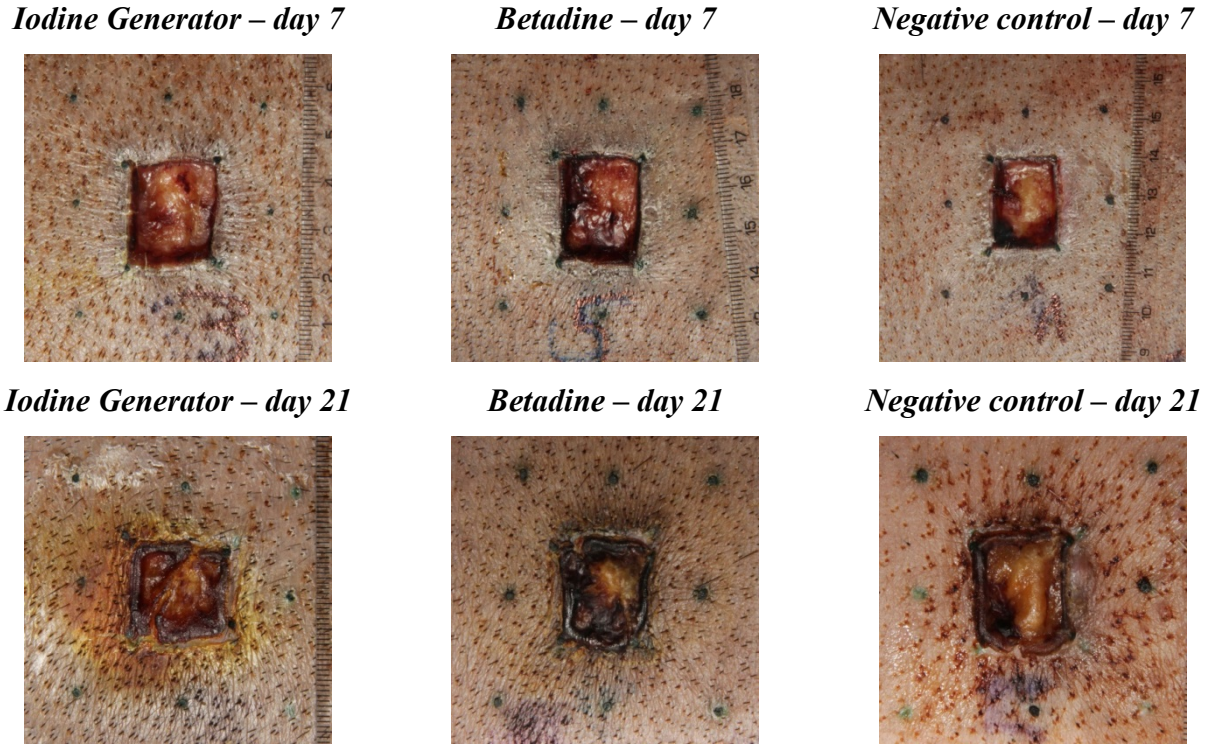


Figure 26. Photos of minipigs' wounds and the adjacent area after treatment with Iodine Generator (KI: 690 g/m^2), 10% Betadine and negative control (a gauze with physiological solution).

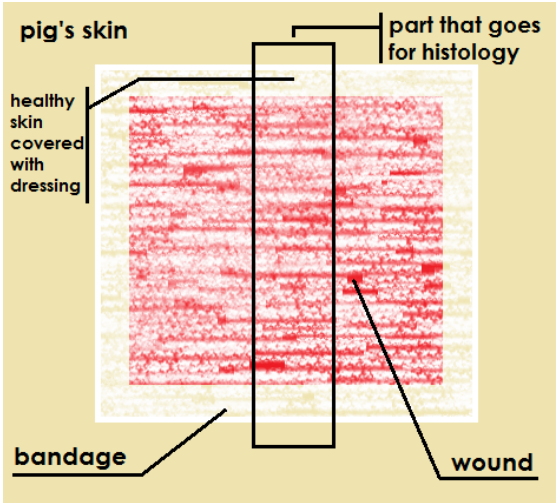
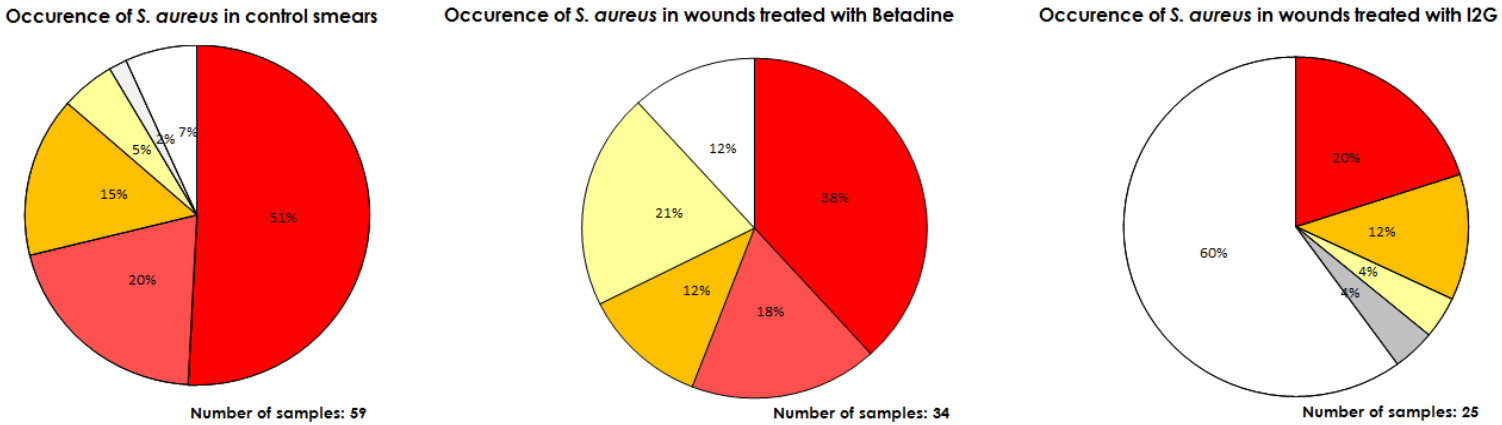
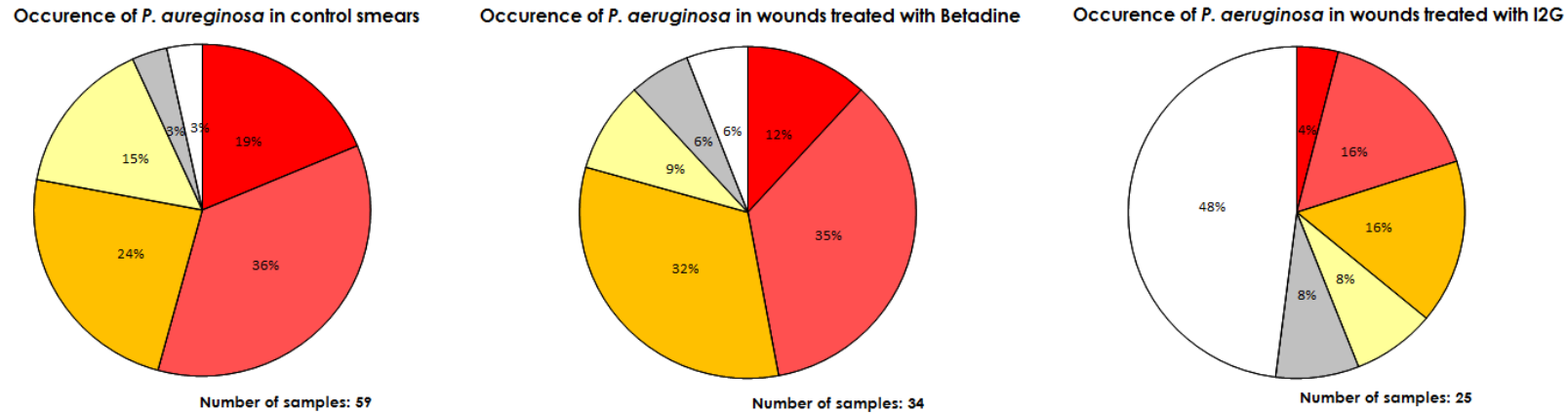


Figure 27. Idea of testing the irritating properties of the dressings containing iodine.

Staphylococcus aureus 120



Pseudomonas aeruginosa 108



Graph 57. Chosen semiquantitive swabs from chronic infected wounds.

4 SUMMARY

During the research there were used three principles of iodine formation *in situ*. First two reactions of potassium iodide with sodium iodate or sodium perborate required significant amounts of acidic starters to achieve a long-term kinetics of iodine release from the dressing. The third one was based on the temperature – dependent mechanism of iodine formation by the oxidation of potassium iodide by dibenzoyl peroxide catalysed by raising the temperature to 37 °C. The reaction based on DBP is not susceptible to the pH values which is a huge advantage of this solution (in contrast to the previously used reactions): lower irritating properties and smaller influence of the wound exudates on the dressing efficacy in biofilm eradication.

During this work there were developed two analytical methods for the determination of the kinetics of iodine release from the wound dressings. First method was based on the reaction of iodine with starch ("starch gels") and the second one was based on monitoring of the current response at two polarizable platinum electrodes to which a constant voltage was applied. Both methods were adapted to the model imitating the wound bed. Moreover, the *in vitro* Lubbock biofilm model used for the determination of the efficacy of wound dressings was modified and used as a second main source of information in prototype choice.

Dozens of variants of wound dressings with Iodine Generator were developed from which one prototype for the *in vivo* study was chosen. During this research there were optimized the constructions of wound coverings, the forms and amounts of active and auxiliary substances. For instance, polyvinylpyrrolidone (PVP) was used as a capacitor of iodine in the bandage. The release of iodine from the dressing during first two days (instead of one that was expected) was significantly inhibited and its release only slightly increased in the further periods of time when compared to the sample without the addition of PVP. The results from the *in vitro* study showed that the addition of PVP did not increase the dressing efficacy in the second and third day of treatment but only decreased the efficacy in the first day so that the incorporation of PVP to the Iodine Generator became useless.

The chosen prototype contains 69 mg KI/cm^2 and 100 mg of 5% Akneroxid Gel/cm^2 (that is 5 mg of dibenzoyl peroxide). Potassium iodide is impregnated (by the lamination technic) into two textile layers (PES 280 g/cm^2) which are supplemented with the gel containing peroxide (Akneroxid, Almirall). When Akneroxid is used in the mentioned amount the dressing is slightly wetted and there is not visible any excess of the gel outside the edges of the absorbing textiles that gives the protection against direct contact of peroxide with an open wound. Polyamide (40 g/cm^2) used as a contact layer has adhesive properties that helps with the biofilm removal. Moreover, PAD catches iodine and forms a barrier for KI loss. Due to the fact that there is no need to add the acidic starters, the skin irritating properties are reduced to minimum.

The prototype is characterized by the long-term kinetics of iodine release (even six or seven days) with the maximum of its amount during first 24 hours and a slow stable release in the next days. The chosen dressing enables the effective biofilm eradication (24 h; *in vitro* Lubbock biofilm model) and a subsequent protection of the model wound from its re-appearance (48 h and 72 h). Its efficacy in biofilm eradication is significantly better than in case of Betadine (PVP-iodine from Egis) and comparable to Iodosorb (cadexomer-iodine from Smith&Nephew).

Further research with the developed wound dressing concerns the *in vivo* study on the porcine model with chronically infected wounds and the establishment of the technological properties of the dressing manufacturing during a half-production.

5 LITERATURE

Akiyama H., Oono T., Saito M., Iwatsuki K.: *Assessment of cadexomer iodine against Staphylococcus aureus biofilm in vivo and in vitro using confocal laser scanning microscopy*, The Journal of Dermatology, Vol. 31, pp. 529-534, **2004**;

Anderson L.B., Reilley C.N.: *Thin-layer electrochemistry: steady-state methods of studying rate processes*, Journal of Electroanalytical Chemistry, Vol 10, Issue 4, 295, **1965**;

Bard A.J., Crayston J.A., Kittlesen G.P., Shea T.V., Wrighton M.S.: *Digital Simulation of the Measured Electrochemical Response of Reversible Redox Couples at Microelectrode Arrays: Consequences Arising from Closely Spaced Ultramicroelectrodes*, Anal. Chem. 58, 2321-2331, **1986**;

Bentley J.P., Fellmann J.H.: *Wound dressing protocol*, WO92/01382, February 6, **1992**;

Bernstein J.E.: *Method of treating acne vulgaris with a composition containing carbamide peroxide*, US4,607,101, August 19, **1986**;

Bernstein J.E.: *Method of treating inflammatory acne vulgaris and rosacea with carbamide peroxide*, US7,371,367 B2, May 13, **2008**;

Bjarnsholt T., Kirketerp –Moller K., Ostrup-Jensen P., Madsen K.G., Phipps R., Krogfelt K., Hoiby N., Givskov M.: Why chronic wounds will not heal: a novel hypothesis, Wound Repair and Regeneration, **2007**;

Bjarnsholt T.: *The role of bacterial biofilms in chronic infections*, APMIS, Blackwell Publishing Ltd, **2013**;

Butcher M.: *PHMB: an effective antimicrobial in wound bioburden management*, British Journal of Nursing, Vol. 21, No 12, **2012**;

Chang J., Liu W., Han B., Peng S., He B., Gu Z.: *Investigation of the skin repair and healing mechanism of N-carboxymethyl chitosan in second-degree burn wounds*, Wound Rep Reg, 21, pp. 113-121, **2013**;

Centner V., Massart D.L., de Jong S.: *Inverse calibration predicts better than classical calibration*, Fresenius J Anal Chem, 361: 2-9, **1998**;

Chellquist E.M., Gorman W.G.: *Benzoyl Peroxide Solubility and Stability in Hydric Solvents*, Pharmaceutical Research, Vol. 9, No. 10, **1992**;

Cooper R.: *Biofilms and wounds: much ado about nothing?*, Wounds UK, Vol. 6, No 4, **2010**;

Costerton J.W., Cheng K.J., Geesey G.G., Ladd T.I., Nickel J.C., Dasgupta M., Marrie T.J.: *Bacterial biofilms in nature and disease*, Annu Rev Microbiol., 41:435-64, **1987**;

Costerton J.W. Stewart PS, Greenberg EP: *Bacterial Biofilms:A Common Cause of Persistent Infections*, Science, Vol. 284, **1999**;

Cutting K.F.: *Addressing the challenge of wound cleansing in the modern era*, British Journal of Nursing, Vol. 19, No 11, **2010**;

Debrox: http://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?id=71649; **2013**;

De Cuellar, Blanca Rosa Alvarez Bello, Luis Armando Lopez: *Composition and method for treating skin lesions*, EP0136768, April 10, **1985**;

European Centre for Disease Prevention and Control and European Food Safety Authority (**ECDC**): *Shiga toxin/verotoxin-producing Escherichia coli in humans, food and animals in the EU/EEA, with special reference to the German outbreak strain STEC O104*, Stockholm, **2011**;

Edmonds J.S., **Morita** M.: *The determination of iodine species in environmental and biological samples*, Pure&Appl.Chem., Vol. 70, No. 8, pp. 1567-1584, **1998**;

Food and Drug Administration (**FDA**): *Classification of Benzoyl Peroxide as Safe and Effective and Revision of Labeling to Drug Facts Format; Topical Acne Drug Products for Over-The-Counter Human Use; Final Rule*; Federal Register, Vol. 75, No. 42; March 4, **2010**;

Freise J., Kohaus S., Korber A., Hillen U., Kroger K., Grabbe S., Dissemond S.: *Contact sensitization in patients in chronic wounds: Results of a prospective investigation*, JEADV, 22, 1203-1207, **2008**;

Gilman M.E. et al.: *Povidone-iodine and sucrose wound healing dressing*, WO2011085356(A2), 14 July **2011**;

Gjødsbøl K. et al.: *Multiple bacterial species reside in chronic wounds: a longitudinal study*, IntWoundJ, 3 (3), p.: 225-231, **2006**;

Gnat D., Dunn A.D., Chaker S., Delange F.: *Fast Colorimetric Method for Measuring Urinary Iodine*, Clinical Chemistry 49, No. 1, **2003**;

Green T. R.: *Conveyance of anti-infective activity to wound dressigs*, WO0128600 (A1), 26 Apr. **2001**;

Greener B., Hughes A.A., Bannister N.P., Douglass J.: *Proteases and pH in chronic wounds*, J Wound Care, 14, pp. 59-61, **2005** (abstract);

Hill K.E., Malic S., McKee R., Rennison T., Harding K.G., Williams D.W., Thomas D.W.: *An in vitro model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities*, J Antimicrob Chemother, **2010**;

HPA: *Voluntary Reporting of Staphylococcus aureus bacteraemia in England*, Wales and Northern Ireland, **2009**;

Huang F.L.: *Bacteriostatic porous polyelectrolyte material and its preparation process*, CN1810298 (A), 2 August **2006**;

Hunt T.K.: *Disinfectants, antiseptics and antibiotics*, Iodine and Wound Physiology, Proceedings of a symposium held during the 5th Annual Meeting of the European Tissue Repair Society, Padua, Italy, September **1995**;

Hymes A.C.: *Improved bandage containing a medicament*, US 0 072 251 A2, 16 Feb. **1983**;

ISP brochure: http://www.brenntagspecialties.com/en/downloads/Products/Multi_Market_Principals/Ashland/PVP_-_PVP_VA/PVP_Brochure.pdf, **2013**;

Jirsák O., Kalinová K.: Netkané textilie, script from the Faculty of Textile at the Technical University of Liberec, **2014**;

Khazan M., Azizi F., Hedayati M.: *A review on iodine determination methods in salt and biological samples*, Scimetr 1 (1), **2013**;

Klein et al.: *Stable Benzoyl Peroxide Composition*, US4,387,107, 7 June, **1983**;

Klein P., Sojka M., Kucera J., Pavlik V., Matonohova J., Nemec J., Kubickova G., Slavkovsky R., Danek P., Rozkot M., Velebny V.: *A porcine model of chronic skin wound infected with polybacterial biofilm*, Abstract Book, Free Paper Session, 75, 24th Conference of the EWMA and GNEAUPP, **2014**;

Kolwzan B.: *Analiza zjawiska biofilmu – warunki jego powstawania i funkcjonowania*, Ochrona Srodowiska, Vol. 33, No. 4, pp. 3-14, **2011**;

Koman K. et al.: *Wound treating agent*, US 2006078628 (A1), 13 April, **2006**;

Konto-Ghiorghi Y., Mairey E., Mallet A., Dume´nil G., Caliot E.: *Dual Role for Pilus in Adherence to Epithelial Cells and Biofilm Formation in Streptococcus agalactiae*, PLoS Pathog 5(5), **2009**;

Kučera J., Sojka M., Pavlik V., Szuszkiewicz K., Velebny V., Klein P.: *Multispecies biofilm in an artificial wound bed—A novel model for in vitro assessment of solid antimicrobial dressings*, Journal of Microbiological Methods 103, 18–24, **2014**;

Lacey R.W.: *Antibacterial activity of Povidone-Iodine towards non-sporing bacteria*, App. Micr., Vol. 46, Issue 3, p. 443-449, June **1979**;

Lichtig J., Zaia D.A.M., Valente J.P.S., Oliveira Rezende M.O.: *Biamperometry in the Diffusion Current Region: Consideration For Its Application In Trace Analysis*, Braz. Chem. Soc. 4, 172, **1993**;

Mancl K.A., Kirsner R.S., Dragana A.: *Wound biofilm: Lessons learned from oral biofilms*, Wound Rep Reg, Vol. 21, pp. 352-362, **2013**;

Marciniec B., Dettlaff K.: *Radiation sterilization of drugs*, IAEA, **2008**;

Mehta K.: *Gamma irradiators for radiation sterilizarion*, IAEA, **2008**;

Molan P.C.: *The role of honey in the management of wounds*, J Wound Care, 8(8), pp. 415-8, **1999**;

Niedner R.: *Cytotoxicity and sensitization of povidone-iodine and other frequently used anti-infective agents*, Dermatology 195 (suppl 2), pp. 89-92, **1997**;

Nobuyuki S., Shuji S.: *Dibenzoyl peroxide – containing composition*, JPS6251666, **1987**;

Oli A.K. et al.: *Biofilm formation by multidrug resistan Enterococcus faecalis (MDEF) originated from clinical samples*, J. Microbiol. Biotech. Res., Vol. 2 (2), pp. 284-288, **2012**;

O'Meara S., Cullum N., Majid M., Sheldon T.: *Systematic reviews of wound care management: (3) antimicrobial agents for chronic wounds; (4) diabetic foot ulceration*; Health Technology Assessment, Vol. 4; No. 21, **2000**;

O'Meara S., Cullum N., Majid M., Sheldon T.: *Systematic review of antimicrobial agents used for chronic wounds*, British Journal of Surgery, 2001, 88, 4-21, **2001**;

Orsoni-Segona S., Willcox N.: *Dermatological/cosmetic gels comprising at least one retinoid and benzoyl peroxide*, US20110070274A1, **2011**;

Percival S.L., Bowler P.G.: *Biofilms and their potential role in wound healing*, Wounds 16 (7), **2004**;

Percival S.L., Bowler P.G., Dolman J.: *Antimicrobial activity of silver-containing dressings on wound microorganisms using an in vitro biofilm model*, Int Wound J, 4, 186-191, **2007**;

Percival S.L., McCarty S., Hunt J.A., Woods E.J.: *The effects of pH on wound healing, biofilms, and antimicrobial efficacy*, Wound Rep Reg 22, pp. 174-186, **2014**;

Ph. Int. (*The International Pharmacopoeia)*: *5.8 Methods of sterilization*, Fifth Edition, **2015**;

Philips P.L., Yang Q., Sampson E., Schultz G.: *Microbiocidal effects of wound dressings on mature bacterial biofilm on porcine skin explants*, **poster**, Institute of Wound Research and Otolaryngology, University of Florida, **2010**;

Piergallini R. et al.: *Combination of an oxidant and a photoactivator for the healing of wounds*, US2011/0224599 A1, **2011**;

Rahimi M., Mikkelsen S.R.: *Cyclic Biamperometry at Micro-Interdigitated Electrodes*, Anal. Chem. 83 (19), **2011**;

Reilley C.N., Cooke W.D., Furman N.H.: *Derivative Polarographic Titrations*, Anal. Chem. 23, 1223, **1951**;

Roberts G., Chumley A., Mani R.: *The wound milieu in venous ulcers – further observations*, European Wound Management Association conference, Stuttgart, **2005** (abstract);

Schwartz J.A., Lantis J.C., Gendics C., Fuller A.M., Payne W., Ochs D.: *A prospective, non-comparative, multicentre study to investigate the effect of cadexomer iodine on bioburden load and other wound characteristics in diabetic foot ulcers*, International Wound Journal, **2012**;

Schwarz J., Hill R., Weisspapir M.: *Stabilizator of benzoyl peroxide in solution*, US7153888B2, **2006**;

Sherman M.: *Medical Device Packaging Handbook, Second edition, revised and expanded*, Marcel Dekker, New York, p. 200, **1998**;

Silva Aquino K.A.: *Sterilization by Gamma Irradiation*, www.intechopen.com, Brazil, **2012**;

Singh V.A.: *Bacterial biofilms in wounds* (Editorial), Wound Rep. Reg., Vol. 16, **2008**;

Song J., Zhao C., Guo W., Kang X., Zhang J.: *Theoretical and experimental study of the biamperometry for irreversible redox couple in flow system*, Analytica Chimica Acta, Vol. 470, Issue 2, **2002**;

Sterigenics: *Sterilization Alternatives Gamma Radiation*, www.sterigenics.com, **2015**;

Szuszkiewicz K., Valentová Z., Klein P., Pravda M., Hekrlová V., Velebný V.: *Krytí na rány, produkující jód in situ, a souprava*, utility model CZ 29194 U1, 22 February **2016**;

Takahashi S. et al.: *Solid iodophor preparations and process for producing the same*, EP1044689A1, **2000**;

USPC, The United States Pharmacopeial Convention, Official Monographs, page 2328, **2012**;

Thomson CH.: *Biofilms: do they affect wound healing?*, Int Wound J, Vol. 8, p. 63–67, **2011**;

Todar K.:*Nutrition and growth of bacteria*, Todar's Online Textbook of Bacteriology (http://textbookofbacteriology.net/nutgro_4.html), **2013**;

Trevani A., Andonegui G., Giordano M., Lopez D., Gamberale M., Minucci F.: *Extracellular acidification induces human neutrophil activation*, J Immunol, 162, pp. 4849-57, **1999**;

Uzun M., Anand SC, Shah T.: *The effect of wound dressing on the pH stability of fluids*, J Wound Care, 21(2), Feb **2012**;

von Eiff C, Heilmann C, Peters G.: *New aspects in the molecular basis of polymer-associated infections due to staphylococci*, Eur J Clin Microbiol Infect Dis, 18(12): 843–6, **1999**;

Winston A.E.: *Stabilized peroxide solutions*, US4895875, **1990**;

Yang X., Zhang G.: *The voltammetric performance of interdigitated electrodes with different electron-transfer rate constants*, Sensors and Actuators B – Chemical, Vol. 126, Issue 2, **2007**;

Zhou L.H., Nahm W.K., Badiavas E., Yufit T., Falanga V.: *Slow release iodine preparation and wound healing: in vitro effects consistent with lack of in vivo toxicity in human chronic wounds*, British Journal of Dermatology, 146: 365-374, **2002**.