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Faculty of Chemical Technology

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

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ANNOTATION

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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.

INTRODUCTION

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. 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)

One of the aims of this work was to develop the method of the continous determinaiton of iodine that released from the wound dressing to choose the prototype for the *in vitro* study on biofilm model. 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 and to remove the influence of charging current (Rahimi, 2011).

EXPERIMENTAL PART

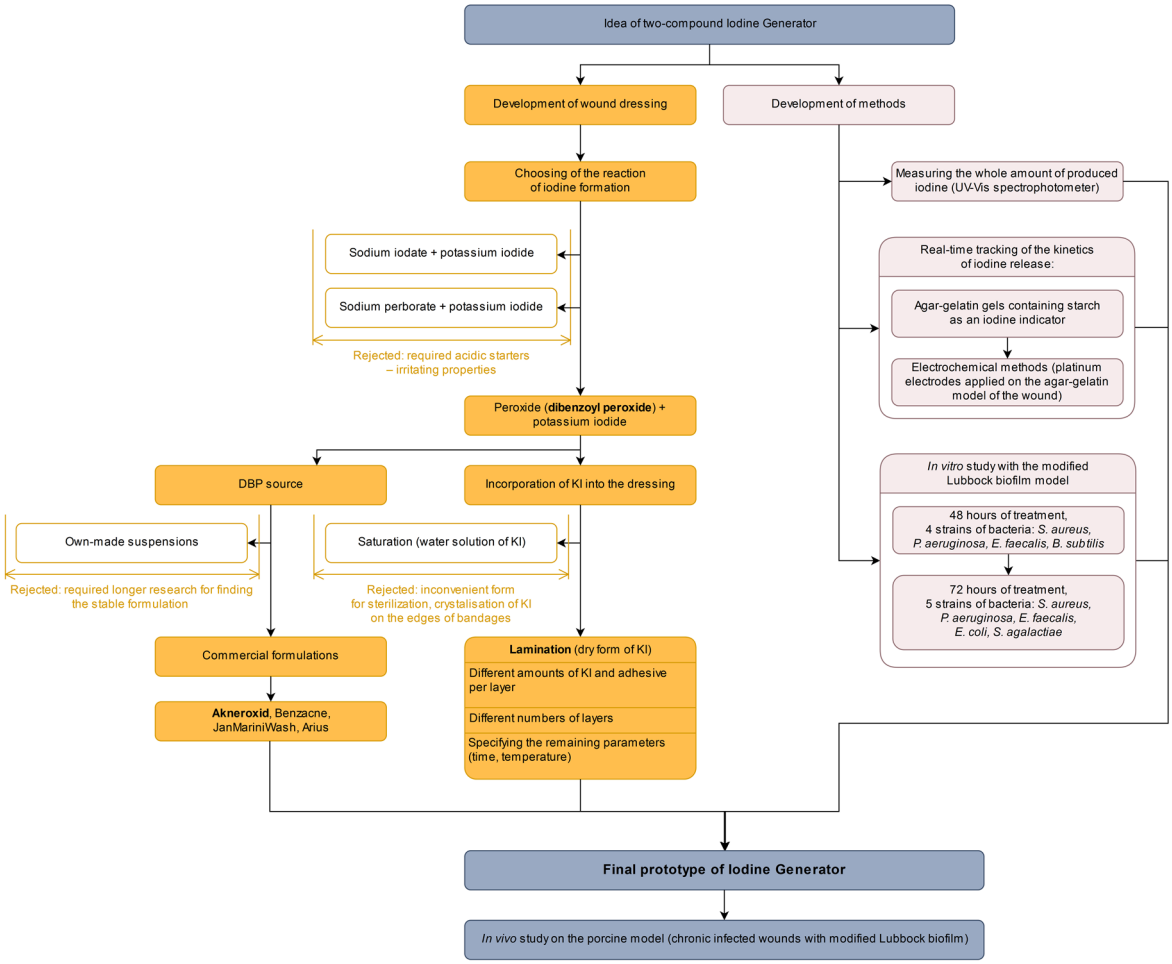
MATERIALS AND EQUIPMENT

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	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/2012/02467/150529051a	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	WTW 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

METHODS

The flowchart (graph 1) that is located below presents the story of Iodine Generator (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.

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 with the addition of adhesive (ethylene vinyl acetate). 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.

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 (DBP) 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).

Continous monitoring of iodine release from wound coverings

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.

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.

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. 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. That is why the elecrochemical method for the continous measurements of iodine release was susequently developed.

Tracking the kinetics of iodine release (electrochemical methods)

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).

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 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.

For further experiments the other type of the printed sensor was used. Sensor CC1.W2 (BVT Technologies, CR) 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. However, 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) 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.

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. 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. 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.

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. 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.

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

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)

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 5 mm 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 1)

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 1)

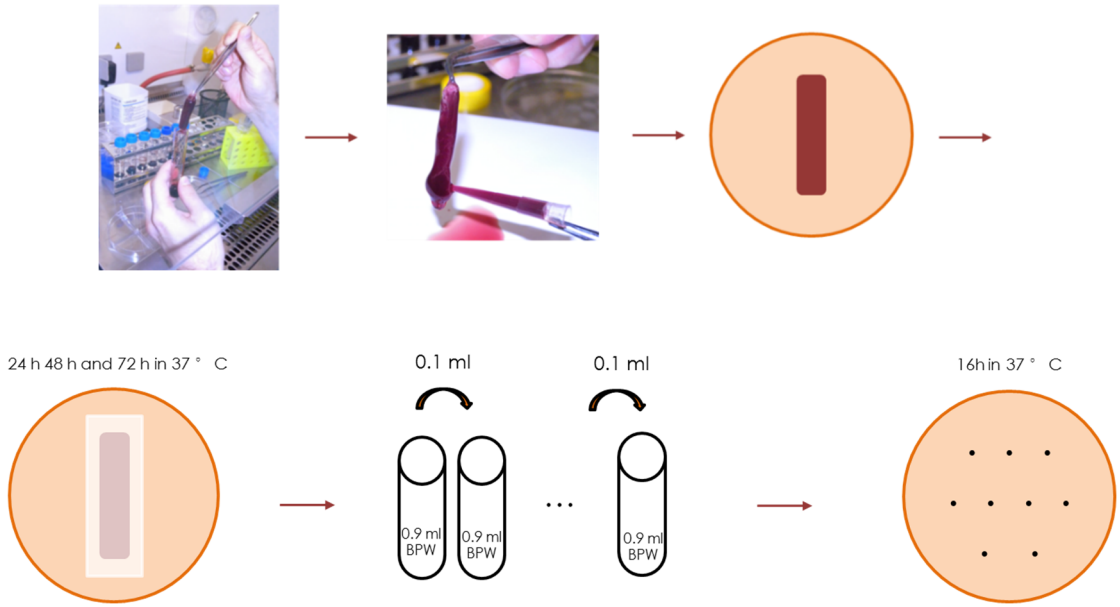


Figure 1. Picture presenting the manipulation with the *in vitro* Lubbock biofilm model: removal of the mature biofilm (after 48 hours of incubation) from the polystyrene tube with the use of tweezers; application of the biofilm to the well present on the Petri dish with a nutrient medium; application 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.

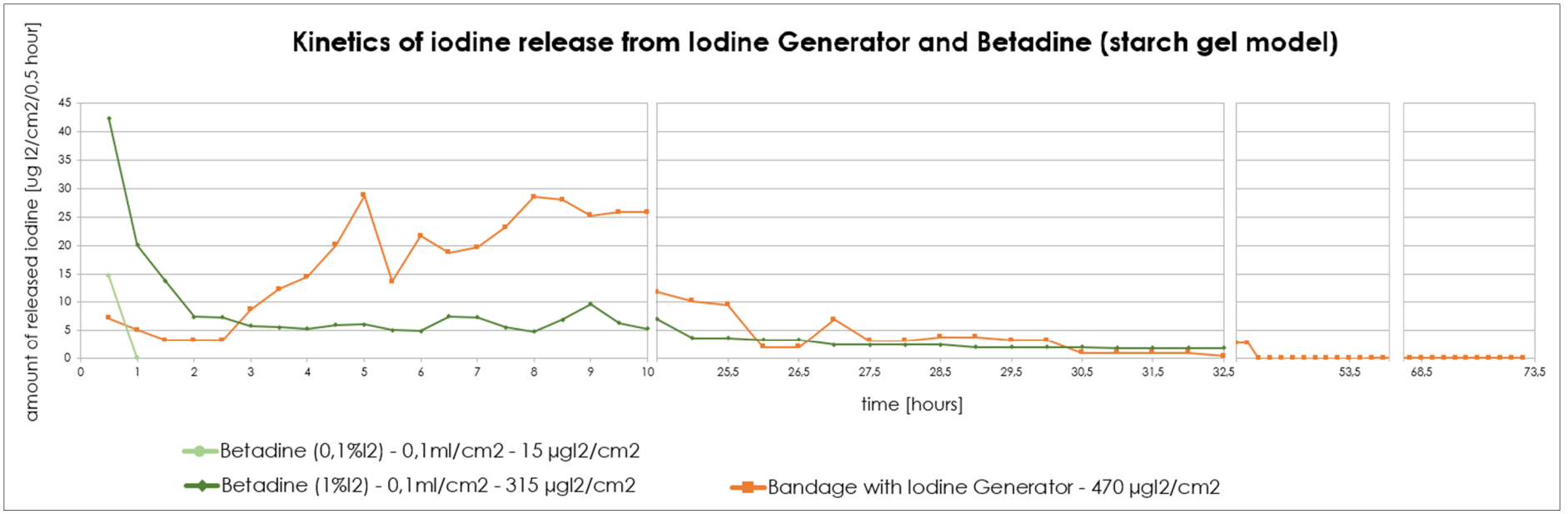
RESULTS AND DISCUSSION

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 the thesis there are 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 show 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. 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. Acidic starters caused the excessive reduction of pH that could cause the irritation of the patients‘ skin. 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. 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. 2 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 2. Kinetics of iodine release from dressing with Iodine Generator (DBP2 (1:9)) and textiles saturated with the solution Betadine (PVP-I_2) in two concentrations.

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 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. Lamination allowed to avoid the formation of iodide crystals after drying of the textiles and simplified the manufacturing process.

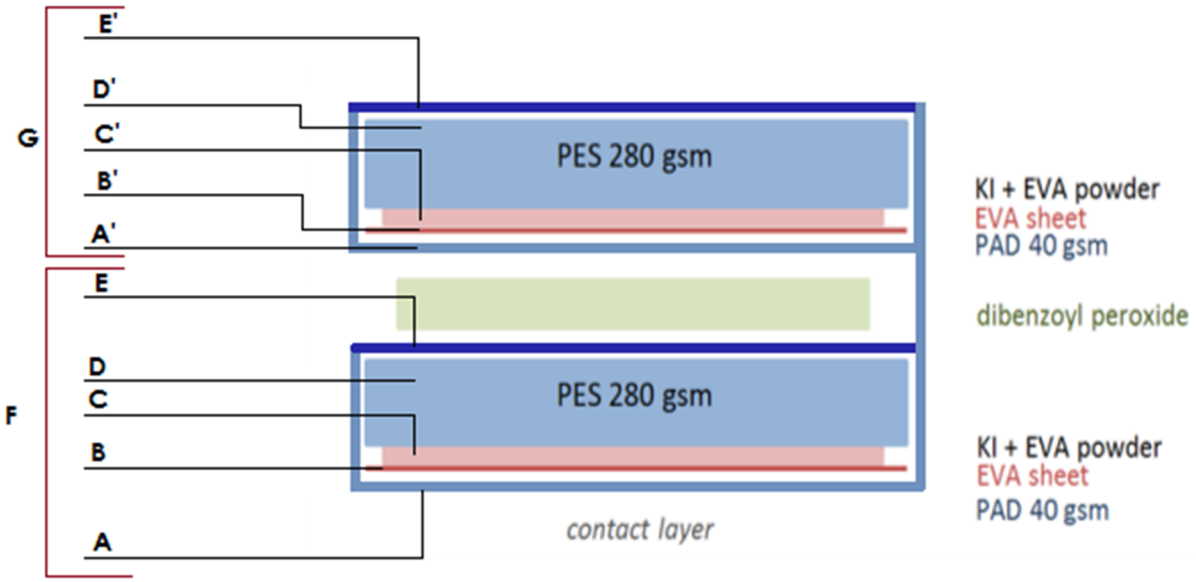


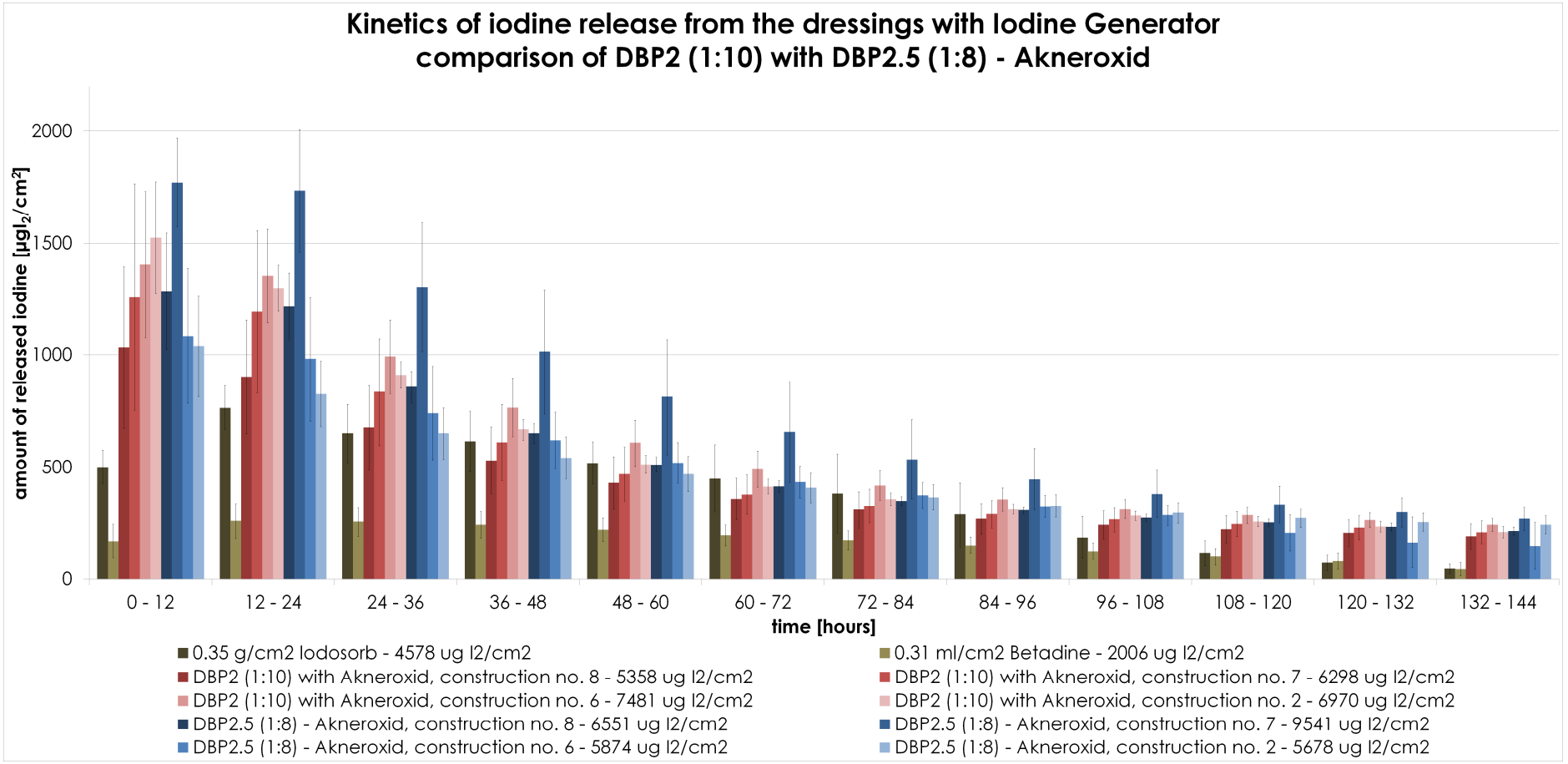
Figure 2. Bandage with the final construction in a form of a sachet.

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 2).

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

From all tested dressings with different sources of DBP, variant DBP 2 (1:10) with 69 mg of potassium iodide per cm^2 and 5 mg of dibenzoyl peroxide per 1 cm^2 (with Akneroxid Gel used as a DBP source), being sufficiently effective in biofilm eradication (graph 4), became the prototype that was subsequently directed for the *in vivo* study on the porcine model.

The performed experiments 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. This growth allowed the chosen wound covering (DBP2 (1:10)) to be totally effective in *in vitro* biofilm eradication (graph 4). In case of using 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)) there was observed the slight increase of iodine release in case of the dressing containing more DBP (graph 3).



Graph 3. Comparison of the kinetics of iodine release from dressings: DBP 2 (1:10) and DBP 2.5 (1:8).

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

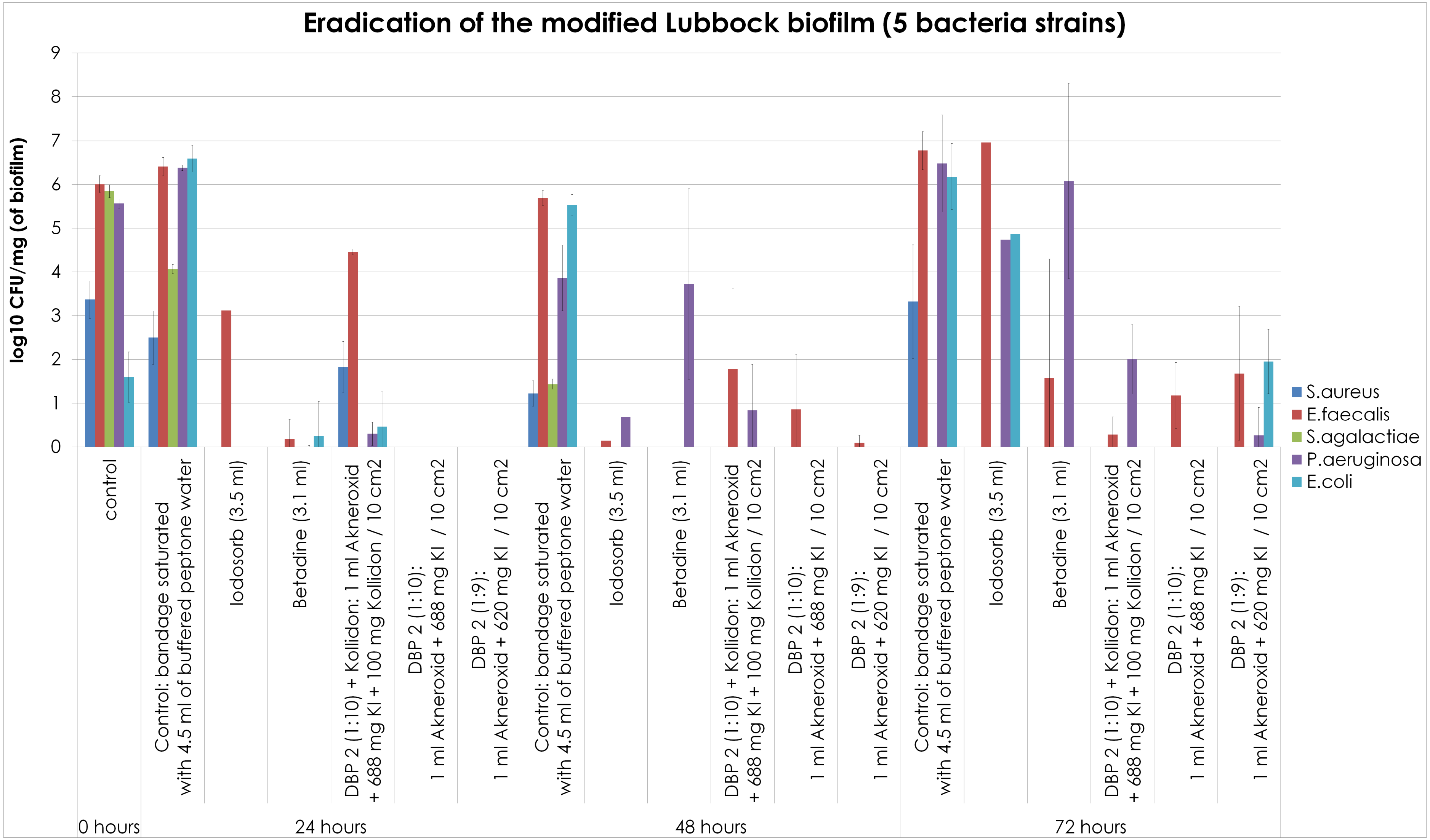
BIOFILM INHIBITION AND ERADICATION – PERFORMED EXPERIMENTS

The following paragraph presents chosen 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*.

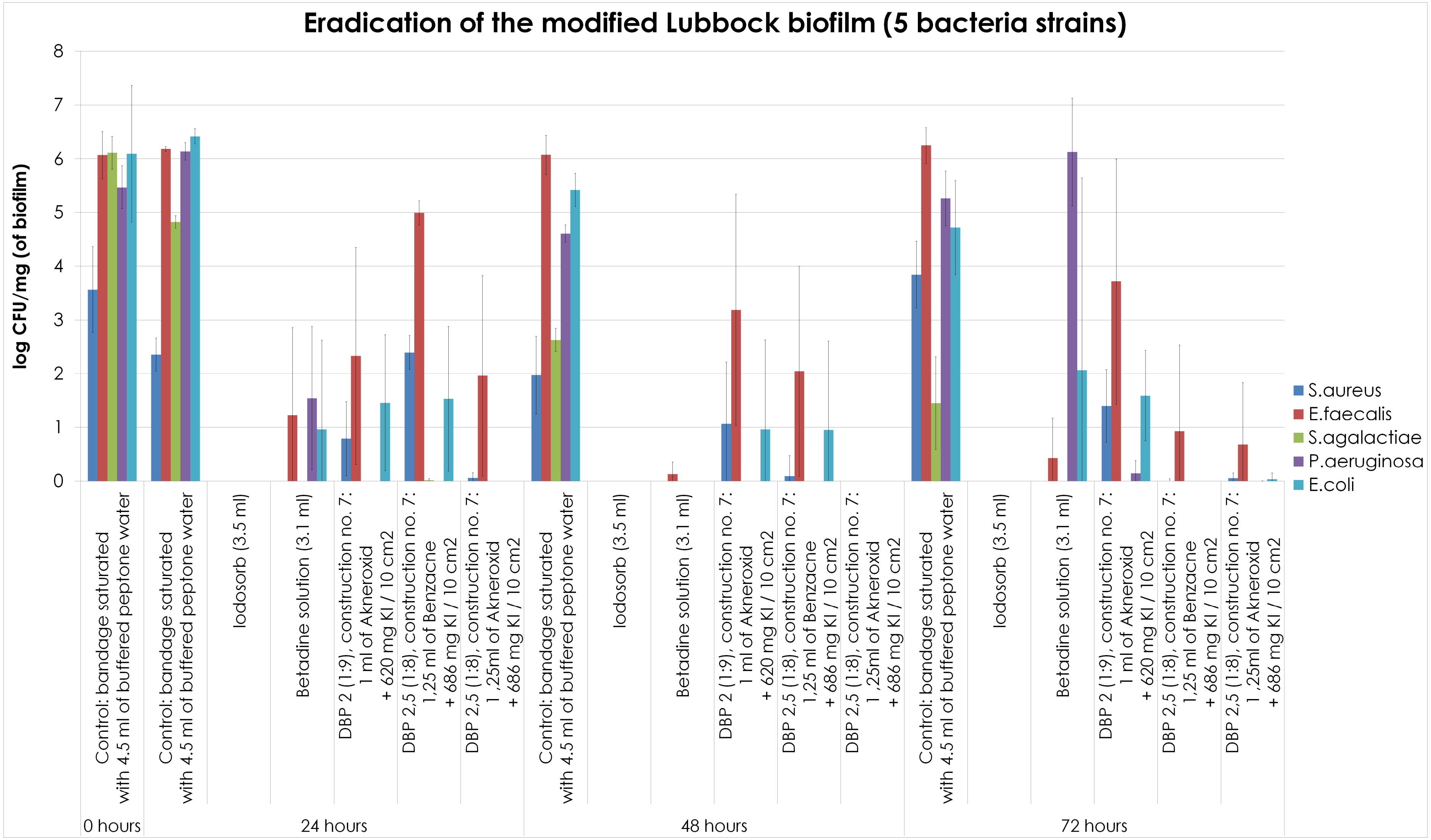
In the second model there were always used at least two controls: negative control (textile saturated with buffered peptone water) and a positive control (Betadine and/or Iodosorb). After 72 hours of incubation the biofilm was weaker and not as firm as after 24 or 48 hours (for negative control). However, it was more stable than the biofilm from the previous model.

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 Iodosorb was extremely effective during the experiment no. 9. It totally eradicated biofilm; there was no regrowth of bacteria even after 72 hours of treatment. (graph 5)

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*. 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. (graphs 4 and 5) 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 4. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-5 (modified).



Graph 5. The bacterial population counts 24, 48 and 72 hours after the application of the bandages with Iodine Generator. Test BF-9 (modified).

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 (graphs 4). 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. 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 4) 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.

Results concerning the kinetics of iodine release from the commercially available compositions coincide with data obtained from both *in vitro* models of biofilm. When Iodosorb was used, the kinetics of iodine release was comparable to Iodine Generator (DBP2 (1:10) with Akneroxid, 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 3). 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.

CONCLUSIONS

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.

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