Original Paper

Blood Purification

Blood Purif 2005;23:339–348
DOI: 10.1159/000086870

Received: November 16, 2004
Accepted after revision: April 19, 2005
Published online: July 7, 2005

A New Procedure Allowing the Complete Removal and Prevention of Hemodialysis Biofilms

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Key Words
Biofilm · Biomass detachment · Disinfection · Hemodialysis · Silicone tubing

Abstract
Most currently used disinfectants for dialysis machines have a good bactericidal efficacy on biofilms but leave dead cells on the surface. This contributes to the regrowth of biofilm and the release of pyrogens. A new anti-biofilm procedure consisting of sequential treatment combining enzymes and detergents is able to detach adherent cells. The efficacy of this procedure was assessed both in vitro and in reality. For in vitro studies, a biofilm model was set up. Studies were also performed in reality in a clinically used dialysis machine. Biofilm removal was first monitored by image analysis. Then, the biomass was detached by scraping and quantified by plate counts and endotoxin level measurement. Treated samples were compared to untreated control samples. The procedure led to the complete detachment of the biomass, both in vitro and in the reality situation. The aim of this procedure is to replace or complete the usual disinfection methods for medical devices.

Introduction

It has been clearly demonstrated that biofilms develop inside hemodialysis systems, notably on the inner surface of the silicone tubing of dialysis machines [1–3]. Biofilms are known to be a permanent source of recontamination [4] by releasing pyrogens that are allowed to cross the dialysis membrane and cause chronic inflammatory diseases among patients on dialysis [5]. It has now been established that to prevent infections biofilms must be banished from the surface of dialysis systems, as well as any medical device. Unfortunately, no available cleaning and/or disinfection procedure presents the necessary efficacy.

Dialysis machines are disinfected between each dialysis session, using products such as bleach, peroxyacetic acid, glycolic acid or citric acid. Various procedures have been recommended by different manufacturers depending on the product, the contact time and the temperature. Actually, heating the disinfectant solution is current practice, and reverse osmosis water heated at 90°C is also used as a disinfectant. However, previous studies have demonstrated that although the currently used disinfection procedures for dialysis machines have a good bactericidal efficacy on biofilms, they leave dead bacterial cells on the surface of the tubing [6]. This contributes to the release of pyrogens (mainly produced by dead cells) and favors the regrowth of new biofilm by increasing the

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Table 1. Different treatments tested in the study

Frequency	Preventive treatment	Corrective treatment
Regular use (between each dialysis session)	detergent 1%, 10 min, 85°C 15-min rinse	no regular corrective treatment
Periodical/single use	enzymes 0.1%, 10 min, 37°C 15-min rinse, then detergent 1%, 10 min, 85°C 15-min rinse	enzymes 0.1%, 20 min, 37°C 15-min rinse, then enriched detergent 1.5%, 10 h, 85°C 30-min rinse

roughness of the surface and feeding the bacteria. We concluded that the less efficient treatments were those performed at high temperature that 'bake' the biofilm on the surface, improving biomass adherence and endotoxin release. We realized that bacterial kill was necessary but not sufficient and that the use of a disinfectant that leaves biomass residuals on the treated surfaces was not the best way to eradicate the biofilms inside dialysis machines. Previous work showed that coverage reduction, i.e. the detachment of the biomass, is in fact the main parameter to take into account. Unfortunately, the conventional treatments tested were unable to completely detach the biomass from the surface.

These observations motivated the development of an effective procedure for biofilm detachment. A thorough literature survey led to the selection of products with enzymatic and detergent properties [7–13]. Sixty-five chemical mixtures were tested in vitro for their efficacy on biofilm detachment and only one among all the combinations tested gave promising results. This combination has been optimized to comply with the operating conditions of dialysis machine disinfection.

This paper describes the process for assessing the efficacy of this new anti-biofilm procedure both in vitro and in a reality situation. This efficacy was first assessed on different multi-species biofilms grown with the help of a biofilm reactor system mimicking the dialysis machine. Then, the efficacy and feasibility of the procedure was validated inside a dialysis machine in a dialysis facility (AURAL, Lyon, France).

Material and Methods

Anti-Biofilm Procedure

The anti-biofilm procedure tested is a new cleaning procedure called Pronetron® (Kazieltys Laboratoire, Lyon, France) that can be integrated in the process of dialysis disinfection. It is based on the sequential and synergetic action of the two following products:

Pronetron α®, the powder of a multi-enzymatic mixture containing amylases (60,000 European Pharmacopoeia units (EPU)/g), lipases (80,000 EPU/g) and proteases (3,000 EPU/g), and

Pronetron β®, an alkaline detergent solution (pH 10) containing hydrates, phosphates, polyphosphates and sodium hydroxide.

Moreover, an enriched formulation of this detergent solution (Pronetron β*Plus*®) is used for stronger cleaning. It contains non-ionic surfactants and 0.1% of sodium hypochlorite after dilution.

Two treatments are available: a preventive (or 'light') treatment, to be used between each dialysis session to remove adherent cells that have attached during the session, and a corrective (or 'enriched') treatment for 'one-step' cleaning to remove thicker biofilms.

The anti-biofilm action is related to the enzyme/detergent combination, but the preventive anti-adherence activity can be maintained for many months by using the detergent solution alone. However, the periodical use of the enzyme/detergent combination, even for a preventive action, is essential. The corrective treatment always includes the enzyme/enriched detergent combination. Enzymes are used first, then rinsed and followed by the detergent solution.

The treatments tested in this study are indicated in table 1. The concentrations of the products given (percentages) are the final concentrations after dilution. The same concentrations were used for in vitro experiments (manual dilutions) and for experiments in reality (dilution by the machine).

In vitro Studies

Biofilm Reactor System

The model used for the in vitro assessment of the efficacy of the procedure was similar to the one we previously developed while studying the anti-biofilm efficacy of the currently used disinfection procedures [6]. Briefly, the model consisted of a continuously stirred tank reactor supplied with a non-sterile dialysate made with sterile dialysis concentrates (Baxter, USA) and reverse osmosis water naturally contaminated by *Pseudomonas fluorescens, Flavimonas orizyhabitans* and *Stenotrophomonas maltophilia* providing a total level of 150 colony-forming units (CFU)/ml and 1.2 endotoxin units (EU)/ml. These levels were in accordance with current AAMI standards but a little higher than the upper limit of the European Pharmacopoeia standards [14, 15].

The dialysate was maintained at 37°C and circulated through a 1.5-m recycle loop made of silicone tubing (Masterflex® 7015, 5 mm ID) at a flow rate of 500 ml/min provided by a peristaltic pump (Masterflex® L/S).

This model was used to grow 5 different biofilms (biofilms 1, 2, 3, 5 and 6). The growth of all biofilms except biofilm 2 was accelerated by the addition of nutrients provided by a 1/50-diluted Luria Bertani broth (BD Difco).

Treatments

Two sets of experiments were performed: 'one-step' and 'multistep' experiments. A flow chart describing the different experiments is shown in figure1.

'One-Step' Experiments. For 'one-step' experiments, the products were used only once, subsequent to biofilm growth, to determine the removal activity of both preventive and corrective treatments. The complete preventive treatment (with the enzyme/detergent combination) was tested on biofilms 1–3. The corrective treatment was tested on biofilm 3. In these experiments, the biofilm reactor system was allowed to run until a mature biofilm develops on the surface of the silicone tubing (a mature biofilm is defined as confluent microcolonies separated by water channels). Then, the tubing covered with the biofilm was cut into 10-cm-long segments. Three samples were randomly selected to undergo treatment by the test procedure, and three samples were kept untreated as controls.

These experiments were completed by assessment of the efficacy of the corrective treatment on a real clinical biofilm sample taken from the connection between the water distribution loop and a dialysis machine in a dialysis center (biofilm 4). This sample was 1 year old and had never been treated by any disinfectant. It contained 10^3 CFU/cm^2, 115 EU/cm^2, and its coverage was 80%.

'Multistep' Experiments. For the 'multistep' experiments, performed on biofilms 5 and 6, the biofilm reactor system described above was slightly modified. Three tubing samples (10 cm in length) were connected together and the non-sterile dialysate, supplemented with Luria Bertani broth, was run through them for 4 h. Then, the system was stopped, the tubing was disconnected and attached to another peristaltic pump to undergo disinfection treatment. The samples were then reconnected and the contaminated media circulated for 4 h more. Three untreated 'control' samples were run in parallel. Two simulated dialysis sessions and 3 disinfection sessions were performed alternatively everyday. The system was stopped after the last disinfection. At night and over the weekend, the tubing samples were allowed to sit dry and static at room temperature. This system was run in this manner until a mature biofilm developed on the untreated samples.

Sample Treatments. Samples to be treated were attached to a system made of 2 tubes and a peristaltic pump. The different chemicals were run through the samples at a flow rate of 500 ml/min in a close recirculating system for a definite contact time specific to the product tested. The temperature of the chemical during treatment was controlled with a water bath. After each treatment, the samples were rinsed at room temperature at a flow rate of 500 ml/min with reverse osmosis water.

Reality Studies (Dialysis Facility)

Machine Equipment

A dialysis machine (Fresenius 4008B) was disconnected from the dialysis water distribution loop of the clinic and re-equipped to be used in a closed system. This new system was a recirculating loop continuously fed with contaminated dialysate. A Y-shaped inlet valve was used to alternatively draw in the dialysate or reverse osmosis rinse water. During the experiments the dialysate was allowed to recirculate in the close recycle loop at 600 ml/min, while at the beginning and the end of the experiments the rinse reverse osmosis water could be flushed through the system and out to waste at the same flow rate. The pre-made dialysate and reverse osmosis water were contained in 10- and 20-liter containers, respectively.

Dialysate and Bacterial Cultures

A second dialysis machine connected to the dialysis water distribution loop was used to prepare 7 liters of fresh dialysate before each dialysis session using acid (Standard acid 377A, Fresenius) and bicarbonate concentrates (Fresenius).

The reverse osmosis water used to prepare the dialysate as well as to rinse the machine was tested for contaminants prior to use and was shown to contain 23 CFU/ml and <0.005 EU/ml with *S. maltophilia* and *Acinetobacter baumannii* being the main bacterial isolates. The fresh dialysate contained 50 CFU/ml and 0.08 EU/ml. These levels are in accordance with current AAMI and the European Pharmacopoeia standards [14, 15]. The dialysate was inoculated with bacterial cultures prepared in 50-ml culture tubes (Fischer Bioblock) and made of 50% non-sterile reverse osmosis water and 50% Luria Bertani broth. After a 72-hour incubation, the number of culturable living cells reached $5 \cdot 10^9$ CFU/ml. Depending on the expected final bacterial concentration in the assay, a definite volume of bacterial culture was used to inoculate the dialysate.

Disinfection Treatments

For both preventive and corrective treatments, the detergent solution was drawn up by the 'UF pump' at the bottom of the machine, usually used for disinfection. The product was diluted by the machine to 1/25, leading to the required final concentration (table 1). Enzymes were drawn up by the pump, usually used for acetic acid or bleach, in front of the machine. The powder was manually pre-diluted to 1/3 with sterile water and then diluted by the machine to 1/35, leading to a final concentration of 0.1%.

For each trial, one external tubing (1 meter in length) was disconnected from the system during disinfection, so as to retain the biofilm for the purposes of an experimental control. During the trial and at the end of each trial, 3 external and 3 internal treated tubing samples (10 cm in length) and 3 untreated tubing samples were taken to monitor biofilm growth and determine the efficacy of the treatments (external tubing samples are the connections between the generator and the dialysis membrane; internal tubing samples are from the inside of the generator).

The study included the testing of both preventive and corrective treatments. Preventive treatments were assessed in three trials. In each case, the disinfections were performed between simulated 3.5- to 4-hour dialysis sessions. The machine was used for 2 dialysis sessions/day, after which it was disinfected using the anti-biofilm procedure. Between each dialysis session, the machine was disinfected with the detergent solution alone. At the end of the trial, the machine was disinfected with the enzyme/detergent combination. On the evenings and weekends, the dialysis machine was filled with reverse osmosis water subsequent to the last disinfection.

The first trial was performed using a highly contaminated dialysate to mimic operation in a contaminated system, and therefore the most rigorous challenge possible. Initially the dialysate con-

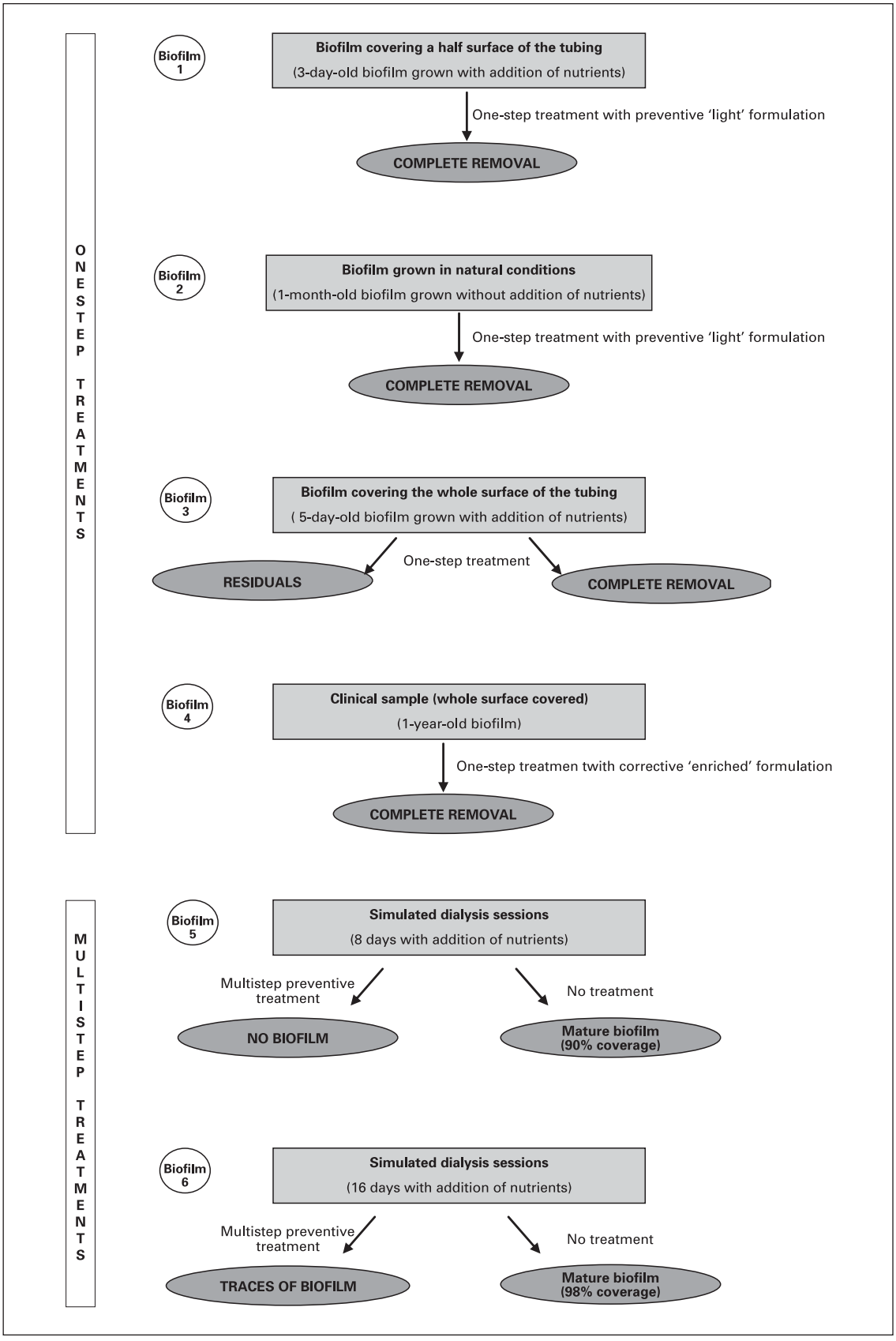
ONE STEP TREATMENTS
Biofilm 1
Biofilm covering a half surface of the tubing
(3-day-old biofilm grown with addition of nutrients)
One-step treatment with preventive 'light' formulation
COMPLETE REMOVAL
Biofilm 2
Biofilm grown in natural conditions
(1-month-old biofilm grown without addition of nutrients)
One-step treatment with preventive 'light' formulation
COMPLETE REMOVAL
Biofilm 3
Biofilm covering the whole surface of the tubing
(5-day-old biofilm grown with addition of nutrients)
One-step treatment
RESIDUALS
COMPLETE REMOVAL
Biofilm 4
Clinical sample (whole surface covered)
(1-year-old biofilm)
One-step treatmen twith corrective 'enriched' formulation
COMPLETE REMOVAL
MULTISTEP TREATMENTS
Biofilm 5
Simulated dialysis sessions
(8 days with addition of nutrients)
Multistep preventive treatment
No treatment
NO BIOFILM
Mature biofilm (90% coverage)
Biofilm 6
Simulated dialysis sessions
(16 days with addition of nutrients)
Multistep preventive treatment
No treatment
TRACES OF BIOFILM
Mature biofilm (98% coverage)

Table 2. In vitro studies (n = 3): characteristics of the 6 different biofilms used in the study (untreated samples; mean ± SD)

Biofilm	Addition of nutrients (LB broth)	Growing time, days	Coverage %	CFU/cm^2	EU/cm^2
1	yes	3	60 ± 7	$10^4 \pm 1.7 \cdot 10^3$	1,040 ± 230
2	no	30	50 ± 13	$10^3 \pm 560$	40 ± 4
3	yes	5	98 ± 1	$10^8 \pm 2 \cdot 10^7$	35,200 ± 6,430
4	clinical sample	1 year	80 ± 5	$10^3 \pm 340$	115 ± 12
5	yes	8	90 ± 3	$2 \cdot 10^7 \pm 5 \cdot 10^6$	6,600 ± 860
6	yes	16	99 ± 0.5	$3 \cdot 10^9 \pm 5 \cdot 10^8$	65,000 ± 12,860

tained approximately $5 \cdot 10^7$ CFU/ml (the initial bacterial level at the beginning of the dialysis session) by adding 4 ml of bacterial culture to the 7 liters of fresh dialysate. The total dialysis duration time was 21 h (3 days with 2 dialysis sessions/day). This duration time was chosen in accordance with the in vitro experiments (a mature biofilm developed within 3 days).

In order to simulate real clinical dialysis conditions, a second trial was performed in which the 7 liters of dialysate were used without any addition of bacterial culture.

For corrective experiments, the untreated tubing from the first preventive trial covered by the thickest biofilm was reconnected to the dialysis machine and treated with the corrective procedure.

The 2 last experiments examined the preventive efficacy of peroxyacetic acid and citric acid used instead of the Pronetron® procedure. The trial using the highly contaminated dialysate ($5 \cdot 10^9$ CFU/ml) was repeated twice, 21 h each, first using peroxyacetic acid (37°C, 10 min) and then citric acid (85°C, 10 min).

Samples Analysis

Three main parameters were measured to assess the efficacy of the tested procedure versus untreated controls: coverage reduction, culturable bacteria reduction and endotoxin level reduction. The methods used for biofilm analysis were those described by Marion-Ferey et al. [16] and are summarized below.

Quantitative Measurement of the Coverage

Tubing samples were stained by either a 0.25% crystal violet solution (BD Difco) or the bacterial viability kits (L7012, Molecular Probes) allowing the discrimination between the living cells (green) and dead cells (red or yellow). Then, the inner surface of the tubing was imaged with an optical microscope and an attached CCD camera (model ST-133, Princeton Instruments). The surface coverage was then determined using the image analysis software Scion Image. The treated samples and untreated controls were compared to determine the efficacy of the treatments. For each experiment, the coverage of the controls was arbitrarily set to 100%. The quantification limit of the method was 0.05%.

Some samples were additionally analyzed with a confocal scanning laser microscope (Model TCS-NT, Leica Microscope) to provide 3-dimensional images and structural information.

Fig. 1. Flow chart describing the experimental set-up (in vitro studies).

Culturable Bacteria Quantification

The biofilm was first detached from the surface of the tubing by scraping with the help of a 'mechanical biofilm scraper'. Then the culturable bacteria were enumerated in the resulting bacterial suspension by CFU counting on R_2A agar (Reasonner's medium) after a 7-day incubation period at room temperature. The quantification limit of the method was 0.1 CFU/cm^2.

Endotoxin Level Measurement

After detachment by scraping, pyrogens were quantified in the bacterial suspension using the LAL chromogenic kinetic assay [17]. The quantification limit of the method was 0.005 EU/cm^2.

Interpretation of Results

The efficacy of the treatment was assessed by comparison between treated and untreated samples. Samples in which neither biofilm bacteria nor toxins were found, or coverage was measured, were labeled as 'below or equal to the detection limit' of the method.

Results

In vitro Studies

Biofilm Growth

The biofilm reactor systems that we developed for in vitro studies allowed the growth of five different mature biofilms on the control tubing samples. The characteristics of these biofilms (growth conditions and biomass quantification) are described in table 2.

'One-Step' Experiments

Biofilms 1 and 2 were completely removed from the surface of the samples treated by the preventive procedure, whereas the same treatment left a few adherent cells on the surface of the tubing 3 covered by a thicker biofilm. The corrective treatment performed on biofilms 3 and 4 led to their complete detachment from the surface of the tubing samples.

Quantitative results showing the efficacy of the preventive and corrective treatments used as a 'one-step'

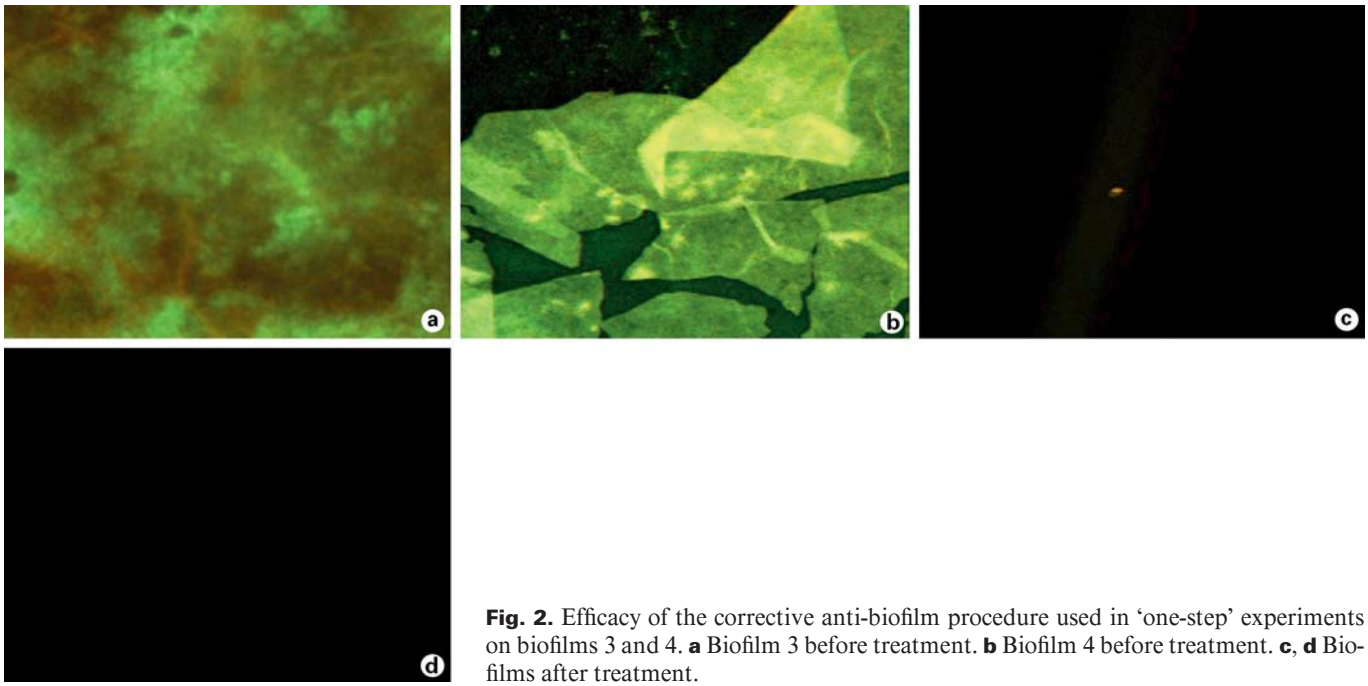

Fig. 2. Efficacy of the corrective anti-biofilm procedure used in 'one-step' experiments on biofilms 3 and 4. **a** Biofilm 3 before treatment. **b** Biofilm 4 before treatment. **c**, **d** Biofilms after treatment.

Table 3. In vitro studies (n = 3): quantitative results showing the efficacy of the tested procedure when used in 'one-step' or 'multistep' experiments

Biofilm	Treatments	Experiments	Coverage %	CFU/cm^2	EU/cm^2
1	preventive	one-step	<0.05	<0.1	<0.005
2	preventive	one-step	<0.05	<0.1	<0.005
3	preventive	one-step	15 ± 3[a]	9 ± 2	0.7 ± 0.2
3	corrective	one-step	<0.05	<0.1	<0.005
4	corrective	one-step	<0.05	<0.1	<0.005
5	preventive	multistep	<0.05	<0.1	<0.005
6	preventive	multistep	4 ± 1	<0.1	0.4 ± 0.15

[a] Means ± SD.

treatment on biofilms 1–4 are given in table 3 and shown in figure 2.

'Multistep' Experiments

The growth of biofilm 5 was completely inhibited on the samples treated repeatedly by the preventive procedure. Conversely, a mature biofilm developed on the controls, as shown in table 2. The biofilm 6 control tubing samples were covered by a very thick biofilm containing a large amount of slime and living cells (more than 10^9 CFU/cm^2). Only a few adherent dead cells remained on the surface of the samples repeatedly treated by the preventive procedure. Quantitative results showing the efficacy of the preventive treatment used repeatedly on biofilms 5 and 6 are given in table 3.

Reality Studies

Preventive Treatments

Trial Using a Contaminated Dialysate. Bacterial colonization of 1,120 CFU/cm^2 and 45 EU/cm^2 developed on the untreated samples after only 2 dialysis sessions. After 16 h and 10 min, the corresponding biofilm contained $2 \cdot 10^5$ CFU/cm^2 and 998 EU/cm^2; after 21 h of simulated dialysis sessions, the surfaces of the untreated samples were covered by a thick mature biofilm containing $1.4 \cdot 10^8$ CFU/cm^2 and 46,410 EU/cm^2.

Subsequent to treatment with the detergent solution, the inner surfaces of the treated samples showed a few isolated adherent cells (coverage 0.4%) that were then removed after treatment with the enzyme/detergent combination. Quantitative results are given in table 4, and the corresponding pictures are shown in figure 3.

Trials Using a Fresh Dialysate. The biofilm that grew on the control sample developed more slowly, so the total experimental length was increased to 106 h (approximately 26 dialysis sessions of 4 h each). The quantitative values corresponding to biofilm development as a func-

Fig. 3. Efficacy of the preventive anti-biofilm procedure used in a dialysis machine highly contaminated with a dialysate containing approximately $5 \cdot 10^7$ CFU/ml. **a** Untreated tubing. **b** Treated external tubing. **c** Treated internal tubing.

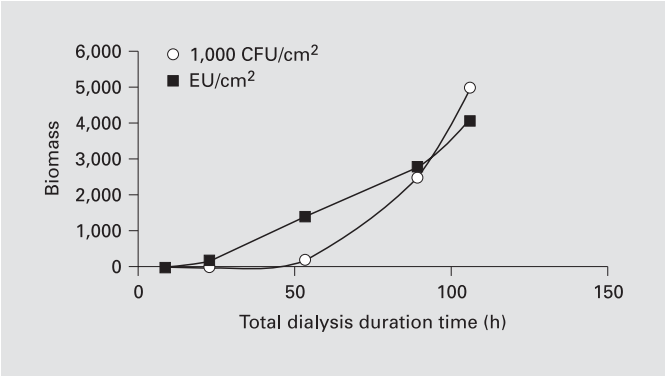

Fig. 4. Biofilm development on untreated tubing during in vivo experiments as a function of time (trial using a fresh dialysate).

Table 4. Reality studies (n = 3): quantitative results of the different trials on a real dialysis machine (mean ± SD)

Trial	Untreated tubing		Treated tubing		
	CFU/cm^2	EU/cm^2	CFU/cm^2	EU/cm^2	% coverage
Preventive treatment (contaminated dialysate)	$1.4 \cdot 10^8 \pm 3 \cdot 10^7$	46,410 ± 7,430	**<0.1**	**0.005 ± 10^3**	**0.05**
			<0.1	*0.005 ± 10^3*	*0.05 ± 0.01*
Preventive treatment (fresh dialysate)	$5 \cdot 10^6 \pm 10^5$	2,920 ± 698	**<0.1**	**0.005 ± 10^4**	**<0.05**
			<0.1	*0.005 ± 5.10^4*	*<0.05*
Corrective treatment	$1.4 \cdot 10^8 \pm 3 \cdot 10^7$	46,410 ± 7,430	**<0.1**	**<0.005**	**<0.05**
			<0.1	*<0.005*	*<0.05*
Preventive treatment with peroxyacetic acid	$10^6 \pm 2 \cdot 10^5$	4,900 ± 850	**<0.1**	**4.4 ± 0.2**	**35 ± 3.6**
			0.1 ± 0.1	*6.3 ± 0.4*	*50 ± 6*
Preventive treatment with citric acid	$1.7 \cdot 10^6 \pm 2.6 \cdot 10^5$	4,700 ± 880	**0.2 ± 0.1**	**3.8 ± 0.2**	**35 ± 5**
			0.2 ± 0.1	*6.1 ± 0.5*	*50 ± 4*

Bold type = External tubing; italic type = internal tubing.

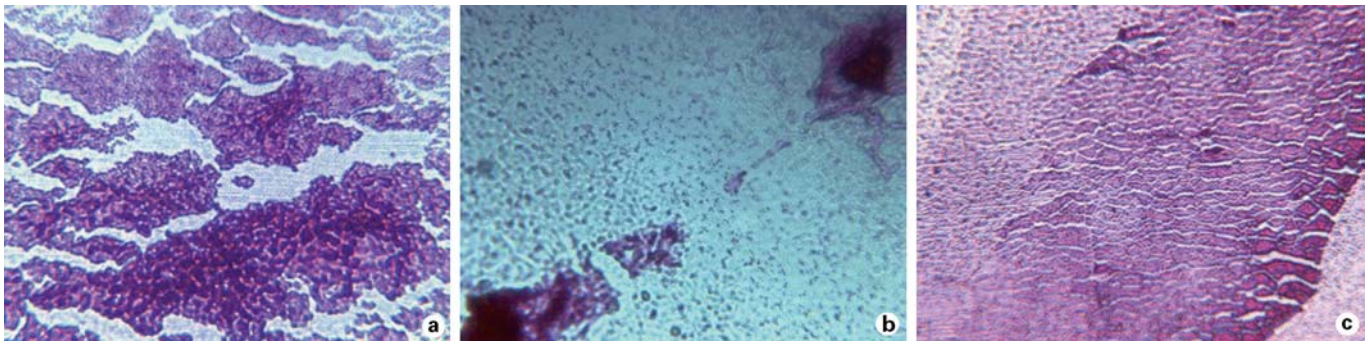

Fig. 5. Preventive action of peroxyacetic acid on biofilm (in vivo experiments). **a** Untreated tubing. **b** External tubing treated by peroxyacetic acid. **c** Internal tubing treated by peroxyacetic acid.

tion of time are given in figure 4. It illustrates what could happen inside a never disinfected dialysis generator after 1 month.

A few isolated adherent cells began to appear after 53 h of dialysis on samples treated by the detergent alone. No more cells could be found at the end of the trial subsequent to treatment by the enzyme/detergent combination. Table 4 gives the quantitative results of the preventive efficacy of the anti-biofilm procedure in this last trial.

The dialysate from the 10-liter container was analyzed before the dialysis session, and after 4 h of recirculation. The level of microorganisms and pyrogens in the fresh dialysate was in accordance with the American and European standards, but it had increased significantly by the end of the dialysis session (approximately 5,000 CFU/ml and 175 EU/ml). This was due to the recirculation of the dialysate for 4 h at 37°C and to a known biofilm that developed inside a portion of the water inlet pipe that was never disinfected. Once again the increase in the contamination level with time represents the worst conditions for testing.

Corrective Treatment

Subsequent to corrective treatment, no more biofilm could be found on the inner surface of the tubing previously covered by the thickest biofilm ($1.4 \cdot 10^8$ CFU/cm^2, 46,410 EU/cm^2). Table 4 shows the corrective efficacy of the anti-biofilm procedure on the removal of this biofilm.

Treatments Using Current Disinfectants

Peroxyacetic Acid. After 21 h of simulated dialysis, the inner surface of the untreated tubing was determined to be covered by a biofilm of 10^6 CFU/cm^2 and 4,900 EU/cm^2. After treatment by peroxyacetic acid, 3 external tubing samples were analyzed and determined to contain a biofilm of 35% surface coverage, made up of mostly dead cells (<0.1 CFU/cm^2) and containing 4.4 EU/cm^2. Three tubing samples taken from the inside of the machine at the end of the trial had a 50% biofilm coverage. Table 4 and figure 5 show the preventive action of peroxyacetic acid on biofilm.

Citric Acid. After 21 h of dialysis, the inner surface of the untreated tubing was covered by a biofilm similar to previous experiment ($1.7 \cdot 10^6$ CFU/ cm^2 and 4,700 EU/cm^2). After treatment by citric acid, 3 external tubings were analyzed and determined to contain a biofilm of 0.2 CFU/cm^2, 3.8 EU/cm^2 and 35% coverage. Similar to the peroxyacetic acid results, the 3 internal tubing samples still had a 50% coverage. Table 4 shows the quantitative results related to the preventive action of citric acid on biofilm.

Efficacy of the Anti-Biofilm Procedure on Existing Biofilms. The corrective Pronetron® procedure was applied to remove the biofilm previously developed on the generators disinfected with peroxyacetic and citric acids. After treatment, no more biofilm could be found on the surface of the tubing.

Discussion

This study assessed the anti-biofilm efficacy of a new anti-biofilm procedure both in vitro and in reality. The results highlight the great efficacy of this procedure for the removal of mature biofilms using the corrective treatment, and for the prevention of bacterial colonization

using the preventive treatment. The specificity of this procedure is its ability to detach the different components of the biofilm from the contaminated surface. Our hypothesis is that enzymes digest most parts of the extracellular matrix, preparing the action of the detergent solution. Then, the detergent detaches the remaining biomass and kills the bacteria, so that the whole biofilm including pyrogens is removed. The synergetic combination of both products applied in this order is essential to obtain the optimal detachment efficacy. When used as a preventive treatment, the detergent alone can face the adherent cells. The few cells that attach to the surface of the tubing during the dialysis session are not sufficient to create a mature biofilm within 3 or 4 h. However, the use of a conventional disinfectant between each dialysis session leaves adherent cells that finally accumulate on the surface and form a mature biofilm within time. Even though the detergent is more efficient than disinfectants for the removal of adherent cells, it is not sufficient by itself to detach 100% of those cells. This is the reason why the addition of enzymes is needed from time to time.

This study shows that the tested procedure is able to detach as well as to prevent thick biofilms containing more than 10^8 CFU/cm^2. We decided to test this procedure on biofilms thicker than those usually found inside dialysis machines. It showed its high efficacy under even the worst conditions, so it can guarantee the perfect cleanness of the machine under usual operating conditions.

The bacterial resistance that develops by the regular use of the same disinfectant is not a problem with this new procedure because of the cleaning step which removes bacteria from the system preventing resistance development. A regular use of the procedure is possible and does not involve the use of other conventional disinfectants.

The use of enzymes, detergents and/or disinfectants for biomass detachment has been suggested previously, however the efficacy of this procedure has never been shown. The idea was first proposed by researchers studying biofilm development on dental systems (tooth, dental water lines). Le Magrex et al. [7] tested different disinfectants on dental biofilms, and in their conclusions they mentioned interest in biomass detachment with enzymatic treatment. This idea was continued by Jacquelin et al. [8] who showed the synergetic action of enzymes and phenolic disinfectant. They concluded that enzyme treatment had to be performed prior to the action of the disinfectant [8]. This is in accordance with the procedure described here. But the enzymes were tested separately and were only selected among the glycosidases. The results showed no complete biomass detachment. Similar results were obtained by Johansen et al. [10].

Most enzymes tested by these authors were tested in our laboratory in vitro and showed a similar low efficacy (data not shown). Additionally, we also tested many different detergent formulations in combination with our enzyme mixture, but none was as efficient as the selected alkaline detergent solution. The thorough examination of previous patents concerning the association between enzymes and detergent for biofilm removal led to the same conclusions. The enzymatic formulations tested were not complex enough and the detergent was not suitable to allow a complete biofilm removal.

In addition to its ability to fight against biofilms, this procedure presents many other advantages. We demonstrated that the detergent solutions (enriched or not) have bactericidal, virucidal and fungicidal properties in accordance to the European Standards for disinfection [18] when used at their respective final concentrations (table 1) for 10 min at 85°C (data not shown). The detachment properties of the products are not affected by temperature. Additionally, this procedure will not 'bake' the biomass on the surface of the tubing like other products used at high temperature [6] because the biomass is detached and eliminated.

As opposed to all classical disinfectants (acids, bleach, etc.), both enzyme and detergent products are odorless. The detergent solution, which is the most regularly used product, has a low toxicity (not irritant for skin and eyes, LD_{50} IV = 5 g/kg). Both enzyme and detergent products have been tested for their compatibility with 13 different spare parts of a dialysis generator (e.g. electric floodgates, seals, conductivity probes, silicone tubing, pumps, connectors) according to the French standard for corrosion [19]. Similar spares were treated with sterile distilled water at the same temperature (85°C) in parallel (controls). Spares were observed using scanning electron microscopy and no significant differences were found between controls and treated samples.

Only a 10-min rinse is needed between enzymes and detergent because the enzymes are completely and immediately decomposed by the detergent and the high temperature of the treatment. After a final 15-min rinse, the quantification of organic and inorganic carbon by high-performance chromatography showed no more residue, proving the complete elimination of both products. The use of specific semiquantitative sticks (proteins for the enzymes, pH for the detergent solution and chlorine for the enriched detergent solution) have been validated for

use as a bedside method to measure the residual level of the products.

Disinfection habits have been taken into account to include this procedure in the disinfection process of dialysis machines. In order not to increase the duration of the process, the use of the detergent alone (Pronetron β®: final concentration after dilution 1%, contact time 10 min, temperature >85°C, rinsing time 20 min) is recommended between each dialysis session. Then, as a precaution, it is recommended that the enzyme mixture (Pronetron α®: final concentration after dilution 0.1%, contact time 10 min, temperature 37°C, rinsing time 20 min) in combination with the enriched formulation of the detergent solution (Pronetron β*Plus*®: final concentration after dilution 1.5%, contact time 10 min, temperature >85°C, rinsing time 20 min) be used every 6 months on machines equipped with dialysate ultrafilters, and every 3 months on other machines.

The problem of biofilm development in dialysis systems is still seriously considered by the nephrologists and the dialysis machine manufacturers. Many researchers are currently searching for solutions to this problem, but conventional disinfectants remain the only products available. The idea of biomass detachment is a very new approach and represents an existing and viable solution to the problem of biofilm persistence. The use of this procedure in combination with other preventive methods against biofilm development, such as systems producing highly purified water and dialysate ultrafiltration, could provide the highest microbiological security for patients on dialysis [20, 21].

Complementary developments are currently being studied to apply this new anti-biofilm procedure for the disinfection of dialysis water treatment systems. It could be interesting to associate this chemical treatment from time to time with new physical disinfection treatments, including UV radiation and ozone, that seem to have a preventive anti-biofilm efficacy on water treatment systems [22]. That way, the complete cleanliness of the inner surface of the hydraulic system could be maintained.

The use of this procedure will also be extended to many other fields such as the disinfection of dental water lines and endoscopes, and the cleaning of processing systems in the food industry.

Acknowledgements

We are grateful to Dominique Raclet, Stéphane Jakubowski and Sébastien Fournier from the Technical Department of the association AURAL for their technical assistance.

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