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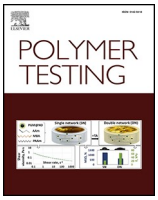

Controlling endotoxin contamination in PDMS-based microfluidic systems for organ-on-chip technologies

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A B S T R A C T

In the past decade, organ-on-chip (OoC) systems have gained significant attention as advanced platforms for replicating the physiological microenvironments of various organs. These microfluidic devices allow *in vitro* cell cultures at the microscale, integrating biophysical and biochemical cues that traditional cell culture models cannot reproduce. A critical aspect of their functionality is sterilization, which is necessary to eliminate bacterial contamination. However, conventional sterilization methods often fail to remove bacterial endotoxins, such as lipopolysaccharides (LPS) from Gram-negative bacteria. These endotoxins are potent pyrogens that can induce fever and profoundly alter cellular behavior, thereby compromising the reliability and accuracy of OoC models. This issue is particularly challenging for OoC systems fabricated with hydrophobic materials like polydimethylsiloxane (PDMS), which readily adsorb endotoxins.

In this study, we developed an analytical method to quantify endotoxin levels in PDMS-based microfluidic devices using a Limulus Amoebocyte Lysate (LAL) assay. We tested two groups of devices: those made with PDMS from a batch opened for over a year ("1-year-old PDMS"), and others made with PDMS from a batch opened for just one month ("1-month-old PDMS"). We also compared contamination levels after 1 h and 1 week post-sealing by oxygen plasma treatment. Storage time (period from sealing to testing for endotoxin) displayed critical for contamination level, revealing that oxygen plasma treatment is effective in reducing endotoxin from PDMS surfaces. This result was also confirmed by FTIR analysis.

Our findings emphasize the critical need for rigorous contamination control in the manufacturing of OoC systems to ensure they are not only sterile but also endotoxin-free. Achieving this dual standard is essential for maintaining the reliability and performance of these innovative platforms in biomedical research and therapeutic development.

1. Introduction

During the recent decade, organ-on-chip (OoC) systems have been extensively utilized to replicate physiological microenvironment of several organs such as the gut, heart, liver, bone, kidney, lung and brain [1–3]. An OoC is a microfluidic device allowing for *in vitro* cell cultures organized at the microscale with the incorporation of biophysical/biochemical signals, whereas traditional cell culture models cannot [4]. Sterilizing these microfluidic devices before using them is mandatory to remove any bacterial contamination [4]. While the common sterilization methods employed in the laboratory setting, such as UV or ethanol treatment, can successfully destroy microbial contaminants, are not indicated to remove or inactivate bacterial endotoxins [5].

Endotoxins, such as lipopolysaccharide (LPS) found in the outer membranes of Gram-negative bacteria, are referred to as pyrogens because they can induce fever when introduced into the human body [6]. A single *Escherichia coli* contains about 2 million LPS molecules per cell, that shed into their environment in small amounts when they are actively growing, and in large amounts when they die [7,8]. Given the widespread use of bacteria for manufacturing therapeutic drugs, vaccines, and pharmaceutical formulations, endotoxin contamination poses a significant risk in this industry. To address these issues, international regulatory bodies that oversee public health have established guidelines for acceptable endotoxin concentrations in various medical products. These include oral and injectable medications, water used in hospitals, and devices implanted in the body. The Limulus Amoebocyte Lysate (LAL) assay is one of the most widely used methods for detecting endotoxins and is approved by most of the international regulation agencies [9]. The LAL test has also been utilized for testing endotoxin also in further contexts, such as in food industry [10], in nanomaterials [11], and to verify safe work environments in ranching and farming [12].

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As pervasive environmental contaminants, endotoxins are not limited to medical supplied but can be detected in all chemicals and glassware and in almost all raw materials involved in a biomedical laboratory. Its high thermostability makes it resistant to the majority of the sterilization methods usually applied (e.g. standard autoclaving) [13]. In the past, Gobert et al. found that in the laboratory environment where biomaterials are synthesized and their biocompatibility assessed, endotoxin contamination was hard to avoid and advocated routine testing of endotoxin on biomaterials and reagents [14]. Nevertheless, the issue of potential endotoxin contamination in biomaterials intended for clinical applications is often overlooked, resulting in inaccurate bioactivity interpretations and possibly causing unexpected health problems. In particular, endotoxin contamination of biomaterials can significantly impact the reliability of new 3D models. Recently, Heinrich et al. demonstrated the effects of high levels of endotoxins in commercially-available gelatin on the macrophage-cancer cell crosstalk in a 3D bioprinted co-culture model [15]. They showed that endotoxin contamination in a 3D *in vitro* model can significantly alter intra- and intercellular communication and thereby drug efficacy, which might lead to misinterpretation of the potency and safety of the tested compounds. In general, endotoxins in *in vitro* models can interfere with cellular signaling pathways, resulting in increased or decreased cellular proliferation, differentiation, and apoptosis. Such contamination can cause significant deviations in experimental data, affecting the reproducibility and accuracy of the results [8,16,17].

Likewise, endotoxins can have a significant influence on cell behavior and the reliability of *in vitro* models based on OoC. Because of their hydrophobicity, soluble endotoxins tend to have strong affinities for other hydrophobic materials, especially some plastic products used in the laboratory [8,18]. Mostly OoC systems are based on microfluidic devices made of polydimethylsiloxane (PDMS), thus their surfaces are highly hydrophobic. However, the presence of endotoxins in microfluidic devices is an overlooked problem. During the fabrication of microfluidic devices for OoC applications, it is crucial to identify and mitigate potential sources of endotoxin contamination to ensure device sterility and functionality.

In this study, we examined the impact of material age and post-fabrication handling on endotoxin levels in PDMS-based microfluidic devices. Two groups of devices were fabricated: one using PDMS from a batch opened for over a year, which had undergone multiple material withdrawals, and another using PDMS from a batch opened for just one month. All devices were sealed using oxygen plasma treatment, and their endotoxin levels were assessed at two time points: within 1 h of sealing and one week post-sealing. Our findings revealed that devices made from a PDMS batch opened for over a year, despite being stored and handled exclusively in cleanroom environments, showed significant endotoxin contamination. Using FTIR analysis and endotoxin quantification methods (LAL assay), we demonstrated that plasma treatment effectively reduced endotoxin contamination. Notably, oxygen plasma sealing effectively reduced endotoxin levels, particularly when the devices were used within an hour of the sealing process. Conversely, prolonged storage after sealing resulted in a resurgence of endotoxin levels, emphasizing the importance of timely device utilization.

This study introduces an analytical method for quantifying endotoxins in PDMS-based microfluidic devices and highlights key contamination risks during the fabrication process. To mitigate endotoxin contamination, we recommend using fresh PDMS batches, minimizing material exposure, implementing rigorous sealing protocols, and reducing delays between device sealing and application. These strategies are essential for maintaining sterility and reliability in microfluidic devices designed for sensitive biological applications.

2. Experimental section

2.1. Fabrication and classification of microfluidic devices

Microfluidic devices, also known as chips, were fabricated in a cleanroom environment using replica molding in PDMS (Sylgard 184; Dowsil). These devices featured a double-layer design with a single channel measuring 60 μm in height, 5 mm in width, and 30 mm in length [Fig. 1]. To create the PDMS slabs for the microchannel layer and the flat layer, a PDMS pre-polymer mixture (10:1 elastomer base to curing agent, by weight) was degassed and poured onto silicon wafers patterned using optical lithography with SU-8 2075 (Micro resist technology) and onto a Petri dish, respectively, then cured for 15 min at 140 °C. After polymerization, the flat layer was punched with a 1.5 mm diameter biopsy punch to create inlet and outlet holes. The two layers were then sealed together using oxygen plasma treatment (60 % oxygen, 50 % power, 1 min) with a plasma cleaner (Diener electronic).

The chips were divided into two groups: a) those tested within 1 h of sealing ("t_0"), and b) those tested one week post-sealing ("t_7"). Chips were stored in a sterile and closed Petri dish, sailed with parafilm. Additionally, the chips were made using two different batches of PDMS. The first set was fabricated with PDMS from a batch opened for over a year ("1-year-old PDMS"), which had experienced numerous material withdrawals but always in a clean room environment. The second set was made with PDMS from a batch opened for just one month ("1-month-old PDMS"). Finally, all chips in both sets were exposed to UV light for 30 min under a biosafety cabinet before use to ensure sterility.

2.2. Endotoxin extraction from microfluidic devices

We evaluated the microfluidic devices for endotoxin contamination as medical devices. According to the US Food and Drug Administration (FDA) protocol, the recommended procedure for testing endotoxin contamination involves soaking or immersing the device or flushing the fluid pathway with extracting fluid heated to 37.0 ± 1.0 °C, ensuring the fluid remains in contact with the relevant surfaces for at least 1 h (USP <85> Bacterial Endotoxins Test) [19]. Typically, the extracting fluid used is LAL Reagent Water (endotoxin-free pure water). Accordingly, the chips prepared as described earlier (in triplicate for each group) underwent endotoxin extraction from the channel walls by elution for 1 h at 37 °C using LAL Reagent Water. After incubation, the contents of each chip (5 μL of volume) were collected, and the corresponding eluate was transferred into a reaction tube for the LAL assay. The microchannel chips were filled, and the LAL reagent water was collected post-incubation, under a biosafety cabinet to prevent any potential environmental contamination.

2.3. Chromogenic LAL assay

For the quantification of endotoxins, a chromogenic LAL Kit assay (ToxinSensor by Genscript) was performed (under a biosafety cabinet). This assay allows for the measurement of absorbance of the eluates once they have reacted with the kit's reagents, and the calculation of the corresponding concentration of endotoxins (EU/mL) with reference to a calibration curve.

2.4. Contact angle

For the wettability study of the 1-year-old PDMS and 1-month-old PDMS, water contact angles (WCA) were measured with a CAM 200 (KSV Instruments Ltd., Finland) instrument. A 5 μL water drop was generated and deposited on the PDMS slabs and contact angle measurements were acquired. The study was performed on these samples (Ctrl) and on the same samples soon after plasma treatment (at t_0) and after 7 days from plasma treatment (at t_7).

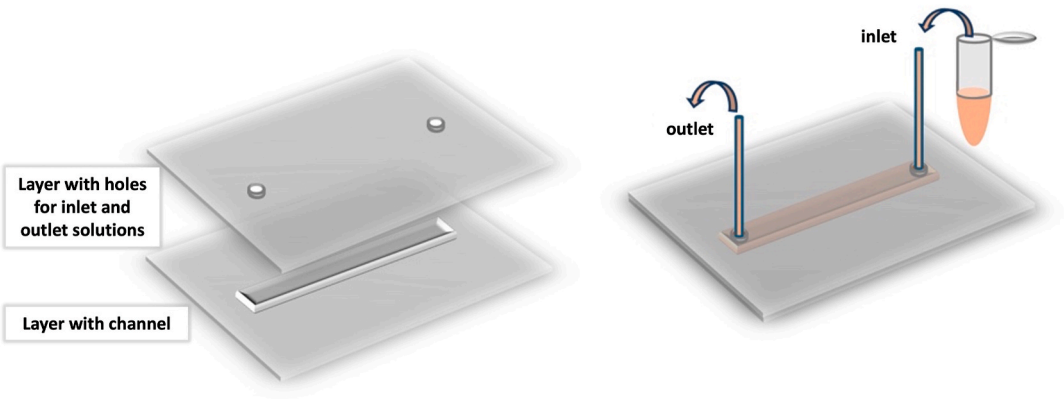

Fig. 1. Scheme of microfluidic devices fabrication. Two PDMS slabs, one with the microchannel pattern and the other with two holes corresponding to the inlet and outlet of the microchannel, are assembled after their inner surfaces are exposed to oxygen plasma treatment.

2.5. FTIR analysis

For the detection of endotoxins on the PDMS surface FTIR spectra were acquired with a PerkinElmer Spectrum One Spectrophotometer equipped with ATR tool (Attenuated Total Reflection mode) with spectral resolution of 4 cm^{-1}. Each spectrum was recorded in the range 4000–800 cm^{-1} and 32 scans were repeated for each measurement. Four PDMS samples were prepared and drops (20 μL) of endotoxin solutions at different concentrations (10, 1, 0.1 and 0.05 EU/mL) were deposited and let dry overnight.

To explore the ability of oxygen plasma treatment to remove surface endotoxin, PDMS samples contaminated with 10 and 1 EU/mL endotoxin solutions were also studied with FTIR measurements before and after oxygen plasma.

2.6. Statistical analysis

All experiments were performed at least three times or repeated in three batches of independent experiments. Data were presented as the mean ± standard deviations (SD).

3. Results and discussion

3.1. Endotoxins detection on microchannels of PDMS-based devices

Due to the small volume of eluates obtained from the chips (samples) and their high dilution, the calibration of the LAL assay was obtained using comparable volumes of standard solutions (SS) of endotoxins at known concentrations (0.05, 0.025, 0.01 EU/mL).

The LAL assay conducted on the eluates from PDMS-based chips revealed the following: i) all chips fabricated from 1-month-old-PDMS were free of endotoxins; conversely, ii) chips fabricated from 1-year-old PDMS exhibited fluctuating levels of endotoxin contamination based on the duration between sealing and testing ("storage time") (Table 1).

Therefore, it was apparent that more withdrawals from a PDMS batch result in heightened endotoxin contamination both in the PDMS batch and on the chip channel surfaces. Specifically, 1-year-old PDMS chips tested one week post-sealing (t_7) had a contamination level of 0.072 (±0.034) EU/mL, while those tested within 1 h of sealing (t_0) had a contamination level of 0.011 (±0.005) EU/mL, indicating a significant increase in contamination levels, corresponding to an almost seven times greater contamination for chips with longer storage time.

Additionally, the WCA readings on PDMS slabs employed in chip production revealed that 1-year-old PDMS exhibited greater contact angle values than 1-month-old-PDMS, regardless of whether the plasma treatment occurred 1 h (t_0) or 1 week prior (t_7) (Fig. 2). Therefore, the water wettability of the surfaces of contaminated devices (made with 1-year-oldPDMS) is reduced compared to the uncontaminated devices (made with 1-month-old PDMS). From these results, it emerges that the wettability degree of plasma-treated PDMS slabs could become a significant parameter for evaluating the quality of PDMS devices in relation to the presence or absence of endotoxins. Conversely, when evaluating WCA values of chips made from 1-year-old PDMS exposed to different storage times, it appears that the plasma treatment applied for sealing affects the contamination levels on the channel surfaces, leading to a temporary reduction that can be reversed.

The phenomenon of hydrophobic recovery in plasma-treated PDMS is well-documented, resulting from the movement of polymer chain segments [20]. The primary objective of plasma treatment is to enhance the hydrophilicity of PDMS surfaces. Oxidative plasma boosts surface energy, thereby improving surface wettability. Nonetheless, the

Table 1
Endotoxin contamination in PDMS-based chips.

Sample	Storage Time	EU/mL
Eluates from 1-month-old PDMS chips	t_0	0
	t_7	0
Eluates from 1-year-old PDMS chips	t_0	0.011 ± 0.005
	t_7	0.072 ± 0.034

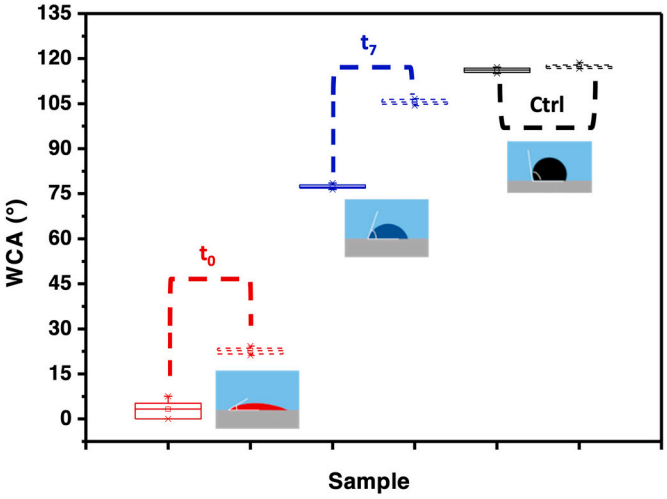

Fig. 2. WCA measurements of 1-month-old PDMS slabs (solid-line box) and 1-year-old PDMS slabs (dashed-line box). Control samples (black) represent untreated PDMS slabs before plasma treatment. t_0 (red) indicates measurements taken within 1 h after plasma treatment, while t_7 (blue) corresponds to measurements taken 1 week post-treatment.

functionalities created on the surface by plasma treatment are not enduring. As a result, the surface's hydrophilic properties diminish within a short period under ambient conditions. The reorientation and migration of newly formed polar groups from the surface can be attributed to the predominantly non-polar gas composition of air, making the interaction of surface polar groups with air energetically unfavorable. In conclusion, we observed that plasma treatment effectively removes endotoxins adsorbed on the channel walls of contaminated chips. However, we hypothesize that due to hydrophobic recovery, endotoxins migrate from the PDMS matrix back to the surface, becoming detectable again after one week of storage.

To the best of our knowledge, there are no universally established regulatory thresholds for endotoxin levels specifically addressing the approval of sterilization processes for PDMS-based microfluidic devices. However, the Limulus Amoebocyte Lysate (LAL) assay is widely employed to detect endotoxin contamination, with regulatory guidelines primarily set for medical devices and injectable pharmaceuticals. The United States Pharmacopeia (USP) and European Pharmacopeia (EP) specify endotoxin limits based on the intended use of the product. For instance, for medical devices that come into contact with blood or cerebrospinal fluid, the accepted limit is typically 0.5 EU/mL or 20 EU per device, respectively (USP <85> Bacterial Endotoxins Test) [19].

Although these guidelines do not directly translate to Organ-on-Chip (OoC) systems, endotoxin contamination in microfluidic devices could significantly impact cell behavior and experimental reproducibility, as previously discussed. Even low endotoxin concentrations (e.g., 50 pg/mL, corresponding to 0.5 EU/mL for the standard LPS from *E. coli* O111:B4) have been shown to modulate inflammatory responses, while higher concentrations (100 ng/mL – 1 μg/mL) can trigger severe inflammatory reactions *in vitro*, potentially compromising the reliability of OoC models [21]. Given the sensitivity of *in vitro* cell cultures to endotoxins, minimizing contamination is crucial for ensuring the validity of experimental outcomes. Further investigations are needed to define appropriate endotoxin thresholds for microfluidic platforms used in biological research.

3.2. FTIR analysis for endotoxin detection on PDMS surfaces

FTIR spectra were acquired to evaluate the presence of endotoxins on the PDMS surface. FTIR is the most used spectroscopic technique for the detection of organic molecules and/or functional groups. On the other hand, this pronounced sensitivity to a wide range of organic molecules makes the procedure of identifying compounds of interest in complex matrices challenging, due to overlapping spectral regions for chemically different functional groups [22]. Therefore, as preliminary tests, the FTIR ability to detect endotoxins on the PDMS surface was checked by a simple procedure.

We prepared four samples on which drops of endotoxin solutions at different concentrations were deposited. The most relevant signals in the endotoxins FTIR spectra are ascribable to the intense vibration signals located in the 3000-2800 cm^{-1} region ($\nu(CH_3)$ and $\nu(CH_2)$), to the typical signals in the amide region (1800-1500 cm^{-1}), to the protein and fatty acid region (also known as mixed region and located at 1500-1200 cm^{-1}), and polysaccharide region overlapped to ν_s PO_2^- (1200-900 cm^{-1}) [23]. Pure PDMS is characterized by main bands at 789–796 cm^{-1} ($-CH_3$ rocking and Si-C stretching in Si-CH_3), 1020–1074 cm^{-1} (Si-O-Si stretching), 1260–1259 cm^{-1} (CH_3 deformation in Si-CH_3), and 2950–2960 cm^{-1} (asymmetric CH_3 stretching in Si-CH_3) [24,25]. The IR spectrum of PDMS is typically dominated by intense signals in the region of symmetric and asymmetric stretching of methyl groups and -Si-CH_3 (2960–2800 cm^{-1}) as well as the corresponding bending (around 790 cm^{-1}). Additionally, the region associated with the stretching of Si-O-Si functional groups (approximately 1074–1020 cm^{-1}) contains many spectral bands [24,25]. Fortunately, the signals attributed to proteins and fatty acids from endotoxins, detectable between 1500 and 1200 cm^{-1}, are minimally affected by PDMS signals, making this region particularly suitable for detecting the endotoxin film. The "mixed region" of endotoxins is a very complex region in which multiple signals coming from amide bonds and ring vibrations can be observed. In particular, those indicated by the arrows in Fig. 3a and located at 1340 and 1360 cm^{-1}, could be unequivocally attributed to the endotoxins. Considering the resolution of the peaks attributed to endotoxins, we can assume that endotoxins cannot be detected on PDMS surface if their concentration is below 1 EU/mL.

After this calibration, 1-year-old and 1-month-old PDMS samples were analyzed. The characteristic peaks reveling consistent contamination levels of endotoxins are not visible, leading us to conclude that the contamination on the surface of both PDMS samples is less than 1 EU/mL (Fig. 3b). This could be considered consistent with our colorimetric LAL assays that determined a maximum contamination level of 0.072 ± 0.034 EU/mL in these samples after the extraction processes.

FTIR has also been used to explore the ability to remove surface endotoxins after oxygen plasma treatment. Both PDMS samples that were contaminated with FTIR-detectable amounts of endotoxins (10 and 1 EU/mL) were analyzed before and after plasma. In both cases, the treatments demonstrate that plasma oxidation removes the first layers of materials adsorbed on surface of PDMS, effecting a cleaning. The treatment is more efficient if a lower concentration of endotoxins is present (no signal is observed at 1340 and 1360 cm^{-1}) (Fig. 3d), and a little less efficient at higher concentration (a weak signal is still present at 1340 cm^{-1}) (Fig. 3c). This result is consistent with the penetration depth of the plasma bombardment and with the presence of contamination layers of different thickness (proportional to the different concentrations of the endotoxin solutions) on the two samples. Adequate adjustment of plasma parameters (e.g. time, power, oxygen level) could lead to a complete removal of endotoxin contamination, although the treatment is time dependent due to hydrophobic recovery bringing endotoxin back from bulk PDMS up to the surface.

Hence, these findings align with the outcomes from LAL assays, which indicated reduced contamination levels in device tested within 1 h from sealing, likely due to the effectiveness of the oxygen plasma treatment in eliminating endotoxins adhered to channel surfaces. Nonetheless, as previously noted, endotoxins became detectable on the channel surfaces after one week from sealing, likely due to the PDMS hydrophobic recovery phenomenon.

4. Conclusions

During this study, we found that PDMS-based microfluidic devices exhibited endotoxin contamination when prepared from a batch of PDMS that had undergone multiple withdrawals over a year, despite being handled in a cleanroom environment. We also observed that the water wettability of the surfaces of contaminated devices is reduced compared to the uncontaminated devices. Then, we demonstrated that contaminated devices showed significantly lower endotoxin levels when tested within 1 h of sealing by oxygen plasma treatment, compared to those tested after one week from sealing. FTIR analysis confirmed that plasma treatment effectively reduced endotoxin levels from surfaces of PDMS slabs. We believe that the reason why devices were more contaminated after one week of sealing was the hydrophobic recovery of the PDMS, which brought again endotoxins from the bulk to the surface. The elution method that we employed to extract and measure endotoxins from the channel surfaces could be further optimized as part of the cleaning protocol before device use. Additionally, to minimize the risk of contamination, we recommend that each withdrawal from PDMS batch be performed under a laminar flow hood and that the hood be regularly cleaned with appropriate cleaning reagents, with strict adherence to sterile techniques. Alternatively, single-use PDMS packaging may be considered for each fabrication session to further reduce contamination risks.

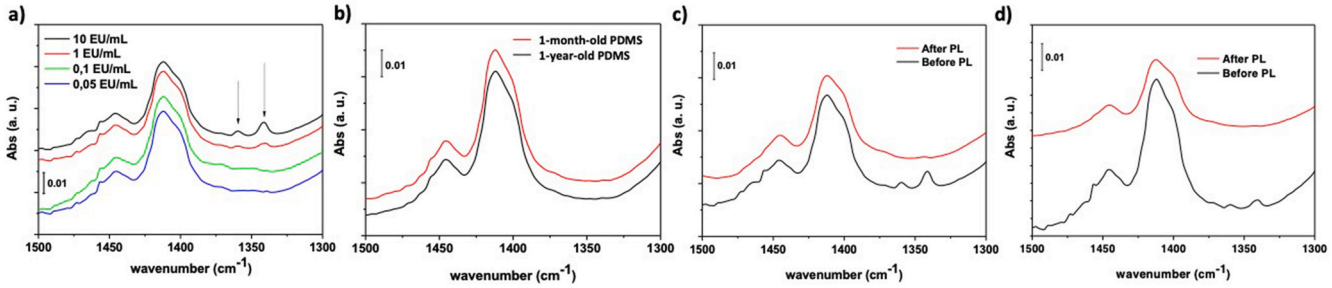

Fig. 3. FTIR spectra of a) calibration samples with added amount of endotoxin solutions of different concentrations; b) 1-month-old and 1-year-old PDMS samples; c) PDMS samples contaminated with a drop of 10 EU/mL endotoxins before and after plasma (PL) treatment; and d) PDMS samples contaminated with a drop of 1 EU/mL endotoxins before and after plasma (PL) treatment. The x-axis represents the wavenumber (cm^{-1}), while the y-axis indicates absorbance (a.u.).

CRediT authorship contribution statement

V. Guarino: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **E. Perrone:** Investigation. **A. Zizzari:** Investigation. **M. Bianco:** Investigation. **G. Giancane:** Investigation, Methodology. **R. Rella:** Resources, Methodology. **M.G. Manera:** Writing – review & editing, Methodology. **V. Arima:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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