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Research paper

Vaporized Hydrogen Peroxide Uptake by Tubing used for Aseptic Fill-Finish Manufacturing of Biopharmaceutical Drug Products

Dilara Ali [a,1], Sarah S. Peláez [a,1], Thomas Lemazurier [a], Ariane Schroeter [a], Michael Adler [a], JaeHwi Bong [a], Oliver Germershaus [b], Hanns-Christian Mahler [a], Andrea Allmendinger [a,c,*]

[a] *ten23 health AG, Mattenstr. 22, 4058 Basel, Switzerland*
[b] *Institute of Pharma Technology, School of Life Sciences, University of Applied Arts and Sciences Northwestern Switzerland, Hofackerstr. 30, 4132 Muttenz, Switzerland*
[c] *Institute of Pharmaceutical Sciences, Department of Pharmaceutics, University of Freiburg, Sonnenstr. 5, 79104 Freiburg, Germany*

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ABSTRACT

Aseptic filling of biopharmaceutical products requires a grade A cleanroom environment, preferably ensured by isolators in grade C surroundings. Isolators are decontaminated before the start of filling processes using vaporized hydrogen peroxide (VHP) and filling starts at pre-defined residual VHP levels (e.g., below 0.5 ppm) depending on product sensitivity towards VHP oxidation. Manufacturing equipment and consumables, including filling assemblies, are exposed to VHP during or after the decontamination cycle or after line interruptions. We studied the VHP uptake by tubing in a lab-scale model isolator to evaluate the impact of tubing properties including contact material, tubing dimensions, suppliers, and VHP exposure (concentration and exposure time). Quantifying the release of H_2O_2 from the tubing into solution using an Amplex Red Hydrogen Peroxide Assay, showed that H_2O_2 concentrations decreased linearly with an increase in wall thickness and increased with higher surface to volume ratio. We further conclude that thermoplastic elastomer and thermoplastic vulcanizate tubing did not show any measurable VHP uptake for the tested conditions, whereas significant VHP uptake occurred in different platinum cured silicone tubing depending on tubing material and supplier. We further verified the results in a GMP manufacturing isolator setting. Based on our findings, we recommend to evaluate VHP uptake of filling tubing used for fill-finish manufacturing in isolators, to reduce the risk of oxidation for active pharmaceutical ingredients or excipients.

1. Introduction

Biopharmaceuticals have revolutionized the treatment of a range of diseases and indications. Due to their in vivo degradation and poor permeability after oral administration or other non-injectable routes of administration, such as inhalation or transdermal application, they are typically administered parenterally by intravenous, subcutaneous or intramuscular injections, and require aseptic filling into their container closure systems.[1] To comply with current regulatory requirements such as those set out in Annex 1, filling preferably takes place in isolators or alternatively in restricted access barrier systems (RABS) to ensure sterility assurance levels. Prior to filling in an isolator, the isolator must be decontaminated, and vaporized hydrogen peroxide (VHP) is widely used decontamination agent.[2] After aeration, the filling process typically starts once the residual VHP concentration declines to a certain level – this will be specific to the facility and possibly also to the product (e.g., ≤ 0.5 ppm). The ability of detection systems to detect residual VHP has therefore received a great deal of attention in recent years.

Residual VHP may be adsorbed by the drug product (DP) solution during the filling process, potentially impacting protein quality as recently reviewed by Meleties et al.[3] VHP might migrate in the DP solution by diffusion into open, filled containers, [4] by exposure of the

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Abbreviations: AUC, Area under the curve; CDR, Complementary-determining region; DP, Drug product; H_2O_2, Hydrogen peroxide; h, Hours; ID, Inner diameter; IS, Inner surface; OD, Outer diameter; OS, Outer surface; PCS, Platinum cured silicone; ppm, Parts per million; PTFE, Polytetrafluoroethylene; RABS, Restricted-access barrier system; RSD, Relative standard deviation; SD, Standard deviation; S, Surface area; t, Time; T, Tubing; T_g, Glass transition temperature; TPE, Thermoplastic elastomer; TPV, Thermoplastic vulcanizate; VHP, Vaporized hydrogen peroxide; V, Volume; WT, Wall thickness.

[*] Corresponding author at: ten23 health AG, Mattenstr. 22, 4058 Basel, Switzerland.
E-mail address: andrea.allmendinger@ten23.health (A. Allmendinger).

[1] The authors contributed equally to this work.

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product at the filling needle, adsorption on empty vials before filling after line interruptions, [4] or by diffusion through single-use components exposed to the decontamination process.[5] If not mitigated by appropriate excipients such as antioxidants, radical scavengers and/or chelators in the formulation, product quality can be impacted by oxidation of surface exposed amino acid residues, and oxidation of amino acid residues may further impair functionality, e.g. if oxidation occurs in the complementarity-determining regions (CDR).[6]

Single-use consumables are typically and widely used in fill and finish operations of parenteral preparations due to their flexible handling as plug-and-play solutions. However, single-use manufacturing consumables such as filling assemblies might be exposed to residual VHP, after the decontamination cycle, leading to VHP migration into the DP solution.[4] Silicone tubing is often used for filling assemblies because of its low cost and suitability, as well as compatibility for filling protein formulations, e.g. as part of peristaltic pump systems.[7] It has gained increasing acceptance for filling biological DPs due to its favorable leachables and extractables (L&E) profile[8] and superior performance with regards to sub-visible particles and low aggregate formation after peristaltic pumping of protein formulations.[9] However, alternatives to silicone tubing are available such as, thermoplastic elastomers (TPE) and thermoplastic vulcanizates (TPV) or fluoropolymer tubing which reduce adsorption of preservatives to the inside of the tubing.[10]

Previous literature has described VHP diffusion through silicone tubing as a source of VHP uptake into a product solution during the filling process.[5] Hubbard et al, studied the uptake of VHP by two types of silicone tubing from the same manufacturer, which differed in the inner diameter (ID) and only slightly in wall thickness (WT). Using a small-scale isolator, the tubing was filled with water, which was shown to be a suitable surrogate for a mAb solution, and exposed to VHP at different levels. The authors concluded that the primary sources of VHP uptake were open, filled vials and the silicone tubing assembly, depending on dimensional parameters.[5] In an additional study, Eisner et al. (2019) studied the influence of processing/sterilization methods on the uptake behavior. They demonstrated that silicone tubing sterilized by autoclaving or gamma irradiation resulted in different VHP uptake patterns, with decreasing uptake rates post autoclaving vs. increased uptake rates post gamma irradiation. However, although silicone tubing is widely used in fill-finish lines, the mechanism of VHP uptake through tubing material is not fully understood and studies of VHP uptake as a function of tubing material and additional properties are lacking.

In the present study, we used a lab-scale model isolator to obtain a systematic understanding of VHP uptake by tubing material used in aseptic filling processes. In this regard, the release of H_2O_2 from the tubing in solution was quantified. Tubing materials from six suppliers were tested to determine the influence of dimensional parameters, and results were verified using an at-scale study in a manufacturing fill-finish RABS setting·H_2O_2 release from the tubing of filling assemblies in solution was quantified during filling operations to verify small-scale results.

2. Materials and methods

2.1. Tubing

Table 1 summarizes the types of tubing used in this study including their dimensional parameters and material properties. 14 different types of tubing were tested dependent on (1) the influence of wall thickness, (2) the influence of inner and outer surface (OS) area, and (3) the influence of material from different manufacturers (M1 – M6) comprising platinum cured silicone (PCS), thermoplastic elastomer (TPE) and thermoplastic vulcanizate (TPV). The manufacturer names are not disclosed due to confidentiality reasons.

2.2. Lab-scale model isolator

A lab-scale model isolator was used as previously described by Švrček et al. (referred to as *Peroxybox)*[11], and adapted for simulated VHP uptake studies for the tubing (Fig. 1). In brief, the model isolator was equipped with an air inlet and outlet, a motor connected to a ventilator and two built-in sample holders for placement of the samples during the incubation process. Sample holders inside the incubation chamber were located at the same horizonal height, one in the front, and one in the back. The VHP concentration in the isolator was controlled with a Dräger Polytron 7000, including a low concentration H_2O_2 LC sensor (Dräger, Lübeck DE). A constant VHP level was obtained using a 30 % (w/w) H_2O_2 solution (Sigma Aldrich, St. Louis, USA), which was diluted to a 0.5 % (v/v) solution with water. The solution was then added on a cotton pad, placed on a metal grid, lying on top of a ventilator, to generate VHP. The adjustment during the incubation time was achieved by adding droplets of the same solution. The temperature (room temperature between 20 – 25 °C) and humidity (40 – 60 %) in the lab-scale isolator were kept constant.

Fig. 2 shows a representative example of VHP concentration during the experiment when tubing was exposed to a defined VHP level for a defined period, targeting a constant level of 0.5 ppm. To compare the VHP exposure between experiments, the cumulative VHP exposure (area under the curve, AUC) as the product of VHP concentration [ppm] * exposure time [min]) [ppm*min] was calculated and later used for

Table 1
Tubing type, dimensions and material properties used for VHP uptake experiments in model isolator. Tubings appearing identical are from the same manufacturer and made from the same material, are sold under different names, suggesting minor differences in their characteristics. Length of tubing was adjusted for specific experiments as described in the method section. (*Data provided by tubing supplier).

Tubing	Manufacturer	Inner Diameter [mm]	Outer Diameter [mm]	Wall Thickness [mm]	Test Length [mm]	Material	Density* [g/cm^3]	Hardness* (Shore A)
T1	M1	4.8	8.0	1.6	100	Platinum cured silicone (PCS)	1.12	50
T2			9.6	2.4			1.12	50
T3			11.2	3.2			1.12	50
T4	M5	0.8	4.0	1.6	160/300		1.10	50
T5	M6	1.2	4.4		100		1.18	60
T6	M5	3.2	6.4		100/75.5		1.10	50
T7	M4	4.8	8.0		100		1.17	65
T8	M1	4.8	9.6	2.4		Thermoplastic vulcanizate (TPV)	0.95	70
T9						Thermoplastic elastomer (TPE)	0.89	65
T10	M3		8.0	1.6			0.89	68
T11	M2					PCS	1.22	80
T12	M3						1.13	50
T13	M4						1.15	50
T14							1.14	50

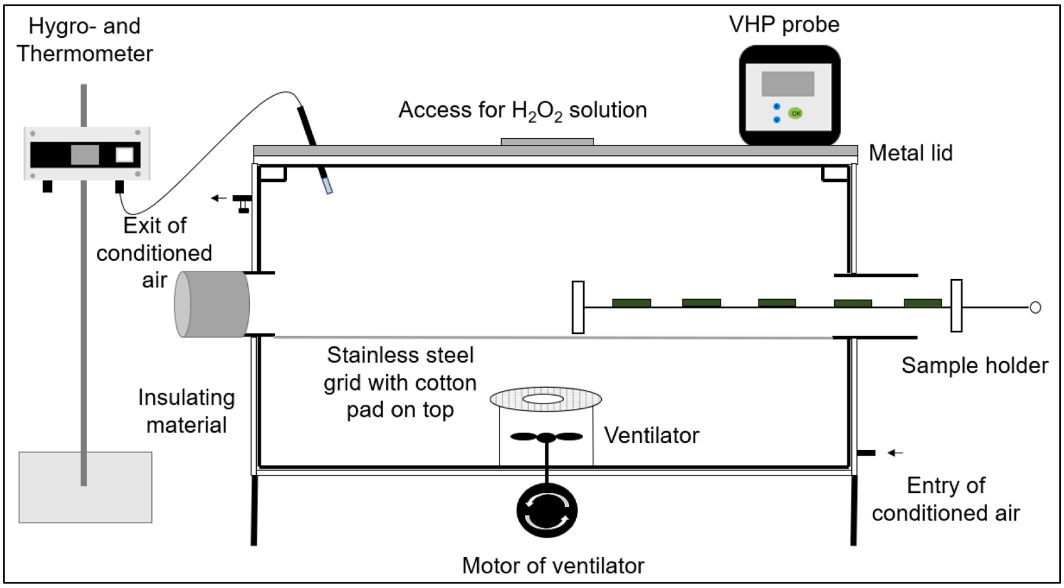

Fig. 1. Lab-scale model isolator to generate controlled VHP concentrations.

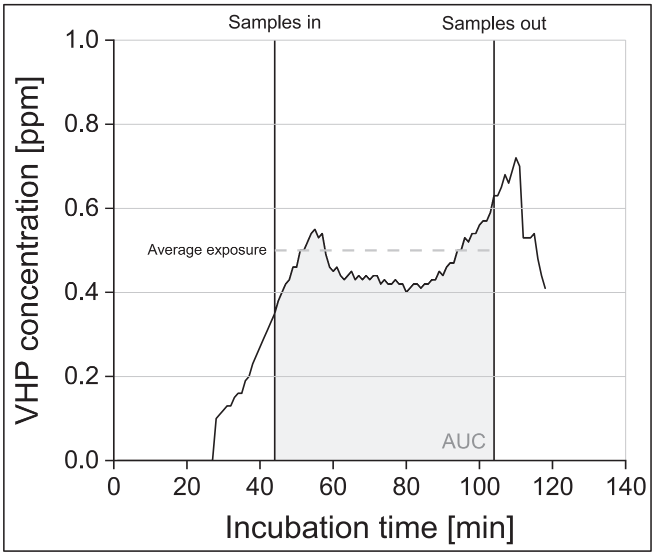

Fig. 2. Representative example of VHP concentration during tubing exposure in the lab-scale model isolator.

normalization of H_2O_2 concentrations. A reference tubing (T7, Table 1) was included in every VHP incubation cycle as a control.

2.3. *Experimental procedure (exposure of tubing)*

For the incubation procedure, each tubing sample was cut to different lengths (e.g., to keep the S/V ratio constant), purged with double the amount of the incubation liquid volume, filled with water, and closed with tubing closure clamps to prevent VHP uptake via the ends of the tubing. When the defined VHP concentration in the lab-scale model isolator was reached, tubing samples were placed in the sample holders and incubated for a defined time. During the incubation time, the VHP concentration was maintained at the specified level. If not stated otherwise, the VHP concentration in the model isolator was maintained at 0.5 ppm for an incubation time of 1 h. Following the incubation cycle, the solution in the tubing was emptied into 2R vials and stored in the crimped vials at – 80 °C until analysis. It was verified that sample storage has no impact on the analytical read-out. Samples were prepared in triplicates ($n = 3$), standard deviation are reported. For the duration of analysis, we verified that H_2O_2 concentrations remained stable from sample pull until the time point of analysis.

2.4. *At-scale VHP uptake study*

VHP uptake by filled vials was studied during filling operations on a GMP filling line with an isolator setting, which had been decontaminated with VHP. 1.2 mL of Milli-Q water was dispensed into 6R vials over a 4-hour period. The filling manifold consisted of five different tubing samples with varying wall thickness (WT = 1.6 – 3.2 mm), material (PCS or TPE) and length, and therefore different holdup volumes. The five different tubing samples were connected to each other by tubing connectors connecting the surge bag to the filling needle (Fig. 3). The holdup volume of each tubing can be calculated based on the dimensions, and the incubated volume of the tubing during the VHP decontamination process can be matched to the order of filled units. Following filling, the vials were stoppered and capped and the samples were stored at –80 °C prior to further analysis. Quantification of hydrogen peroxide (H_2O_2) in the samples was performed using the Amplex Red Hydrogen Peroxide Assay (Thermo Fisher Scientific, Waltham, USA). In total, 162 samples were collected at different time points, including negative controls (without VHP exposure). During the filling process, two production line interruptions occurred: the first lasted for approximately 60 min, and the second for 30 min. VHP concentrations within the isolator were monitored at defined intervals using a Picarro sensor, PI2114 (Picarro, Santa Clara, USA).

2.5. *H_2O_2 quantification and data processing*

The H_2O_2 concentration of the incubated solution was quantified by the *Amplex Red Hydrogen Peroxide Assay Kit* (Thermo Fisher Scientific, Waltham USA) with substitution of the substrate by Amplex UltraRed from the same manufacturer (A36006) to achieve a LOQ of 1 μM H_2O_2. The assay was evaluated for repeatability, specificity, and accuracy, and the LOQ was determined by progressively reducing the H_2O_2 concentration until linearity was lost, and both recovery and repeatability showed unsatisfactory results. The peroxidase assay is a one-step assay using Amplex UltraRed reagent, a fluorogenic substrate for the enzyme horseradish peroxidase (HRP), which can react with H_2O_2 in a 1:1 stoichiometric ratio to produce Amplex UltroxRed (resorufin), a red-

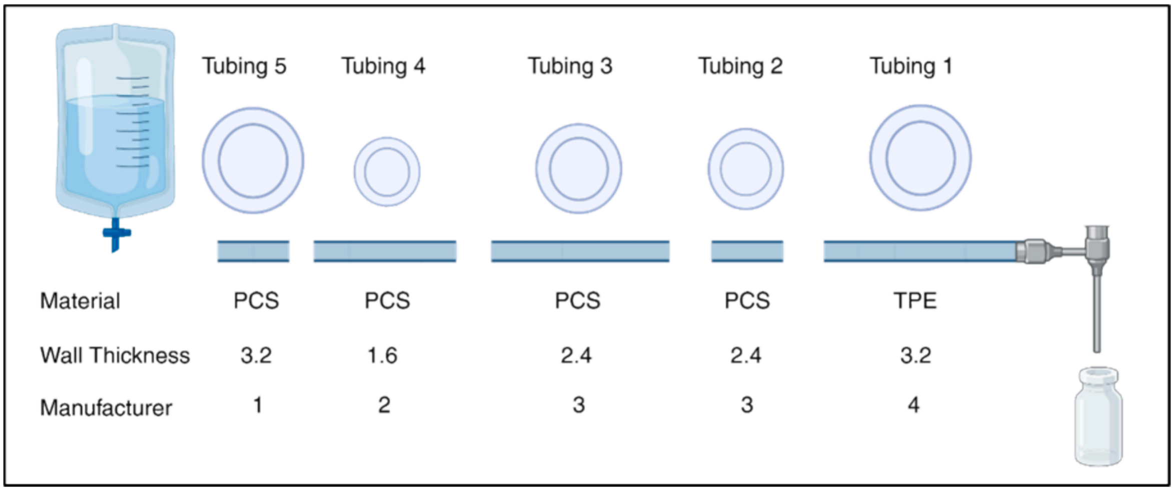

Fig. 3. Set-up of filling manifold of at-scale VHP uptake study. Five tubing samples of different dimensions, materials and lengths (holdup volumes) were used to connect the surge bag to the filling needle.

fluorescent oxidation product, which can be detected by fluorescent or spectrophotometric spectroscopy.[12].

For the assay, reaction buffer (phosphate buffer, pH 7.4), Amplex UltraRed solution and horseradish peroxidase were mixed, added to the samples and controls, and incubated for at least 30 min while shaking at 300 rpm at 37 °C. Samples were diluted with water prior to the measurement using a calibration curve between 0.5 – 10 µM H_2O_2. The fluorescence was measured on a SpectraMax iD5 (Molecular Devices, San Jose US) at 530/590 nm (excitation/emission). H_2O_2 concentrations of the samples were reported in µM, and H_2O_2 concentrations were subsequently converted to ng/mL using a conversion factor of 34.0147. In a final step, the resulting values were normalized by calculating the experiment-specific area under the curve (AUC), which is defined as VHP concentration [ppm] × exposure time [min], to enable data comparability.

2.6. Contact angle measurement

To characterize the wettability of the tubing, dispersive/polar characteristics were assessed by measuring the contact angle of three different solutions (water, diiodomethane, and diethylene glycol) on the tubing's outer surface (OS). Tubing samples were cut into 0.5 mm wide strips, and a 5 µL drop of each solution was applied using a syringe. Contact angles were measured on five samples using a DSA 10 (Krüss, Hamburg DE) instrument. Each solution was measured ten times on every tubing to account for variability. The interfacial tension, along with its respective dispersive and polar components, was determined using the Owens-Wendt-Rabel-Kaelble method, applying error-weighted computation to the contact angle measurements.[13].

2.7. Scanning electron microscopy

The surface structure of the tubing was assessed by the Supra 40VP Scanning Electron Microscope (Zeiss, Oberkochen, DE). The tubing samples were cleaned with nitrogen, cut into approximately 1 cm wide strips, and mounted on a holder using adhesive. They were then coated with palladium for 30 s, resulting in a coating thickness of 2.9 – 4.3 nm. The holder was loaded and evacuated to the appropriate vacuum level. The accelerating voltage was set to 7 kV.

2.8. Statistical analysis

A single-factor ANOVA (Tukey‘s test) was performed to compare statistical significance of H_2O_2 uptake between different tubing samples using *OriginPro 2023.*

3. Results

We established a lab-scale model isolator to study VHP uptake by tubing samples and quantified the release of H_2O_2 from the tubing into solution by using an Amplex Red Hydrogen Peroxide Assay. VHP exposure was between 0.4 – 0.6 ppm for all experiments, unless stated otherwise. A reference tubing (tubing T7, PCS) was used to verify process comparability between each experiment. After normalization to the cumulative VHP exposure, the measured H_2O_2 concentrations of the control samples were verified between 387.7 – 449.1 ng/mL and were not significantly different as tested by a one-way ANOVA.

Sample load and sample position in the chamber did not affect VHP uptake in the established setup (data not shown). Nevertheless, we have randomized sample positions for the tested conditions. The tubing length was adjusted to study the influence of dimensional parameters, such as the inner and outer surface area, while maintaining different surface/volume ratios.

3.1. Correlation of VHP uptake and cumulative VHP exposure

In the first set of experiments, we studied the VHP uptake of silicone tubing (T4, T7) dependent on exposure time at increasing VHP concentrations in the model isolator (Fig. 4 A and B). We tested VHP concentrations between 0.2 to 1.0 ppm and for 30 to 180 min of incubation resulting in cumulative VHP exposure of 6 – 180 [ppm * min]. The VHP uptake in tubing measured by H_2O_2 concentration in solution showed a linear correlation to cumulative VHP exposures (Fig. 4A, R2 = 1.00) operating under sink conditions. We further tested the same tubing with the same WT but different IDs (tubing T4 and T7). Both types of tubing showed a linear correlation (Fig. 4B, R2 = 1.00) of cumulative VHP exposure and H_2O_2 concentration. The tubing with smaller ID (T4) resulted in a higher VHP uptake compared to the tubing with larger ID T7, indicated by a steeper slope in Fig. 4B.

3.2. Correlation of VHP uptake and dimensional parameters

In the next set of experiments, VHP uptake as a function of tubing WT was studied with silicone/ PCS tubing of the same type, derived from the same manufacturer. Three tubing samples (T1 – T3, see Table 1) with different WTs (1.6, 2.4, 3.2 mm) and the same IDs (4.8 mm) were selected. The samples were incubated for one hour at 0.5 ppm and VHP uptake results were normalized by considering the cumulative exposure.

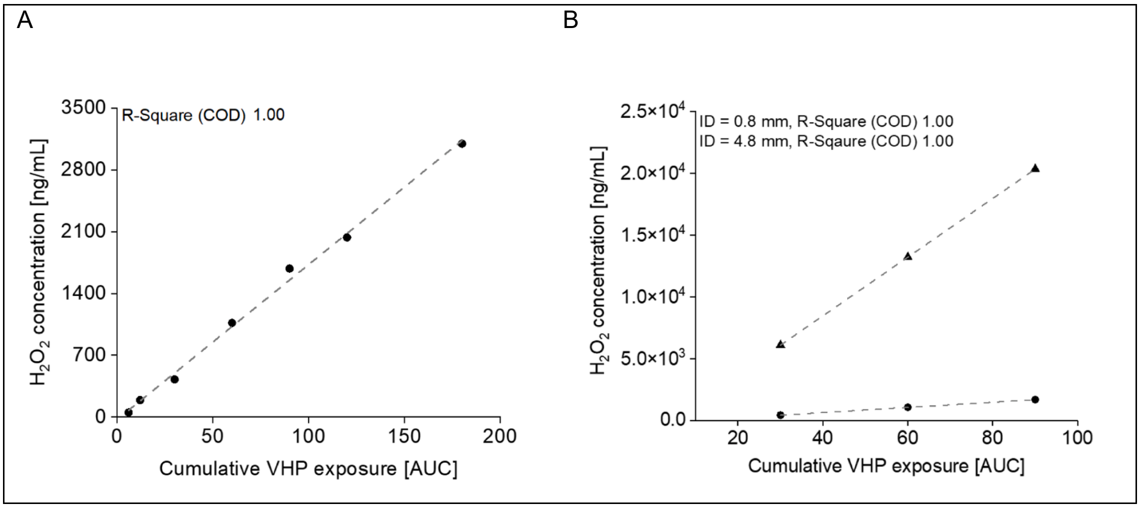

Fig. 4. Linear correlation of VHP uptake determined as H_2O_2 concentration in tubing samples (n = 1) versus cumulative VHP exposure. Cumulative VHP exposure, is calculated as AUC [ppm*min] and derived from different VHP concentrations in the isolator for different incubation times. (A) VHP uptake concentrations in tubing (T7) at different VHP exposures (i.e., 0.2 ppm for 30 min, 0.2 ppm for 60 min, 0.5 ppm for 60 min etc.) are shown with linear fit (R^2 = 1.00). (B) VHP uptake concentrations in two different tubing types with ID 0.8 mm (T4) and 4.8 mm (T7) and same WT, at three different cumulative VHP exposures (0.5 ppm and 1.0 ppm for 30, 60 and 90 min) are shown with linear fit (R^2 = 1.00).

The experiment revealed a negative linear correlation between the wall thickness and VHP uptake/ measured H_2O_2 concentration. As shown in Fig. 5, the VHP uptake in the tubing decreased with an increase in WT.

The influence of the tubing surface area on VHP uptake was tested in two subsequent experiments using PCS tubing T4 – T7 (Table 2). The tubing samples had different inner diameters (0.80, 1.20, 3.18, 4.80 mm) but the same WT (1.6 mm). To achieve comparable surface areas, the tubing length was adjusted to keep either the inner surface area or the outer surface area constant (Table 2). The samples were incubated for one hour at a VHP concentration of 0.5 ppm.

Fig. 6 shows the H_2O_2 concentration for the different tubing dimensions dependent on inner (A) and outer (B) surface/volume ratio. The results show that with an increase in either inner or outer surface to volume ratio, the VHP uptake increases following a sigmoidal function (R^2 = 0.99).

Table 2
Dimensions of tubing used to determine the effect of the surface area on VHP uptake by silicone tubing.

Tubing	WT [mm]	Length [mm]	IS [mm^2]	OS [mm^2]	OS/IS
T4	1.6	160.0	402.1	***2010.6***	5.0
T6		100.0	1005.3	***2010.6***	2.0
T7		81.0	1221.5	***2035.8***	1.7
T4		300.0	***754.0***	3769.9	5.0
T6		75.5	***759.0***	1518.0	2.0
T7		50.0	***754.0***	1256.6	1.7
T5		100.0	377.0	1382.3	3.7

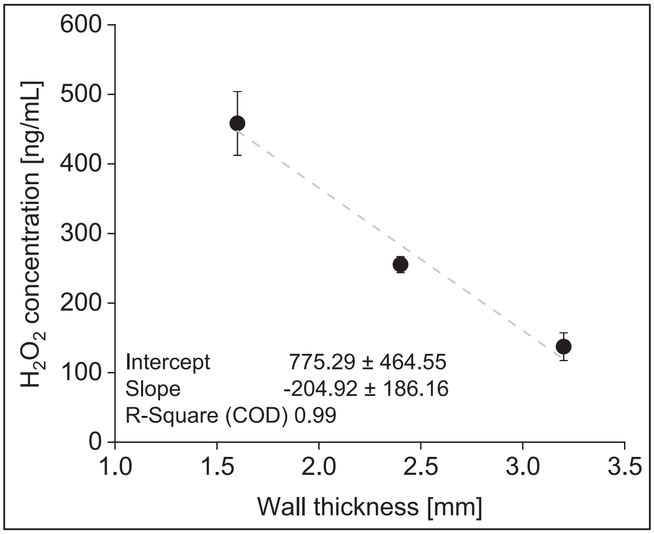

Fig. 5. H_2O_2 concentration versus tubing wall thickness. Measured H_2O_2 concentrations after VHP incubation at 0.5 ppm for 1 h, shown as means ± standard deviation (SD) (n = 3 individual runs) in tubing with different WTs 1.6 mm (T1), 2.4 mm (T2), and 3.2 mm (T3), constant inner surface (IS) areas, and different outer surface (OS) area. Data was normalized by considering the cumulative VHP exposure. A linear fit (R^2 = 0.99) is shown as a dashed line.

As the volume did not show an effect on the VHP uptake, it was eliminated from the equation. As a result, Fig. 7 shows the VHP uptake in correlation to the ratio of OS/IS, following sigmoidal functions (R^2 = 0.99).

3.3. *Influence of tubing material and supplier on VHP uptake*

In the final set of experiments, we studied the effect of different tubing materials from different manufacturers. The VHP uptake was assessed for nine types of tubing (T1 + T7 – T14) as listed in Table 1. The tested tubing samples had equal dimensions, except for the wall thickness of tubing 8 and 9, which had a larger WT of 2.4 mm. Tubing samples were cut to the same length, resulting in equal testing solution volumes. The experiment included six tubing samples of PCS (T1, T7, T11 – T14), two tubing samples of TPE (T9 + T10) and one tubing sample of TPV (T8). The samples were incubated for one hour at 0.5 ppm. We found that tubing made of TPE or TPV showed no detectable VHP uptake in solution, whereas all PCS tubing showed a significant VHP uptake (Fig. 8). The measured H_2O_2 concentrations of T1, T7 and T12 – T14 showed comparable VHP uptake, with no significant difference, whereas tubing T11, a PCS tubing from a different manufacturer (M2), showed a significantly lower VHP uptake, around a 41 % reduction compared to the means observed in the other PCS tubing after an incubation at 0.5 ppm for 1 h.

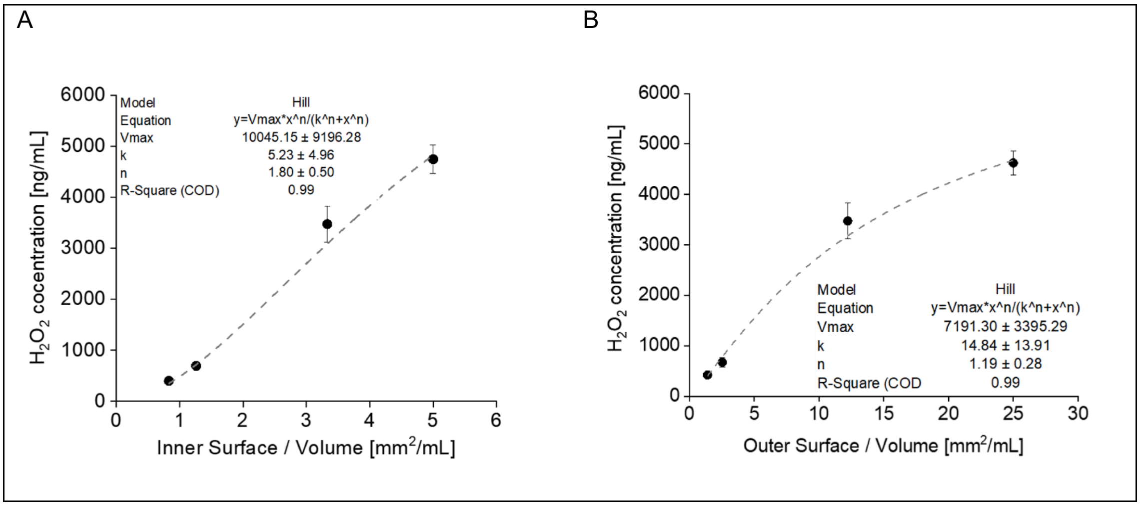

Fig. 6. Correlation of H_2O_2 concentration to (A) inner and (B) outer surface area / volume in PCS tubing T4-T7. The tubings of different OD were adjusted by their length to generate tubing with either constant inner or outer surface area. Each data point represents measured H_2O_2 concentrations after VHP incubation at 0.5 ppm for 1 h as means ± SD of $n = 3$ individual runs. Sigmoidal growth ($R^2 = 0.99$) is shown as dashed line to guide the eye.

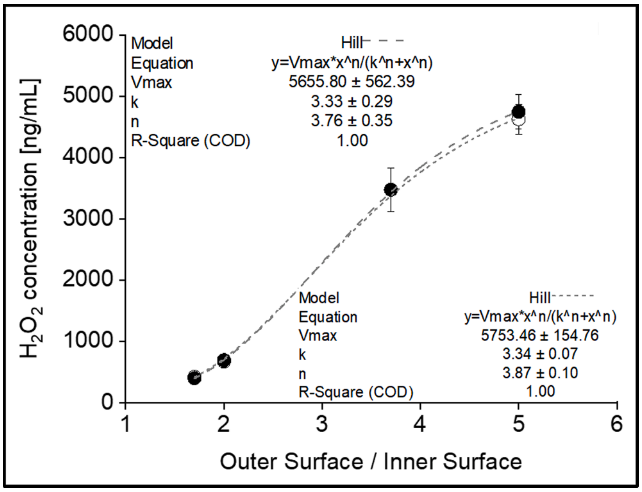

Fig. 7. Correlation of VHP uptake to inner and outer surface area in PCS tubing T4-T7. Each data point represents measured H_2O_2 concentrations after VHP incubation at 0.5 ppm for 1 h as means ± SD of $n = 3$ individual runs, the full black circles being the data set showing the dependance on the inner surface area (R^2 in the left upper corner) and the white circle (R^2 in the left lower corner) showing the dependance on the outer surface area. Sigmoidal growth ($R^2 = 0.99$) for both data sets is shown as dashed line to guide the eye.

3.4. Correlating material properties to VHP uptake of tubing type

In order to elucidate the impact of material properties on VHP uptake by manufacturing tubing, we studied different material properties or collected supplier information comprising (A) density and hardness of tubing material, (B) interfacial tension by contact angle measurements, and C) material and pore structure by scanning electron microscopy.

3.4.1. Density and hardness

The density and hardness of each tubing was analyzed by collecting data from tubing suppliers, with the results summarized in Table 1. There was no difference in VHP uptake for the PCS tubing with densities between 1.10 and 1.18 g/cm^3 and hardness between 50 – 65 (Fig. 8) but a significantly lower uptake for T11, which has the highest density and hardness (1.22 g/cm^3 / 80 Shore A). TPE and TPV tubing had the lowest density (T8 – T10, 0.89 – 0.95 g/cm^3) and highest hardness, however they did not take up any VHP.

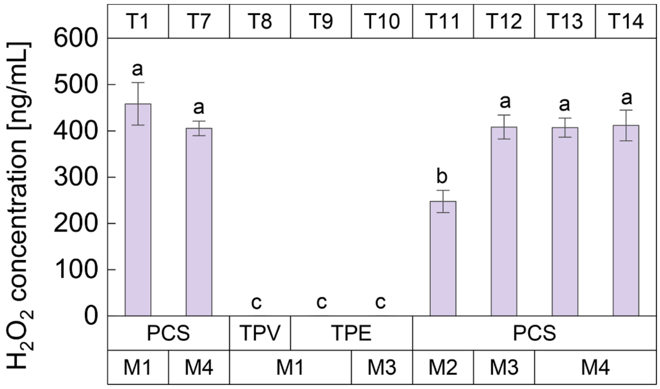

Fig. 8. VHP uptake dependent on tubing material (T1 + T7 – T14). Three different materials (TPV = thermoplastic elastomer, TPV = thermoplastic vulcanizate and PCS = platinum cured silicone) from four manufacturers (M1 – M4) as specified in Table 1 were included. Data is shown as means ± SD of 3 individual experiments. Means highlighted with same letter (a, b, c) are not statistically different from each other ($p < 0.05$), but statistically different from the other groups ($p < 0.0001$), for pairwise comparison of all tubing between groups calculated by ANOVA (Tukey's range test).

3.4.2. Contact angle measurements

To assess the surface lipophilicity of selected tubing samples, interfacial tension was calculated using contact angle measurements as presented in Table 3. The results indicate increasing interfacial tension in the following order: T11 < T10 < T7 < T9 < T1. Higher interfacial tension correlates with greater lipophilicity of the tubing surface. Since the two TPE tubing samples (T9 and T10) did not show any VHP uptake, only the silicone tubing samples are compared. Among these, tubing T11 exhibits the lowest surface lipophilicity and, as shown in Fig. 8, also the lowest VHP uptake. T1 shows the highest interfacial tension and therefore highest surface lipophilicity but, as seen in Fig. 8, comparable VHP uptake to all other tested PCS tubing.

3.4.3. Scanning electron microscopy images

Lastly, scanning electron microscopy images were taken of specified tubing samples (T7, T9, T11, and T12), as shown in Fig. 9 (A – D). The surface images did not reveal distinct structures that could explain

Table 3
Measured interfacial tension of tubing with standard deviations as a result of contact angle measurements of five selected tubing samples. The disperse (D) and polar (P) part of the interfacial tension are also listed in the table.

Tubing	Material	Wall Thickness [mm]	Interfacial Tension (D)	Interfacial Tension (P)	Interfacial Tension [mN/m]
T1	PCS	2.4	20.11	6.78	26.89 ± 2.06
T7	PCS	1.6	21.70	2.80	24.50 ± 1.57
T9	TPE	2.4	22.56	2.48	25.03 ± 1.54
T10	TPE	1.6	21.87	2.03	23.90 ± 1.21
T11	PCS	1.6	19.92	1.79	21.70 ± 1.30

differences in material properties, contributing to variations in VHP uptake.

3.5. At-scale VHP uptake study

To verify the previous findings that VHP uptake and subsequent H_2O_2 release in solution is dependent on tubing material and tubing dimensions, we quantified the H_2O_2 concentration in vials during filling operations at manufacturing scale of 6.5 h (h) interrupted by two filling interruptions of 30 and 60 min (Fig. 10). Filling started after VHP decontamination of the isolator, and Fig. 10 illustrates the residual VHP concentration inside the isolator following an exponential decay measured by a Picarro sensor. Filling started at 0.309 ppm VHP reaching nearly zero (0.011 ppm) by the end of the four-hour filling process.

Fig. 10 further illustrates the H_2O_2 release into water-filled vials depending on filling time. We found an increase in H_2O_2 concentration after few minutes of filling following a distinct pattern, which was the same after both filling interruptions. The filled volume can be correlated to the volume in the specific tubing of the filling assembly as highlighted in the legend of Fig. 10. The H_2O_2 concentration was smallest for the volume in tubing with lowest wall thickness as well as for TPE versus PCS material in line with our previous results. The H_2O_2 concentration pattern was higher after the first line interruption compared to the second following the absolute VHP concentration in the RABS.

4. Discussion

In the present study, we examined the VHP uptake by different tubing samples in a model isolator by measuring the release of H_2O_2 in water incubated in the tubing. The applied method quantifies the H_2O_2 released from the tubing and is thus an indirect measurement indicative for VHP uptake by the tubing. However the absolute VHP uptake in the tubing cannot be assessed by this method as it is a time-dependent

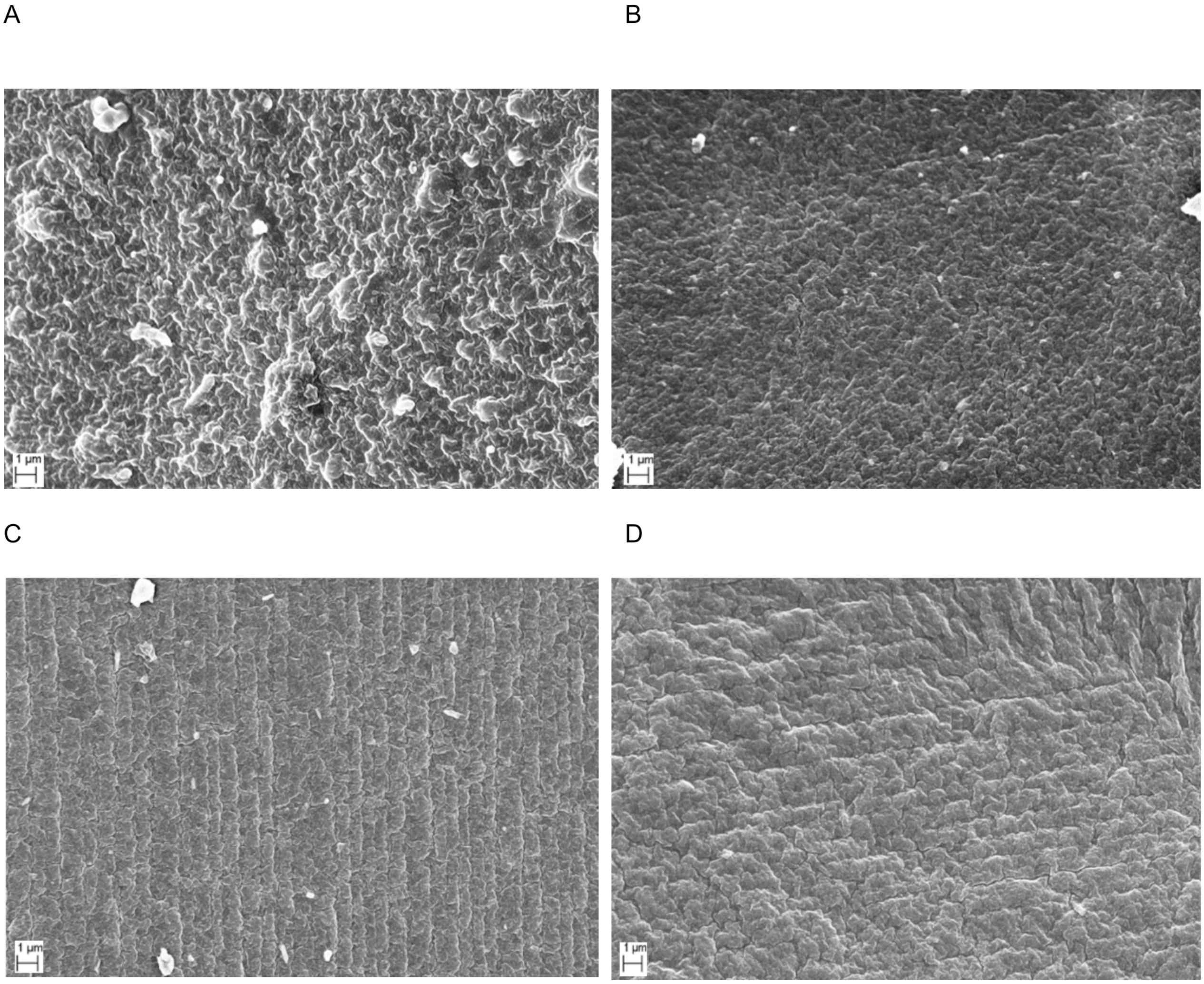

Fig. 9. Representative scanning electron microscopy pictures of (A) TPE tubing T9, (B) PCS tubing T11, (C) PCS tubing T12 and (D) PCS tubing T7 at a magnification of 10.00 K X. Samples were sputtered with a palladium coating.

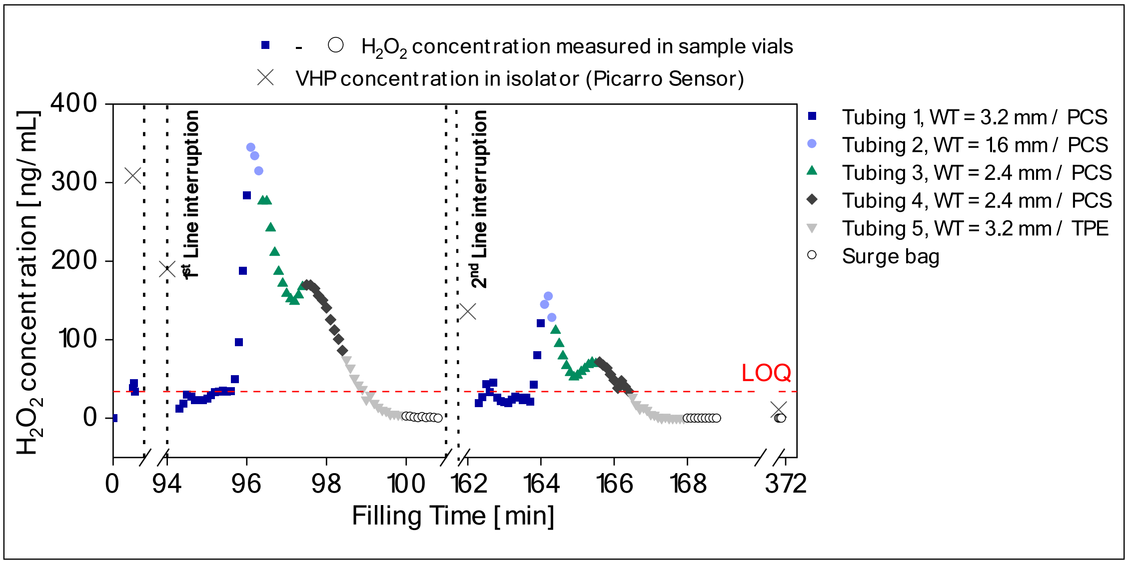

Fig. 10. H_2O_2 release from tubing in water-filled 6R vials on a GMP filling line after isolator decontamination dependent on filling time. VHP concentrations in the isolator were measured by a Picarro sensor·H_2O_2 concentrations in the vials were measured at the beginning of filling and after each of the two line interruptions (the first interruption lasted 60 min, and the second 30 min). Samples are labelled to visualize the relation of the hold-up volume of the individual tubing of the filling assembly, which is transferred to the individual vial at the beginning of filling. The red dashed line represents the limit of quantification (LOQ) for the Amplex assay of 1 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diffusion-controlled process.

We demonstrated that the cumulative VHP exposure quantified as the AUC from measured VHP concentrations in the model isolator was linearly correlated to the measured uptake of hydrogen peroxide (H_2O_2) under the test conditions (Fig. 4). The study covered process conditions from 0.1 to 1.0 ppm and exposure times up to 180 min, resulting in H_2O_2 concentration of up to 20‘317.7 ng/mL. The linear H_2O_2 increase with AUC confirms that the generated data was obtained under sink conditions and considerably below the saturation concentration of H_2O_2 (280,000 ng/mL)[14] in water. Numerical results are in line with previously reported data by Vuylsteke et al. (2019), mechanistically studying the diffusion of H_2O_2 into open primary packaging containers filled with water, which reported a linear increase in H_2O_2 concentrations dependent on VHP concentration, test volume, and incubation time.[4]

4.1. *Influence of tubing dimension*

To elucidate the impact of tubing dimensions on VHP uptake by the incubated sample, we reported a linear decrease in measured H_2O_2 concentration in the samples with increasing WTs. A higher WT constitutes longer pathways for VHP diffusion, which may lead to the observed reduction in VHP uptake in our experiments.

As recently reviewed by Zhang et al. for silicone rubbers, the amount of gas that permeates through a membrane, depends on solution, diffusion and evaporation of the gas under consideration, with the first and last mechanism described by Henry’s law (Equation (1):[15]

$$c = S^{*}p \tag{1}$$

with c being the concentration of gas molecules in rubber, S the solubility of gas in rubber, and p the pressure of gas; and the process of gas diffusion described by Ficks’ first law (Equation (2):[15]

$$F = -D\partial C/\partial x, \tag{2}$$

where F represents the transfer rate per unit area of section, C the concentration of the diffusing substance, x the space coordinate measured in relation to the section, and D the diffusion coefficient.[16]

With regard to our setup, equation (2) describes the relationship between the diffusion path length (∂x) corresponding to the wall thickness (in mm), and the concentration of the diffusing component (∂C), which corresponds to the measured H_2O_2 concentration (in ng/mL). The inverse proportionality between these variables implies that as wall thickness increases, the rate of mass flow decreases, which we have verified in our experiments.

We further investigated the effects of the outer surface area and the inner surface area on VHP uptake separately. First, we tested tubing samples with constant inner surface areas and then with constant outer surface areas by changing the length of the tubing. These experiments showed that the increase in VHP was the same when trying to fix the inner surface or the outer surface by changing the length of the incubated tubing. Therefore, only the ratio of surface area to volume (whether inner or outer) showed a correlation with VHP uptake. Since the same tubing samples were utilized for both experiments, differing only in length, the ratio of outer surface area to inner surface area remained constant.

This emphasizes the significance of the outer surface to inner surface (OS/IS) ratio, which is also reflected in the formula describing the diffusion coefficient of cylinders. Equation (3) describes the diffusion of gases into a hollow cylinder indicating that in steady state the diffusion is dependent on the inner and outer radii of the cylinder:[16]

$$Q_t = \frac{2\pi Dt(C_{2-C_{1)}}}{\ln(b/a)} \tag{3}$$

where Q_t represents the mass of diffusing substance per unit length over a defined time period t, D is the diffusion coefficient (m^2/s), t is the time span under consideration (s), C_1 is the concentration entering at the surface (ng/mL), and C_2 is the concentration in the system (measured as H_2O_2 in ng/mL). Lastly, a and b represent the inner and outer radii (in mm), respectively.

Therefore, the concentration of diffusing gas is a function of the ratio of outer to inner radius and time of exposure.[16] In our study, this is demonstrated by the increasing H2O2 uptake with higher S/V ratios as the volume exhibits sigmoidal growth with an increase of diameter-dependent dimension (ID, OD or WT), and the data was fitted following a sigmoidal function. A sigmoidal growth pattern was chosen as it demonstrates the characteristics of diffusion adequately, by including the change of concentration over time, a potential saturation and the initial lag phase.

4.2. *Influence of tubing material and supplier*

In VHP uptake experiments comparing different tubing materials, PCS tubing exhibited high VHP uptake (ranging from 247.6 to 458.3 ng/mL). In contrast, the TPE and TPV tubing showed no detectable VHP uptake following a one-hour incubation at 0.5 ppm VHP, which is consistent with studies performed by Hubbard et al. (2015). They tested VHP uptake of different tubing (including silicone and PTFE) in a small-scale isolator, by running a loop of tubing into the isolator chamber and simulating product flow path. While holding a constant hydrogen peroxide injection rate (0.1 ppm), they revealed that no hydrogen peroxide was taken up by PTFE tubing.[17] PCS tubing T11 demonstrated a significantly lower VHP uptake (41 % less) compared to all other PCS tubing (T1 + T7 and T12 – T14) (Fig. 8). This highlights the significant impact of material properties, particularly in PCS tubing from different manufacturers or different tubing types, on VHP uptake in solution. In fact, in our manufacturing scale study, we showed that two silicone tubing samples of equal wall thickness resulted in different H_2O_2 patterns in the water-filled vials.

In general, differences in terms of gas permeability of different classes of materials of construction such as TPE, TPV and PCS or for the same < declared > material, as we have observed in this study for PCS tubing from different manufacturers, may be explained by their specific chemical properties.

In our study, we evaluated three macroscopic properties including density and hardness, surface lipophilicity and surface structure.

Following a hypothesis by Kushwah et al. (2020) that variations in VHP uptake may be associated with differences in the lipophilicity of surfaces, they demonstrated that a higher hydrophilicity of glass vial surfaces leading to reduced VHP adsorption could be attributed to competitive behavior between H_2O_2 and water molecules. They hypothesize that more hydrophilic materials limit H_2O_2 vapor adsorption as these materials allow increased adsorption of vaporized water molecules.[18] Contact angle measurements did not have a significant impact on the extent of VHP uptake in our setting, and the contact angle measurements showed that the hypothesis from Kushwah et al. (2020) aligns only partly with the data obtained. Regarding silicone tubing for instance, tubing T11, with the same WT as the reference tubing (T7), exhibited a 41 % lower VHP uptake and demonstrated the lowest lipophilic properties among the measured tubing samples (Table 3). Tubing T1 – T3 displayed the highest lipophilic properties, but when comparing the uptake of these to other tubing with the same dimensions (T7 = 405.33 vs. T1 = 458.26 ng/mL), there was no significant difference.

As a next potential material property to be correlated to the VHP uptake of silicone tubing, the relative densities of the tested tubing as well as their hardness were sourced from manufacturers' brochures as listed in Table 1, within the Materials and Methods section. Notably, only tubing T11 exhibited an apparent decrease in VHP uptake compared to the remaining tubing. Upon comparing the density and hardness of tubing T11 (1.22 g/cm^3 / 80 Shore A) with that of the other silicone tubing (1.12 – 1.17 g/cm^3 / 50 – 65 Shore A), which demonstrated negligible variance, it becomes evident that tubing T11 has the highest relative density and highest hardness. This difference in relative density as well as hardness may signify essential differences in molecular packing or material composition (more filler present), potentially influencing its uptake behavior with VHP. Therefore, the heightened density (hardness) of tubing T11 could serve as a contributing factor to its distinctive VHP uptake behavior, warranting further investigation into the underlying mechanisms driving this observation.

When examining macroscopic SEM images (Fig. 9) of the tubing surfaces, no obvious structural differences were observed. Variations may be due to molecular level differences in the materials and not necessarily limited to surface properties.

We conclude that for our experiments and tubing samples tested, none of the tested properties dictate the VHP uptake alone. We hypothesize a complex interplay between molecular structure and surface properties associated with the overall process of gas diffusion in polymers on a macroscopic level.

The molecular level is mostly described by means of phenomenological models, such as the free volume theory, which describes how penetrant molecules have the tendency to reside in microcavities inside a polymer matrix. This suggests that microcavities can be regarded as parts of the "free volume" between surrounding polymer chains.[15] The shape and size of these microcavities in "rubbery" polymers (amorphous polymers with glass transition temperature below room temperature [19]) and therefore the free-volume distribution, can change much more frequently in comparison to "glassy" polymers (amorphous polymers kept below their glass transition temperature).[20] Specifically, the high flexibility of silicone-oxygen chains provide openings (free volume) that allow gas diffusion.[15]

In addition, the type and formulation of silicone can impact the permeability of silicone rubber, explaining the variation in uptake for the same material (PCS) from different manufacturers (Fig. 8). Zhang et al. (2006) noted that increasing the polarity of pendent groups in siloxane chains, decreases permeability, while crosslink density has little effect. Filler levels reduce permeability in proportion to volume fraction,[21] as fillers increase the enthalpy of gas solution, [15] affecting the polymer matrix and VHP diffusion. Overall, gas diffusivity decreases with higher silica filler, suggesting that increased silicone density may reduce VHP uptake, potentially correlating specific density to permeability.

4.3. *Considerations for fill-finish operations*

While previous reporting about VHP aeration mainly focused on operator safety, with environments considered safe once VHP levels fell below the OSHA permissible exposure limit (PEL) of 1 ppm, Hubbard et al. (2018) specifically studied the impact of VHP on protein quality.[5] Biopharmaceutical molecules such as monoclonal antibodies, and especially their specific solvent-exposed amino acid residues, such as Methionine are inherently sensitive to H_2O_2 oxidation and require stricter contamination controls during filling operations if relevant for drug safety or efficacy. Especially if filling is interrupted, H_2O_2 uptake can increase rapidly, posing risks to formulations and hence on product quality.[5] Hubbard et al. (2018) showed in a laboratory study that residual VHP, even at 0.25 ppm, can diffuse into formulation solutions through tubing, particularly after aeration, leading to significant peroxide uptake. The study showed that the VHP uptake immediately after the aeration phase in commercial manufacturing was sufficiently high to show a negative impact on mAb purity.[5]

With the results from the at-scale VHP uptake study in a manufacturing RABS setting (Fig. 10), we strengthened several key findings related to our mechanistic lab-scale study for both time-dependent and tubing-specific uptake. The exponential decay in VHP concentration, eventually decreasing from 0.309 to 0.011 ppm over four hours from the start of filling operations, highlights the importance of evaluating VHP uptake in manufacturing equipment. The VHP uptake pattern in water-filled vials after line interruptions showed a distinct, though overall decreasing, pattern indicating continuous VHP release from the manufacturing tubing. Moreover, the tubing-specific results underlined that the dimensions and material of the tubing significantly influence the uptake of H_2O_2. As shown Fig. 10, differences in tubing wall thickness (ranging from 1.6 to 3.2 mm) and material composition (PCS or TPE) resulted in varying H_2O_2 concentrations in the corresponding water samples. This confirmed our previous (mechanistic) findings that thicker tubing or tubing made of materials with different permeability may either retain more H_2O_2 or allow slower release into the sample. Therefore, the variations in H_2O_2 uptake associated with tubing material and wall thickness underline the need for further optimization of equipment configurations to ensure uniform exposure and minimal residuals, particularly in larger-scale applications.

It is also worth noting that various other factors associated with the production site can have an influence on the measured H_2O_2 uptake by silicone tubing, for example, tubing pretreatments, time in isolator, and average residual VHP concentration. Eisner et al. (2019) showed that autoclaved tubing absorbed 20 % less H_2O_2 than the non-sterilized control tubing after two days. Tubing autoclaved 14 days prior to testing, somewhat recovered and took up 39 % of the H_2O_2 concentration of the control. As autoclaved tubing is usually used after a few days, this could mean that autoclaving would be a favorable step regarding VHP uptake by silicone tubing. They experimentally verified that the reason for the reduced absorption was reduced water content in the tubing as a result of the drying practice at the end of an autoclaving cycle. In general, free volume is likely to result ingas diffusion and as silicone is hydrophobic, tubing permeability can decrease if water is removed from the free volume of the tubing material.[22] In addition, Eisner et al. reported that gamma-irradiation negatively impacts VHP uptake (increased uptake rates post gamma irradiation). Still, the pretreatment of tubing needs further investigation in relation to performance criteria.[22]

The selection of filling tubing for the fill-finish process is dependent on numerous parameters which will impact product compatibility (wear and tear from filling operations and adsorption phenomenon by formulation components), process performance (e.g., filling accuracy), or handling/operational considerations.[23] Only considering the uptake of VHP, silicone tubing underperform, compared to TPE or TPV. Moreover, Poumarede et al. demonstrated that TPE tubing might be beneficial in terms of oxidation protection of formulation excipients but still they concluded that the inherent rigidity limits their applications for peristaltic pumps.[24] Nevertheless, silicones are frequently selected due to their many advantages such as inherent material stability across a wide temperature range and their resistance to oils, solvents, and chemicals.[25] Most biopharmaceutical DP filling lines use PCS tubing in the product flow path because of its good mechanical properties.[7] As there is little research on the use of TPE/TPV tubing for biopharmaceutical filling processes, additional considerations (e.g. particles shedding, sterilizability, compatibility etc.) might be of interest.

VHP uptake studies and H_2O_2 spiking studies with tubing related to a specific DP are essential to ensure that the VHP uptake during aseptic fill-finish processes does not compromise the quality of the biopharmaceutical DP. If the preferred options is not available or measurements show unacceptable results, changes in the aseptic fill-finish process (like adding CIP and SIP processes) may be considered, as also recommended by Hubbard et al. (2018), to wash away a substantial amount of the residual VHP.[5] In summary, the reported data shows that in the case of silicone tubing, dimensional parameters should be taken into account during VHP uptake studies, and that tubing with the smallest surface to volume area and the largest WT are beneficial if filling accuracy and precision are not impacted.

5. Conclusion

In this study, we established a lab-scale model isolator and investigated the influence of tubing properties on VHP uptake and subsequent H_2O_2 release into incubated water. Results were subsequently verified at manufacturing scale. We showed that a lower level of H_2O_2 was measured in samples of exposed tubing with an increase in wall thickness. In contrast, increasing the surface to volume ratio of the tubing led to higher H_2O_2 concentrations in line with Fick‘s first law of diffusion. Our results demonstrate that tubing with smaller S/V ratio and larger WT result in smaller diffusion coefficients, as tested for PCS tubing, and therefore less VHP uptake.

In addition, we tested the influence of different materials on H_2O_2 uptake in tubing and found pronounced differences likely attributed to different material properties. TPE and TPV tubing did not show any uptake of VHP within the scope of the tested conditions, whereas significant VHP uptake occurred for all PCS tubing. Further, we observed significant differences in VHP uptake in PCS tubing from different manufacturers, but were able to only partly correlate these differences to specific material properties; this is subject of ongoing research.

The selection of the tubing for filling of biopharmaceuticals products is dependent on various parameters including compatibility with the drug product and filling equipment, likely wear and tear, and shear stress. Overall, we recommend that the potential VHP uptake by silicone tubing and subsequent release into the drug product solution should be a key consideration for tubing selection. Alternative tubing materials such as TPEs and TPVs could be suitable for fill-finish processes of biologics if their performance during the filling process aligns with the needs at the production site (e.g., particle shedding). When working with PCS tubing, dimensional as well as the material dependent uptake profiles should be considered to limit VHP uptake.

CRediT authorship contribution statement

Dilara Ali: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sarah S. Peláez:** Writing – review & editing, Visualization, Methodology, Investigation, Conceptualization. **Thomas Lemazurier:** Formal analysis, Conceptualization. **Ariane Schroeter:** Writing – review & editing, Conceptualization. **Michael Adler:** Writing – review & editing, Conceptualization. **JaeHwi Bong:** Investigation. **Oliver Germershaus:** Writing – review & editing, Conceptualization. **Hanns-Christian Mahler:** Writing – review & editing. **Andrea Allmendinger:** Writing – review & editing, Supervision, Resources, Project administration, Formal analysis, Conceptualization.

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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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Fig. 3 “Setup of filling manifold of at-scale VHP uptake study” was created with the help of BioRender: Created in BioRender. Allmendinger, A. (2025) https://BioRender.com/t58n921.

Data availability

Data will be made available on request.

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