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Comparison of Perfluorocarbon Liquids Cytotoxicity Tests: Direct Contact Versus the Test on Liquid Extracts

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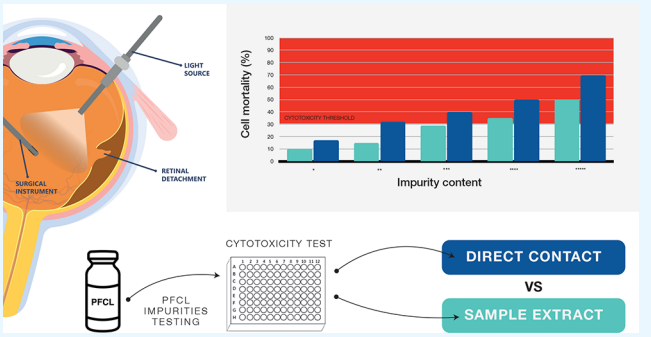
ABSTRACT: The purpose of this study is to compare the in vitro cytotoxicity tests according to the ISO 10993-5 (2009) standards using direct contact and the test on liquid extracts of compounds previously identified as possible toxic impurities in perfluorocarbon liquids (PFCLs) for use in vitreoretinal surgery. Compounds including perfluorooctanoic acid (PFOA), 1*H*-perfluorooctane (1H-PFO), 2*H*-tridecafluoro-2-methylpentane, 1*H*,2*H*-octafluorocyclopentane, and 2*H*,3*H*-decafluoropentane were analyzed by ^{19}F NMR before and after extraction using an aqueous solution and tested by both the direct contact and liquid extract tests in L929, BALB 3T3, and ARPE-12 cells. The concentration that reduced in vitro cell viability by 30%, the cytotoxicity concentration threshold (CC_{30}), was determined for each compound. ^{19}F NMR spectroscopy confirmed the immiscibility of perfluoro-*n*-octane (PFO) and 1H-PFO and the solubility of PFOA with the extraction vehicle. The other samples reacted with the extraction vehicle, releasing fluoride ions. Using the direct contact test, the CC_{30} of PFOA, 1H-PFO, 2*H*-tridecafluoro-2-methylpentane, 1*H*,2*H*-octafluorocyclopentane, and 2*H*,3*H*-decafluoropentane corresponded to 48 124, 50, 14, 8035, and 46 ppm, respectively. The method on liquid extracts did not detect cytotoxicity in three out of five tested compounds, and CC_{30} could not be determined. In conclusion, the in vitro cytotoxicity test by direct contact revealed a positive correlation between cell toxicity and the concentration of the tested substance. Conversely, the test on liquid extracts hardly detected the cytotoxicity of toxic impurities in PFCLs. Thus, only the cytotoxicity test by direct contact, according to ISO 10993-5 (2009), is a sensible and reliable method to detect possible cytotoxic impurities in PFCLs to guarantee patient safety.



INTRODUCTION

Perfluorocarbon liquids (PFCLs), such as fully fluorinated perfluoro-*n*-octane (PFO), are safe and valuable tools in ophthalmic surgery for the management of vitreoretinal diseases, thanks to their physical-chemical characteristics of high gravity, optical clarity, and immiscibility with water.[1−5] Nonetheless, severe episodes of ocular toxicity after using the commercially available PFCLs batches were reported in Spain throughout Europe since 2013.[6−11] The origin of PFCLs toxic effects was thought to be due, among other factors, to the presence of various hazardous impurities whose toxicity was not identified by the manufacturer before the release of the medical device on the market.[6,10] Nevertheless, the manufacturers claimed that the raw material's purity and the finished products' safety were tested by an in vitro cytotoxicity test on aqueous extracts according to ISO 10993-5 (2009).[12]

Thus, the debate arose on the reliability of the cytotoxicity test on extracts versus the in vitro cytotoxicity test by direct contact, both described by ISO 10993-5.[6,8,10] The test on liquid extract does not take into full consideration the hydrophobic and volatile characteristics of the PFCLs[3] and, therefore, may leave undetected the toxicity of some unextracted impurities. The direct contact test, coupled with accurate physical-chemical methods for testing the purity of the raw material (e.g., NMR analyses or gas chromatography techniques), could truly ascertain the complete absence of potentially hazardous impurities in the PFCLs medical devices.[6,7,10,13−16]

Using a fluoride-selective potentiometry, some authors introduced the so-called *H*-value to detect the presence of reactive, incompletely fluorinated impurities and partially hydrogenated perfluoroalkanes capable of inducing toxic side effects due to the release of hydrogen fluorine.[8,17] However, the *H*-value is not identical to the *H* concentration since the "*H*-value only covers the reactive H-containing impurities that

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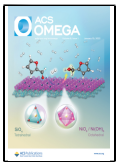

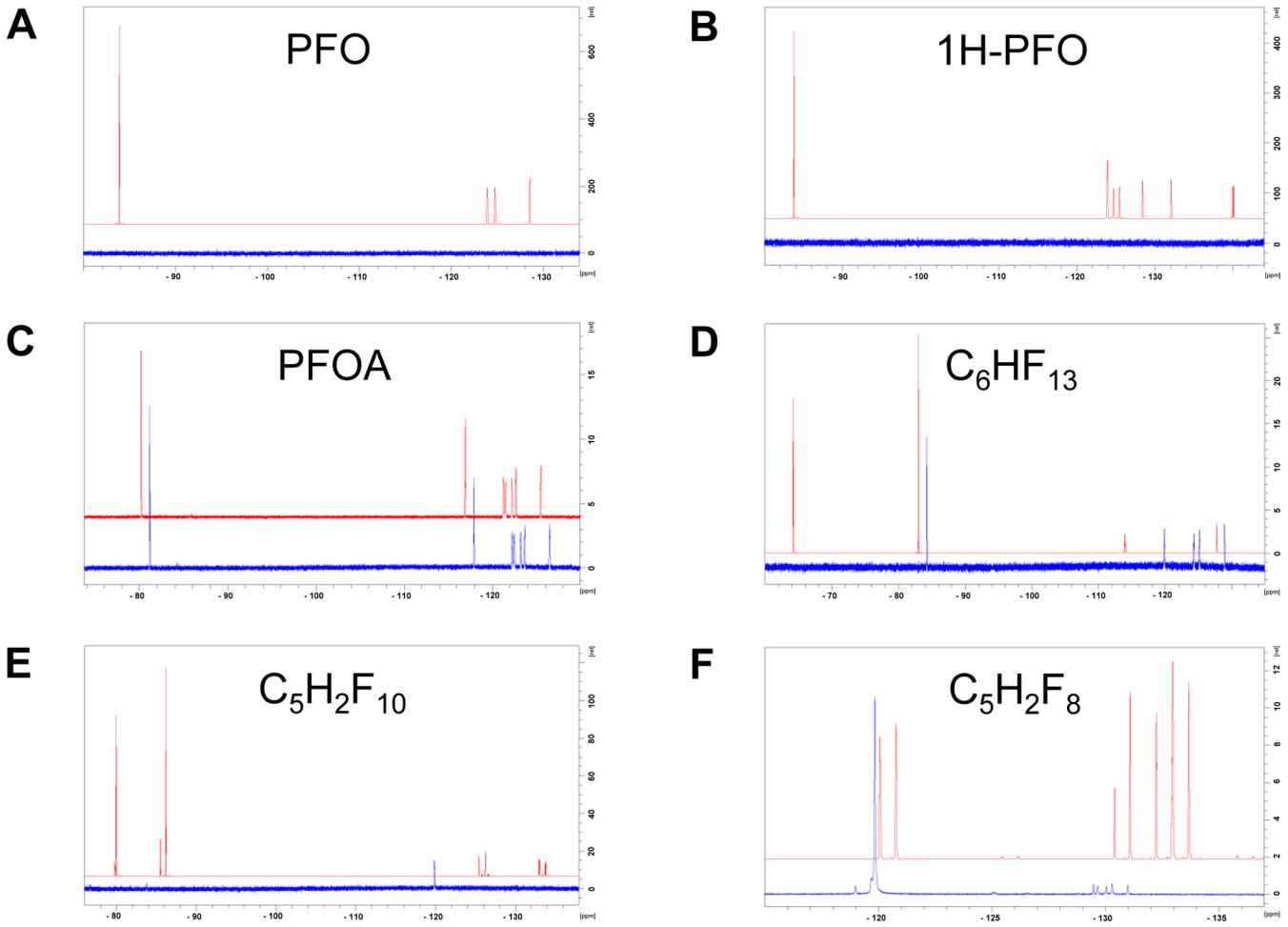

Figure 1. ^{19}F NMR spectra of the PFO diluent and test samples (as pure compounds) before (in red) and after (blue) treatment with DMEM-extracting aqueous solution. (A) PFO; (B) 1*H*-PFO; (C) PFOA; (D) C_6HF_{13}; (E) $C_5H_2F_{10}$, and (F) $C_5H_2F_8$. Please note that to ease viewing both PFOA spectra (Figure 1C), the one obtained after DMEM extraction was deliberately shifted (of 1 ppm) to the right.

only become acute or latent toxic impurities through their reactivity".[8] The potentiometry method does not detect an extensive range of potentially toxic impurities.[16]

This study compared two in vitro cytotoxicity test methods for testing PFCLs: the test by direct contact and the test on liquid extracts, both described in the ISO 10993-5.[12] We tested the cytotoxicity of perfluorooctanoic acid (PFOA) and 1*H*-perfluorooctane (1H-PFO) impurities previously detected in the toxic PFCLs batches[6,11,13] and three reactive hydrogen-containing compounds, including the 2*H*-tridecafluoro-2-methylpentane—C_6HF_{13}, the 1*H*,2*H*-octafluorocyclopentane—$C_5H_2F_8$, and the 2*H*,3*H*-decafluoropentane—$C_5H_2F_{10}$, which are potentially capable of inducing cytotoxic effects.[8,17,18] First, we analyzed the ^{19}F NMR spectra of the tested impurities before and after extraction in an aqueous solution. Afterward, we compared the cell viability of L929, BALB 3T3, and ARPE-12 cell lines using the two cytotoxicity test methods according to ISO 10993-5 (2009).[12] Finally, we conducted dose-response studies using both cytotoxicity test methods on L929 cells to determine for each tested impurity the cytotoxicity concentration threshold (CC_{30}) that reduced the in vitro cell viability by 30% and therefore resulted in being cytotoxic according to the ISO10993-5 (2009).[12]

EXPERIMENTAL SECTION

Preparation of Test Samples. Serial dilutions of PFOA (CAS 335-67-1, Merck, Italy), 1H-PFO (CAS 335-65-9, Fluorochem, UK), C_6HF_{13} (CAS 30320-28-6, UK), $C_5H_2F_8$ (CAS 828-35-3, Fluorochem, UK), and $C_5H_2F_{10}$ (CAS 138495-42-8, UK) were prepared by adding PFO (purity > 99%, AL.CHI.MI.A Srl, Italy) as a diluent and continuously stirring for 30 min at room temperature.

Preparation of Liquid Extracts of Test Samples. The extraction of test samples was performed according to ISO 10993-5 (2009)[12] and as described by Menz et al.[8] by mixing 2.0 g of each test sample diluted in PFO with 6.6 mL of the Dulbecco's Modified Eagle's Medium (DMEM) extraction vehicle containing 10% fetal calf serum (FCS, Sigma-Aldrich) at 37 °C for 24 h. The extraction of the test samples containing the PFOA was performed in the absence of FCS, as recommended by the ISO 10993-5 (2009)[12] for polar substances; however, FCS was added to the DMEM vehicle before application to the cells in the cytotoxicity test. After extraction, each sample underwent centrifugation at 5000 rpm for 15 min: the water-insoluble phase was discarded, and the aqueous phase was used for the cytotoxicity test on the extracts.

NMR Analysis of the Test Samples and Liquid Extracts. NMR spectra were acquired on a Bruker AVANCE

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III HD spectrometer (1*H*: 400 MHz) with a QNP probe (Bruker BioSpin, Germany) using Wilmad coaxial NMR tubes (inner tube: 2.97 mm × 1.96 mm, O.D. × I.D.) for perfluoroalkanes and Wilmad NMR tubes (5 mm) at 298 K. The data acquisition and processing were performed using the Topspin 3.0 software (Bruker BioSpin). Before the data acquisition, the sample was well-tuned and matched manually. The acquisition parameters used for performance tests were set as follows: pulse sequence, zgflqn; relaxation delay (D1), 2 s; spectral width (SW), 200 ppm centered at −100 ppm; data acquisition time (AQ), 0.9 s; dummy scans (DS), 8; number of scans (NS), 8 for perfluoroalkanes or 1800 for buffer solutions; and spinning, OFF. RG was automatically set by Topspin software.

The obtained NMR spectra were processed by multiplying with exponential (0.3 Hz line broadening) and zero filling. The phases were manually corrected, and a fifth-order polynomial corrected the baseline. The NMR spectra of the neat perfluoroalkanes were obtained by acquiring eight repeats. To evaluate the presence of perfluoroalkanes in the DMEM-extracting vehicle, the NMR spectra were obtained by acquiring 1800 repeats. Therefore, the spectra of each tested perfluoroalkane before and after treatment with DMEM were reported with different scales (Figure 1).

^{19}F NMR analysis was also used to evaluate the presence of perfluoroalkyl or fluoride contaminants in the DMEM buffer used in different experiments (data not shown).

Preparation of Cell Cultures. The connective mouse tissue L929 cell lines [NCTC clone 929 (L cell, L929, a derivative of Strain L)] were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The L929 cells were grown in DMEM with high glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate (Sigma-Aldrich) and supplemented with 10% iron-fortified bovine FCS and 1% penicillin−streptomycin (Sigma-Aldrich), in 75 cm^2 culture flasks at 37 °C ± 1 °C, 90% ± 10% humidity, and 5.0% ± 1.0% CO_2/air. The murine fibroblast cells BALB 3T3 clone A31 (ATCC CCL163) and the human retinal pigment epithelial cell line ARPE-19 (ATCC CRL-2302) were obtained from the American Tissue Culture Collection and were grown as previously described by Romano et al.[19,20] For each cell line, at 80% confluence, the cells were removed from the flasks, and suspensions containing 1.0 × 10^5 cells/mL were seeded into 96-well microtiter plates and incubated at 37 °C ± 1 °C, 90% ± 10% humidity, and 5.0% ± 1.0% CO_2/air for 24 h with their respective DMEM growth medium (the vehicle).

Cytotoxicity Test by Direct Contact. The direct contact cytotoxicity test was performed using a validated method according to ISO 10993-5 (2009)[12] described by Romano et al.[19,20] using the BALB 3T3 and ARPE-19 cell lines for the 1H-PFO test sample only. The previously used method assessed the risk and excluded possible test failure due to evaporation of PFCLs during testing.[19] The same procedure was used with the L929 cell line for all the test samples. The culture medium was removed and replaced with 250 μL of a fresh vehicle, and 50 μL of the test sample or control was gently applied to the cell layer.[19] Samples of ultrapure PFO were used as negative controls, and samples of 1H-PFO at a concentration of 12.5% (w/v) in PFO were used as positive (cytotoxic) controls. All test samples and controls were kept in direct contact with the cell layer for 24 h at 37 °C.

Cytotoxicity Test on Liquid Extracts. This test was carried out in compliance with ISO 10993-5 (2009)[12] and as described by Menz et al.[8] For each cell line, the culture medium was removed and replaced with 300 μL of each test sample extract. Afterward, 300 μL of latex gloves (6 cm^2/mL) and polypropylene (1.0 g/5 mL) extracts, corresponding to positive (cytotoxic) and negative (non-cytotoxic) controls, respectively, and test samples were applied to the cells for 72 h at 37 °C.

Cytotoxicity Measurements and Statistics. The MTT-based TOX-1 in vitro toxicology assay kit (Sigma-Aldrich Corp.) was used to test the ARPE-19 cell line's viability as previously described by Romano et al.[19,20] The Neutral Red-based (NRU) TOX4 toxicology assay kit (Sigma-Aldrich) was used to assess the viability of the BALB 3T3 cells,[12,19] and the BCA staining was performed as a measure for cytotoxicity in the L929 cell cultures.[8,12,21,22]

For all cell lines, the mean percentage of cell viability was calculated as follows

$$\text{mean \% of cell viability} = \text{mean}\frac{\text{OD test sample} - \text{OD blank}}{\text{OD vehicle} - \text{OD blank}}\%$$

The mean percentage of cell viability and standard deviation of the mean (SD) were calculated for each tested sample concentration and control.

According to ISO 10993-5 (2009)[12] and ISO 10993-12,[22] evident cytotoxicity is defined as an effect leading to an inhibition of cell growth of more than 30% compared to that induced in cultures treated with the control vehicle. As previously reported, theoretical values of cytotoxic concentration (CC_{30})[20] and 50% inhibitory concentration (IC_{50}),[12] corresponding to the concentrations that would reduce 30 and 50% of cell viability in L929 cells, respectively, were calculated for impurities including 1H-PFO, PFOA, C_6HF_{13}, $C_5H_2F_{10}$, and $C_5H_2F_8$ from the fitted regressions. The differences in percent relative viability between two cell lines were compared using the nonparametric Mann−Whitney *U* test.

RESULTS

^{19}F NMR Analysis. The detection of perfluoroalkanes in the DMEM-extracting vehicle used in the cytotoxicity test on liquid extracts was performed by ^{19}F NMR spectroscopy.

Figure 1 shows that the ^{19}F NMR spectra of neat tested perfluoroalkanes were characterized by the presence of a strong peak in the −80 to −95 ppm range split into three peaks with an area in the ratio 1:2:1 attributable to the $-CF_3$ moiety (Figure 1A−E). Peaks with coupling patterns were observed in the −110 to −140 ppm range and attributable to either CF_2 or CF moieties (Figure 1A−E). Figure 1A,B shows that the extracts of PFO and 1H-PFO did not show the presence of fluorine signals confirming the immiscibility of the DMEM-extracting vehicle with the selected perfluoroalkanes. On the other hand, the ^{19}F NMR spectrum of the PFOA extract showed the presence of fluorine signals, superimposable to those of a reference PFOA in an aqueous solution (Figure 1C), confirming the solubility of PFOA in water and thus its extraction.

Surprisingly, the ^{19}F NMR spectra of C_6HF_{13} (Figure 1D), $C_5H_2F_8$ (Figure 1E), and $C_5H_2F_{10}$ (Figure 1F) extracts were characterized by the presence of a peak at about −120 ppm attributable to the fluorine anion and by the presence of signals in the region of mono-, or di-substituted carbon fluorides not superimposable to the parent compounds (Figure 1D−F).

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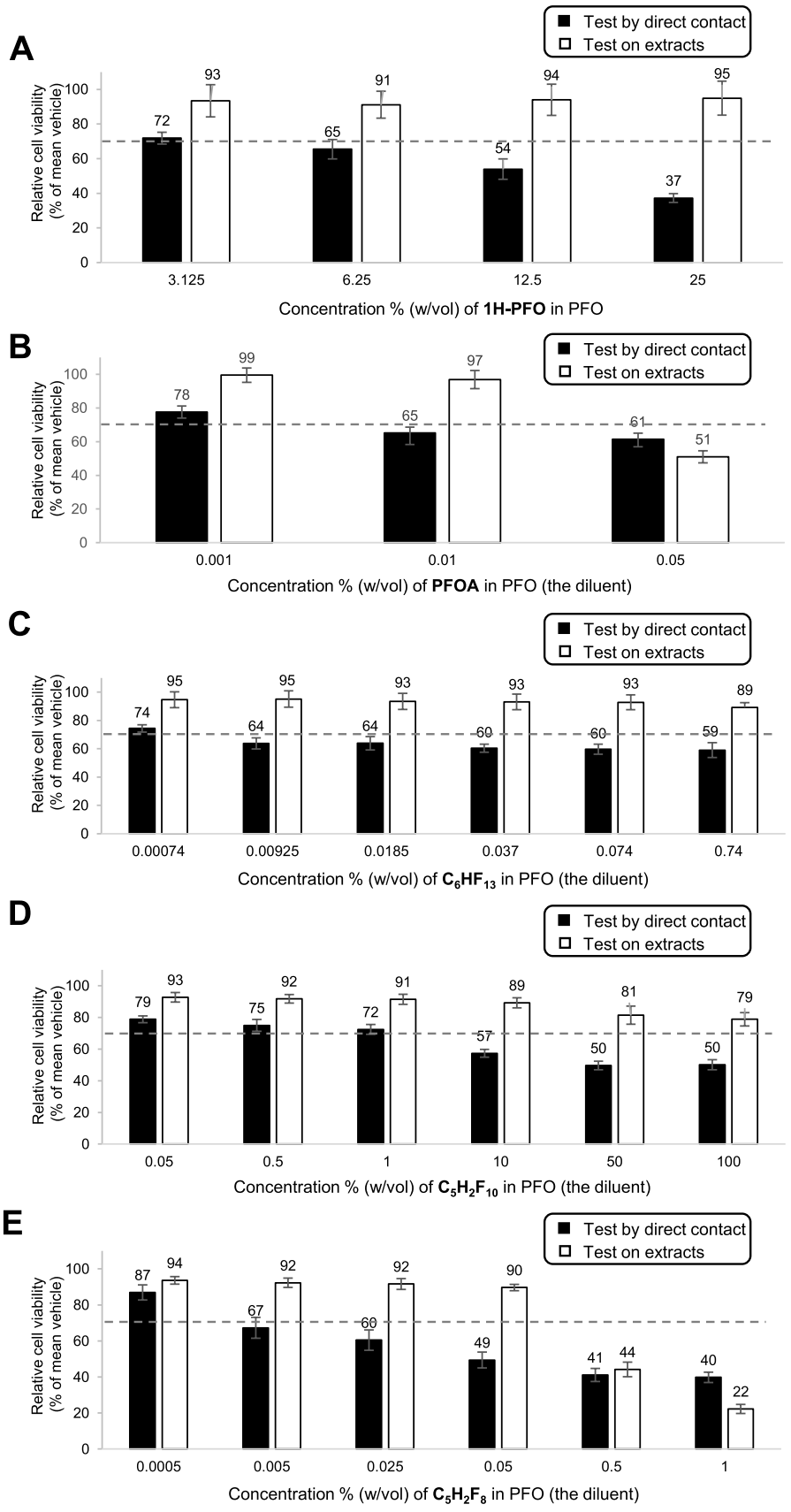

Figure 2. Cytotoxicity testing results of cell viability in L929 cell lines using the direct contact procedure (full bars) and the test on liquid extracts (empty bars), according to ISO 10993-5 (2009)[12] obtained with the different test sample concentrations in PFO: (A) 1H-PO; (B) PFOA; (C) C_6HF_{13}; (D) $C_5H_2F_{10}$, and (E) $C_5H_2F_8$. The dashed line indicates the cytotoxicity threshold at 70%. For a statistical evaluation of differences between the two procedures, please refer to Table S1 in the Supporting Information.

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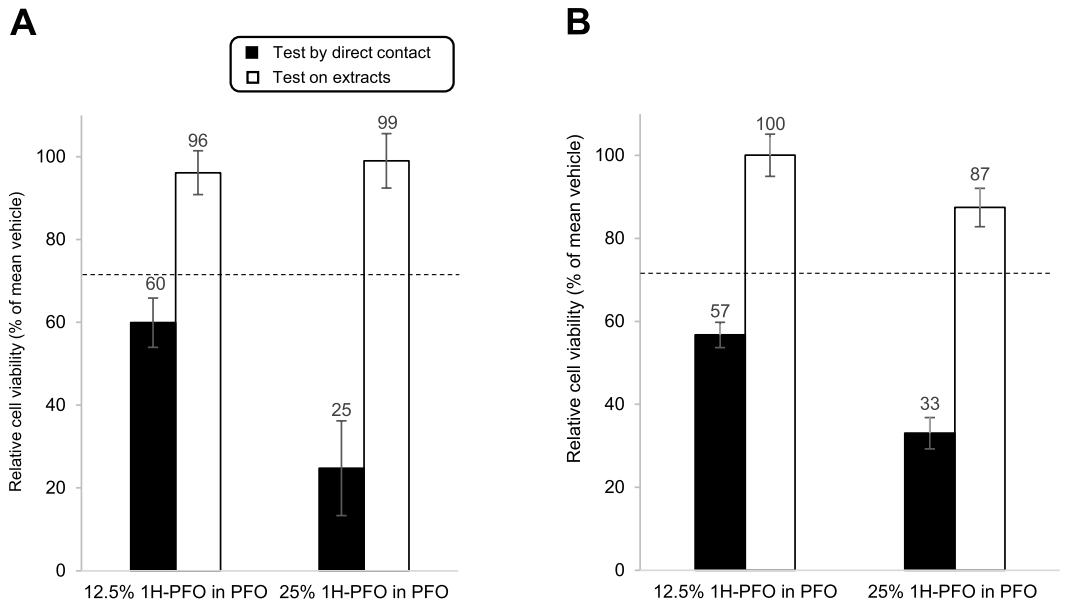

Figure 3. Cytotoxicity testing results of cell viability in (A) BALB 3T3 and (B) ARPE-19 cell lines using the direct contact procedure (full bars) and the test on extracts (empty bars), according to the ISO 10993-5 (2009)[12] standards obtained with two concentrations of 1H-PFO in PFO (%, w/v). The dashed line indicates the cytotoxicity threshold at 70%. For a statistical evaluation of the differences between the two procedures, please refer to Table S2 in the Supporting Information.

Table 1. Estimated CC_{30} and IC_{50} of Test Sample Values Obtained with the Two In Vitro Cytotoxicity Test Procedures in the L929 Cell Line[a]

	test by direct contact		test on extracts	
tested impurity	CC_{30} (ppm)	IC_{50} (ppm)	CC_{30} (ppm)	IC_{50} (ppm)
1*H*-perfluorooctane(1H-PFO)	48,124	212,187	ND	ND
perfluorooctanoic acid (PFOA)	50	5830	324	517
2*H*-tridecafluoro-2-methylpentane (C_6HF_{13})	14	101,343	ND	ND
2*H*,3*H*-decafluoropentane ($C_5H_2F_{10}$)	8035	789,899	ND	ND
1*H*,2*H*-octafluorocyclopentane ($C_5H_2F_8$)	46	1140	2078	4167

[a]ND: not determinable. CC_{30} is the concentration that reduces 30% of cell viability. IC_{50} is the concentration of each compound which inhibits 50% cell viability. CC_{30} and IC_{50} were calculated from fitted regressions of concentration−response curves.

These data suggested that the tested perfluoroalkanes may have reacted with nucleophiles present in the DMEM-extracting vehicle, forming new compounds and releasing fluoride ions, according to the reaction described by Menz et al.[8]

In Vitro Cytotoxicity Tests: Comparison between the Direct Contact Test and the Test on Liquid Extracts. According to ISO 10993-5 (2009)[12] direct contact cytotoxicity test, a sample is cytotoxic when it causes a reduction of cell viability greater than 30%.

When the concentration of 1H-PFO increased (Figure 2A), the cytotoxicity test by direct contact revealed a decrease in the L929 cell viability. Only the less concentrated 1H-PFO sample (3.125% w/v) was not cytotoxic, as it showed viability above the 70% limit. Liquid extracts at all test concentrations did not affect the cell viability (Figure 2A). Concerning the PFOA samples (Figure 2B), only its concentration of 0.001% was not cytotoxic when tested with the direct contact method, whereas all the higher concentrations reduced the cell viability below 70%; instead, only 0.05% PFOA sample extracts induced cytotoxicity in the test on extracts. The *H*-reactive C_6HF_{13} test samples at all concentrations tested by the extraction method resulted in being not cytotoxic, showing cell viability higher than 70% (Figure 2C); instead, the direct contact method revealed that all the C_6HF_{13} samples caused cytotoxicity with the only exception of the lowest tested concentration (0.00074% C_6HF_{13}). The *H*-reactive $C_5H_2F_{10}$ was not cytotoxic when tested by the extraction method (Figure 2D); instead, when using the direct contact test, the cell viability decreased depending on the $C_5H_2F_{10}$ concentration, resulting in being cytotoxic at concentrations higher than 1% (Figure 2D). The *H*-reactive $C_5H_2F_8$ was cytotoxic except for the lowest concentration (0.0005% in PFO) when using the direct contact test (Figure 2E); instead, the H-reactive sample became cytotoxic at concentrations ranging between 0.5 and 1% when using the test on extracts (Figure 2E). A statistically significant difference in cell viability was observed between the two testing procedures for all the conditions (for more details, see Table S1 in the Supporting Information).

Figure 3 illustrates the % of cell viability obtained in direct contact or liquid extract tests in BALB 3T3 (Figure 3A) and ARPE-19 cell lines (Figure 3B) after the application of previously tested samples containing 25 and 12.5% 1H-PFO in PFO. Similar to the L929 cells, the tested samples of 1H-PFO were cytotoxic by direct contact test and not cytotoxic by the test on liquid extracts in both BALB 3T3 and ARPE-19 cell lines (Figure 3A,B).

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The mean cell viability values between the two test methods significantly differed for all tested concentrations in both cell lines (see Table S2 in the Supporting Information).

Cytotoxic Concentration 30 (CC_{30}) and 50% Inhibitory Concentration (IC_{50}) in L929 Cells. Figure S1 in the Supporting Information illustrates the tested samples' concentration−response curves obtained with the direct contact and "on extracts" cytotoxicity test in the L929 cell line. Table 1 reports the cytotoxic concentrations 30 (CC_{30}) and 50% inhibitory concentration (IC_{50}), representing the concentrations that reduced the in vitro cell viability by 30 and 50%, respectively. CC_{30} and IC_{50} values were calculated from fitted regressions of concentration−response curves. The CC_{30} and IC_{50} values obtained from the direct contact test for each tested compound notably differed from the values obtained with the test on extracts (Table 1).

DISCUSSION AND CONCLUSIONS

Our study compared in vitro cytotoxicity testing by the direct contact test and the liquid extract test (concentration−response study) of PFCL samples containing PFOA and 1H-PFO impurities previously detected in the toxic PFCL batches[6] and reactive hydrogen-containing impurities.[9,13,17] We assessed the suitability of the liquid extraction method by NMR analysis of the liquid extracts for the presence of PFCLs. In addition, we compared the CC_{30} values (the concentration that reduced the in vitro cell viability by 30%, which is defined as cytotoxic) for each compound calculated from the fitted regressions for both methods.

The dose−response studies on L929 fibroblasts showed discordant cytotoxicity outcomes based on the used method for all the tested compounds, confirmed by the statistically significant differences in cell viability between the two testing methods. In addition, the cytotoxicity of the 1H-PFO sample was evaluated in the BALB 3T3 and ARPE-19 cell lines, by way of example, and the results obtained in L929 fibroblasts were also confirmed in these two cell lines. In the present study, the L929 cell line was selected for comparison of the data with previously published tests on PFCL extracts,[8,11] the BALB 3T3 cell line was included as the most frequently used cell line for the cytotoxicity tests and is indicated as the reference cell line in ISO standards 1993-5 (2009),[12] and the ARPE-19 cells were included as they are derived from the human retina, and thus allow mimicking the conditions of use of PFCLs in an operating theater.

In order to properly compare our tests with those performed in the literature, we followed the procedures described for the validated in vitro cytotoxicity test by direct contact described by Romano et al.[19] and those described by Menz et al.[13] for performing the cytotoxicity test on liquid extracts. In our study, all tested compounds were diluted at different concentrations in PFO to simulate a possible PFCL medical device containing impurities. Only the direct contact method detected the cytotoxicity of 1H-PFO, C_6HF_{13}, and $C_5H_2F_{10}$, while the test on liquid extracts also noticed the cytotoxicity of PFOA and $C_5H_2F_8$. However, the extract method detected the PFOA and $C_5H_2F_8$ cytotoxicity only at the highest concentrations.

The cytotoxicity test on liquid extracts of test samples implies that the tested samples are miscible with an aqueous extracting vehicle. Due to the immiscibility of perfluorocarbons with aqueous solutions, the test on liquid extracts may not detect water-insoluble toxic impurities. Instead, using the test by direct contact, the PFCL-containing or any potentially poisonous impurity is brought in direct contact with the cells.

The ^{19}F NMR spectroscopy showed the total absence of 1H-PFO and PFO in DMEM-extracting vehicles due to their immiscibility in an aqueous solution. Consequently, the 1H-PFO was absent in liquid extracts applied to the cells during the cytotoxicity test of liquid extract; thus, the 1H-PFO cytotoxicity was not detected at any extract concentration. On the contrary, 1H-PFO was cytotoxic when tested by direct contact, except for the lowest tested concentration.

The ^{19}F NMR analyses of the DMEM extract of the three hydrogen-containing compounds C_6HF_{13}, $C_5H_2F_{10}$, and $C_5H_2F_8$ revealed the presence of a peak attributable to the fluorine anion and other newly formed peaks not superimposable to the parent compounds. We hypothesize that this is due to the reactions of the reactive hydrogen present in the perfluoroalkanes with nucleophiles present in the extracting vehicle according to the reaction described by Menz et al.,[8] forming water-soluble compounds. While the direct contact method detected the cytotoxicity of C_6HF_{13}, $C_5H_2F_{10}$, and $C_5H_2F_8$ at most of the tested concentrations, the extracts of C_6HF_{13} and $C_5H_2F_{10}$ did not induce cytotoxicity at any concentration, and only the $C_5H_2F_8$ extract was cytotoxic at the highest tested concentration. As indicated by the $C_5H_2F_8$ DMEM extract ^{19}F NMR spectrum, its cytotoxicity was induced by the newly formed compound that appeared after the extraction treatment. The original compounds were immiscible in an aqueous solution, and the cytotoxicity obtained with the test on extracts was due to newly formed substances that were not the original compounds. These findings demonstrate that the cytotoxicity test on liquid extracts may produce false-negative results. In contrast, the direct contact cytotoxicity test allowed the detection of all tested compounds, including reactive hydrogen-containing compounds.

Different from the latter compounds, the ^{19}F NMR spectroscopy showed that PFOA was present in the DMEM-extracting vehicle; however, the test on liquid extracts showed the PFOA cytotoxicity at the highest concentration only (i.e., 0.05%).

CC_{30} and IC_{50} could not be calculated for 1H-PFO, C_6HF_{13}, and $C_5H_2F_{10}$ with the extraction method due to the flat dose−response curves. CC_{30} and IC_{50} calculated from the fitted regressions showed marked differences between the two methods; high CC_{30} values obtained for the extraction method would suggest low cytotoxicity of the PFOA and $C_5H_2F_8$, underlining contrasting results compared to the direct contact method. As previously reported,[20] the parameter used to evaluate the cytotoxicity derives from specific ISO 10993-5 (2009)[12] and ISO 10993-12[22] norms that consider a cut-off of 70% in cell viability for designating noncytotoxic versus cytotoxic test sample for both direct contact and liquid extract tests.

These findings confirm what was previously observed by Pastor et al.[6] and Srivastava et al.,[10] who pointed out that the in vitro cytotoxicity test conducted on liquid extracts of test samples, even when performed under ISO 1993-5 (2009)[12] is a poorly reliable procedure as compared to the direct contact test. This may be explained by the immiscibility and/or dilution of the tested samples in an aqueous extracting vehicle, leading to false negative cytotoxicity test results. However, since the ISO standards describe various procedures, the crucial step is selecting and validating the most suitable

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method considering the physicochemical characteristics of the tested samples.[6,19] Only three studies have compared the direct contact and the extractive cytotoxicity test methods.[6,8,9] In all of them, samples of the PFCL batches that caused severe episodes of ocular toxicity in Spain were used. The ocular toxicity of those PFCL batches was likely due to the combined effect of all contaminants rather than one specific substance.[11,13] In this study, we focused on the cytotoxicity of particular impurities, which were previously assessed by the direct contact test.[11,16] To properly compare our tests with those performed in the literature, we followed the procedures described for the validated in vitro cytotoxicity test by direct contact described by Romano et al.[19] and those described by Menz et al.[8] for performing the cytotoxicity test on aqueous extracts. These analyses, coupled with the qualitative ^{19}F NMR spectroscopy to evaluate the solubility of the tested substances in the DMEM-extracting solution propaedeutically to the cytotoxicity in vitro assays were, to our knowledge, never performed before.

According to ISO 16672 (2020) on Ophthalmic implants—ocular endotamponades[23] any identified contaminant should be reduced to a level that the health risk associated with the contaminant is considered acceptable. We have previously determined the cytotoxicity threshold of contaminants in PFCLs described as possible causes of severe adverse reactions in patients.[20]

Our findings in the direct contact test agree with those obtained by Ruzza et al.[16] who studied the extent of ARPE-19 and BALB 3T3 cell mortality after applying 1H-PFO and PFOA at concentrations close to those tested in this study. The only exception was the lack of toxicity of PFOA at a concentration of 0.0028% on BALB 3T3 cells, which might be related to better resistance to that compound in those cells than the L929, which in our study had a toxicity limit (CC_{30}) of 14 ppm. Furthermore, the toxicity threshold assessed for PFOA in this study in L929 cells was lower than that evaluated by Srivastava et al.[11] who found that PFOA at 0.06 mM was close to the toxicity limit in ARPE-19 cells, which corresponded to 25 ppm. However, in this case, it is possible that the difference between the two studies was related, in addition to the different sensitivity of the cell lines to the toxic agent, to the different testing conditions (e.g., sample size, contact time, and the number of replicates).

The toxicity of PFCLs is often caused by incomplete fluorination of hydrocarbons, with reactive hydrogen-containing impurities and unsaturated carbon bonds. The first clues on that date the early 90s.[24,25] Later on, Menz et al.[8] used physical-chemical determination of partially hydrogenated perfluoroalkanes through ion-selective potentiometry after digestion of perfluorocarbon liquid[5,26] to determine the so-called *H*-value, defined as the ppm content of reactive partially hydrogenated perfluoroalkanes, to which an *H*-value of less than 10 ppm was attributed as the safety threshold by Menz et al.[8] However, only the nonlinear semifluorinated alkanes—the so-called *H*-reactive compounds—can release hydrogen fluoride according to the mechanism mentioned above.[18] Thus, this might be the reason, among others, why the importance of determining the *H*-value cannot be overemphasized.[8] As stated by Dresp.[27] "The *H*-value is exclusively a measure for reactive under-fluorinated compounds and other toxic compounds must be determined via other chemical−physical detection methods".

We showed that the in vitro direct contact cytotoxicity test could provide direct information on cytotoxicity, including the traces of impurities contained in PFCLs, independently from substance identification and quantification. This points out the primary role of in vitro cytotoxicity tests in assessing the safety of medical devices, provided that the testing methods are critically evaluated, selected, and validated considering the physicochemical characteristics of the tested samples.

In conclusion, our study demonstrated that only the in vitro cytotoxicity test conducted using the direct contact test according to ISO 10993-5 (2009)[12] could detect cytotoxicity, while the same compounds were not cytotoxic using the test on liquid extract. The in vitro direct contact cytotoxicity test represents a reliable tool to assess the safety of the PFCLs before the product batch can be released on the market, while the liquid extract method is not a reliable approach to recapitulate the potential toxicity of PFCLs in patients due to possible false negative results. The cytotoxicity test by direct contact according to the ISO 10993-5 (2009)[12] standard is the most sensible and the only truly reliable detection method to detect any potential cytotoxicity in PFCLs and, thus, to guarantee maximal safety for the patients; on the contrary, the test on the liquid extract should be avoided when compounds immiscible in aqueous solutions are evaluated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04697.

Relative cell mortality, relative cell viability, concentration, and response curves for cell mortality of the tested impurity samples (PDF)

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Notes

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■ REFERENCES

(1) Bourke, R. D.; Cooling, R. J. The stability of perfluoro-n-octane during vitreoretinal procedures. *Arch. Ophthalmol.* **1996**, *114*, 537−544.
(2) Elsing, S. H.; Fekrat, S.; Green, W. R.; Chang, S.; Wajer, S. D.; Haller, J. A. Clinicopathologic findings in eyes with retained perfluoro-n-octane liquid. *Ophthalmology* **2001**, *108*, 45−48.
(3) Yu, Q.; Liu, K.; Su, X.; Xia, X. Perfluorocarbon liquids: its application in vitreoretinal surgery and related ocular inflammation. *BioMed Res. Int.* **2014**, *2014*, 250323.
(4) Chan, Y. K.; Lu, Y.; Czanner, G.; Wu, J.; Cheng, H. C.; Hussain, R.; Sakamoto, T.; Shum, H. C.; Wong, D. In vitro experiment to elucidate the mechanism of the 'soft shell technique' for preventing subretinal migration of perfluoro-octane. *Br. J. Ophthalmol.* **2017**, *101*, 389−394.
(5) Leisser, C.; Varsits, R.; Findl, O. Does perfluoro-n-octane use in 23G vitrectomy for retinal detachment surgery affect the integrity of the ellipsoid zone? *Eur. J. Ophthalmol.* **2016**, *26*, 639−642.
(6) Pastor, J.; Coco, R. M.; Fernandez-Bueno, I.; Alonso-Alonso, M. L.; Medina, J.; Sanz-Arranz, A.; Rull, F.; Gayoso, M. J.; Dueñas, A.; Garcia-Gutierrez, M. T.; Gonzalez-Buendia, L.; Delgado-Tirado, S.; Abecia, E.; Ruiz-Miguel, M.; Serrano, M. A.; Ruiz-Moreno, J. M.; Srivastava, G. K. Acute retinal damage after using a toxic perfluoro-octane for vitreo-retinal surgery. *Retina* **2017**, *37*, 1140−1151.
(7) Méndez-Martínez, S.; Calvo, P.; Rodriguez-Marco, N. A.; Faus, F.; Abecia, E.; Pablo, L. Blindness related to presumed retinal toxicity after using perfluorocarbon liquid during vitreoretinal surgery. *Retina* **2018**, *38*, 1856−1864.
(8) Menz, D.-H.; Feltgen, N.; Menz, H.; Müller, B.-K.; Lechner, T.; Dresp, J.; Hoerauf, H. How to ward off retinal toxicity of perfluorooctane and other perfluorocarbon liquids? *Invest. Ophthalmol. Visual Sci.* **2018**, *59*, 4841−4846.
(9) Dresp, J. H. Benchmarking different brands of perfluorocarbon liquids. *Graefes Arch. Clin. Exp. Ophthalmol.* **2020**, *259*, 21−27.
(10) Srivastava, G. K.; Alonso-Alonso, M. L.; Fernandez-Bueno, I.; Garcia-Gutierrez, M. T.; Rull, F.; Medina, J.; Coco, R. M.; Pastor, J. C. Comparison between direct contact and extract exposure methods for PFO cytotoxicity evaluation. *Sci. Rep.* **2018**, *8*, 1425.
(11) Srivastava, G. K.; Andrés-Iglesias, C.; Coco, R. M.; Fernandez-Bueno, I.; Medina, J.; García-Serna, J.; Dueñas, A.; Rull, F.; Pastor, J. Chemical compounds causing severe acute toxicity in heavy liquids used for intraocular surgery. Regul. *Regul. Toxicol. Pharmacol.* **2020**, *110*, 104527.
(12) Biological evaluation of medical devices-Part 5: tests for in vitro cytotoxicity, 2009. ISO 10993-5. https://www.iso.org/obp/ui/#iso:std:iso:10993:-5:en (accessed July, 2022).
(13) Menz, D. H.; Feltgen, N.; Lechner, T.; Menz, H.; Müller, B. K.; Dresp, J.; Hoerauf, H. Hydrofluoric acid and other impurities in toxic perfluorooctane batches. *Transl. Vis. Sci. Technol.* **2019**, *8*, 24.
(14) Coco, R. M.; Srivastava, G. K.; Andrés-Iglesias, C.; Medina, J.; Rull, F.; Fernandez-Vega-Gonzalez, A.; Fernandez-Bueno, I.; Dueñas, A.; Pastor, C. J. Acute retinal toxicity associated with a mixture of perfluorooctane and perfluorohexyloctane: failure of another indirect cytotoxicity analysis. *Br. J. Ophthalmol.* **2019**, *103*, 49−54.
(15) Coco-Martin, R.; Andrés-Iglesias, C.; Srivastava, G. K.; Lagos-Rodriguez, J.; Ruiz-Tevah, M.; Diaz-Cardenas, M. R.; Fernandez-Bueno, I.; Garcìa-Serna, J.; Garcia-Gutierrez, M.; Garcia-Layana, A.; Carlos Pastor, J. Intraocular toxicity caused by MEROCTANE perfluorocarbon liquid. *Sci. Rep.* **2021**, *11*, 599.
(16) Ruzza, P.; Gatto, C.; Ragazzi, E.; Romano, M. R.; Honisch, C.; Tóthová, J. H-content is not predictive of perfluorocarbon ocular endotamponade cytotoxicity in vitro. *ACS Omega* **2019**, *4*, 13481−13487.
(17) Meinert, H.; Roy, T. Semifluorinated alkanes − A new class of compounds with outstanding properties for use in ophthalmology. *Eur. J. Ophthalmol.* **2000**, *10*, 189−197.
(18) Meinert, H.; Knoblich, A. The use of semifluorinated alkanes in blood substitutes. *Biomater. Artif. Cells Immobil. Biotechnol.* **1993**, *21*, 583−595.
(19) Romano, M. R.; Ferrara, M.; Gatto, C.; Ferrari, B.; Giurgola, L.; D'Amato Tóthová, J. Evaluation of cytotoxicity of perfluorocarbons for intraocular use by cytotoxicity test in vitro in cell lines and human donor retina ex vivo. *Transl. Vis. Sci. Technol.* **2019**, *8*, 24.
(20) Romano, M. R.; Gatto, C.; Giurgola, L.; Ragazzi, E.; D'Amato Tóthová, J. Toxicity threshold of perfluorocarbon liquids for intraocular use: dose-response assessment of in vitro cytotoxicity of possible contaminants. *Transl. Vis. Sci. Technol.* **2021**, *10*, 24.
(21) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76−85.
(22) International Organization for Standardization. Biological Evaluation of Medical Devices-Part 12: Sample Preparation and Reference Materials, 2021. Geneva, ISO 10993-12:2021(En). https://www.iso.org/obp/ui#iso:std:iso:10993:-12:ed-5:v1:en (accessed July 18, 2022).
(23) International Organization for Standardization. Ophthalmic implants-Ocular endotamponades. ISO 16672:2020. https://www.iso.org/standard/70806.html (accessed July 18, 2022).
(24) Chang, S.; Sparrow, J. R.; Iwamoto, T.; Gershbein, A.; Ross, R.; Ortiz, R. Experimental studies of tolerance to intravitreal perfluoro-n-octane liquid. *Retina* **1991**, *11*, 367−374.
(25) Sparrow, J. R.; Ortiz, R.; MacLeish, P. R.; Chang, S. Fibroblast behavior at aqueous interface with perfluorocarbon, silicone, and fluorosilicone liquid. *Investig. Ophthalmol. Vis. Sci.* **1990**, *31*, 638−646.
(26) Chang, S.; Simpson, R. N. Impure perfluorocarbon liquids: a preventable tragedy. *Retina* **2017**, *37*, 1019−1020.
(27) Dresp, J. Letter of response to "Safety of perfluorocarbon liquids cannot be the retinologist's responsibility. *Graefes Arch. Clin. Exp. Ophthalmol.* **2021**, *259*, 1073−1074.

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