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Drug-eluting silicone hydrogel for therapeutic contact lenses: Impact of sterilization methods on the system performance

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ABSTRACT

Although contact lenses are promising platforms for ocular drug delivery and have been extensively studied for that purpose, the influence of sterilization methods on these systems remains barely investigated. In this work, a silicone-based hydrogel was produced and loaded with different ophthalmic drugs: levofloxacin, chlorhexidine, diclofenac and timolol. The drug release profiles, along with several material properties, were evaluated before and after sterilization by three different methods steam heat, γ-irradiation and ozone gas. Independently of the sterilization method used, the results of the swelling and mechanical properties tests strongly indicate the occurrence of specific drug-polymer interactions promoted by the sterilization. In general, these interactions led to a decrease on the amount of drug released. It is shown that γ-irradiation and ozone led to significant degradation of all of the drugs used in this study. Thus, it was concluded that steam heat is the sterilization method with less impact on the devices. More importantly, the present work shows that the development of efficient and functional drug delivery devices for ophthalmic purposes cannot be done independently of a careful analysis of the influence of the sterilization procedures and methods on the degradation of these polymeric systems as a whole.

1. Introduction

Hydrogels are three-dimensional polymeric chains with the ability to uptake and retain water or biologic fluids [1]. They are widely used as components for biomedical implants and devices [1,2]. Sterilization is a mandatory step in the production of implants and of many of these devices to ensure their safety for users. However, the effects of sterilization methods on the intrinsic properties of hydrogels remain understudied, which delays the development of new and more effective products. The sensitive nature of these soft biomaterials, renders their sterilization a particularly challenging task for the biomedical industry [3].

There are several terminal sterilization methods that have been used for hydrogel based devices. According to the nature of the sterilizing agent, they can be grouped as physical, chemical, and physicochemical methods [4]. Steam heat and dry heat are the most used within the category of physical methods [5]. They are quite simple and relatively inexpensive. However, their application is limited to heat resistant hydrogels (e.g. silicone based, acrylic based). Sterilization can also be achieved by exposing the hydrogels to radiation, like gamma radiation (GR). GR is highly penetrating, allows operating at low temperature and does not leave any chemical residues [6]. The associated drawbacks are related with the elevated cost and complexity of the process, which requires well-trained staff and special facilities, and with its unsuitability for radio-sensitive materials [7]. Concerning the chemical methods, they are divided in methods that use liquids and gases/vapours [4]. Liquids like alcohols, phenols and aldehydes can be used as sterilizing agents. Alcohols are volatile and do not leave residues, but phenols and aldehydes may be toxic, corrosive and/or irritating [8]. Thus, the Food and Drug Administration (FDA) recommends that the use of those liquids shall be limited to situations where other

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conventional methods cannot be used. Regarding gas sterilization, the most common agents are ethylene oxide (EtO) and hydrogen peroxide (HP). Although EtO is highly efficient and quite adequate for heat and radiation sensitive materials [8], it raises concerns regarding the toxic and carcinogenic nature of EtO residues, which have to be removed, increasing the processing times [8]. In turn, HP is a highly oxidizing agent and its use must be carefully pondered to avoid the material's degradation or harmful changes in properties relevant for the considered application [9]. Recently, new methods have been studied as possible alternatives to the already existent, e.g. plasma, ozone and supercritical fluids have deserved special attention and revealed advantageous in specific cases [10].

Overall, sterilization can lead to degradation of the material, promote further crosslinking of polymers or even induce toxic effects [11–14]. In the case of hydrogels, the presence of water in the structure can aid in the breakdown of chemical bonds and therefore act as a promoter of possible alterations in the material [15].

The development of drug delivery devices using hydrogels has been extensively pursued, which can be easily confirmed by the considerable volume of published work on this topic (e.g. see [16,17] for reviews). Particularly, eye contact lenses (CLs) are hydrogel-based devices that have raised great interest as they are recognized as promising platforms for topical ocular drug delivery, capable of increasing drugs bioavailability in the eye in at least 50% when compared to eyedrops (1–5%) [18]. Because of the close contact with the cornea, these devices are required to be sterile [19]. According to manufacturer's information, the most common methods used at industrial level for the sterilization of these devices are steam heat and gamma irradiation. Other methods, such as plasma, have been used to simultaneously achieve sterilization and treat the surfaces to enhance patient comfort through the decrease of bacterial adhesion, promotion of protein- and cell-repelling properties and improvement of wettability[20–22]. However, there is an evident lack of knowledge in the literature concerning the possible effects of terminal sterilization methods on such type of devices loaded with drugs. For these systems, the sterilization processes become even more critical because, besides being able to compromise the biomaterial integrity, affecting in an adverse way important properties for its performance, phenomena such as drug degradation, loss of activity and changes in the release profile may also occur [23,24].

In this work, a comparative evaluation of the effects of conventional sterilization methods (steam heat (SH) and γ-irradiation (GI)) and of an alternative method (ozonation (OZ)) was conducted, concerning a hydrogel loaded with drugs, intended for therapeutic soft CLs. For that, a silicone-hydrogel (SiHy) containing hydroxyethyl methacrylate (HEMA) and [tris(trimethylsiloxy)silyl]propyl methacrylate (TRIS) was produced and soaked in different solutions of drugs (levofloxacin, chlorhexidine, diclofenac and timolol) commonly used for the treatment of ocular diseases [25–28]. We show that the development of efficient and functional drug delivery devices for ophthalmic purposes cannot be done independently of the establishment of the adequate sterilization process and procedure.

2. Materials and methods

2.1. Silicone-hydrogel preparation

The SiHy was prepared according to a previously reported method [29]. Briefly, the silicone monomer 3-tris(trimethylsiloxy)silylpropyl 2-methylprop-2-enoate (TRIS, Sigma-Aldrich), the hydrophilic additive N-vinylpyrrolidone (NVP, Merck), 2-hydroxyethyl methacrylate (HEMA, Sigma-Aldrich) and the crosslinker ethylene glycol dimethacrylate (EGDMA, Sigma-Aldrich) were added to prepare a mixture with concentrations of 0.94 M, 3.58 M, 1.53 M, and 30 mM, respectively. The mixture was then degassed by ultra-sounds (5 min) and bubbled with a stream of nitrogen for 15 min, after which the initiator 2,20-azobis(2-methylpropionitrile) (AIBN, Sigma-Aldrich) was added (final concentration 10 mM). The obtained mixture was stirred for additional 10 min to ensure complete homogenization, and then injected into a mold consisting of two silanized glass plates separated by a teflon spacer. The glasses were previously silanized according to the procedure described by Vasquez et al. [30], i.e. immersion in a 2% solution of dimethyldichlorosilane (Fluka) in carbon tetrachloride (Riedel-de Haën) for 1 h, followed by rinsing with dichloromethane (Sigma-Aldrich) and drying with nitrogen. The SiHy was thermopolymerized at 60 °C for 24 h and then washed over 5 days, by soaking in distilled and deionized water (renewed 3 times a day), in order to remove unreacted monomers. The hydrated samples (approx. 0.35 mm in thickness) were cut into discs with 10 mm of diameter or strips with $\sim 11 \times 5\ mm^2$, dried overnight in an oven at 40 °C and kept in a closed recipient until further use.

2.2. Drug loading

The dry SiHy samples were loaded by soaking in the drug solutions (1 mg/mL) for 5 days, at 35 °C: 6 mL in the case of the discs and 4.2 mL for the strips, to keep the ratio sample area/solution volume 0.13. Drug solutions were prepared by dissolving appropriate amounts of levofloxacin (LVF, Sigma-Aldrich), diclofenac sodium (DCF, Sigma-Aldrich) or timolol maleate (TML, kindly provided by Edol) in saline solution (NaCl 0.9%, Sigma-Aldrich). Chlorhexidine diacetate monohydrate (CHX, AppliChem) was dissolved in water due to its limited solubility in saline solution.

2.3. Silicon-hydrogel characterization

2.3.1. Optical transparency

A Thermo Scientific – Multiscan Go spectrophotometer was used for the transparency studies. Measurements of the transmittance of visible light (wavelength range from 400 to 700 nm) through the hydrated hydrogel discs, both in water and drug solutions, were performed before and after the sterilization procedures. For that, the pre-equilibrated hydrated samples were fixed on the surface of a quartz cuvette. Measurements were done in triplicate using as blank the cuvette filled with saline solution.

2.3.2. Swelling capacity

The swelling capacity of the SiHy was measured by placing the dry discs (initial weight, W_0) in test tubes containing 6 mL of distilled and deionized water, NaCl 0.9% solution or the different drug solutions, at 35 °C. At pre-determined time intervals, the samples were taken out, carefully wiped with absorbent paper and immediately weighed (W_t). The procedure was repeated until W_t became constant (final weight). The equilibrium swelling capacity (SC) was then calculated using Eq. (1):

$$SC = \frac{Wt - W_0}{Wt} \times 100 \quad (1)$$

Samples were also weighed immediately after each of the different sterilization procedures, to infer upon their effect on the swelling capacity. The mass of drug present within the loaded SiHy samples was considered negligible (<3.5%) when compared to the mass of absorbed water. All tests were performed at least in triplicate.

2.3.3. Mechanical properties

The mechanical properties of the hydrated hydrogel samples (strips) were evaluated through tensile tests using a Texture Analyzer, with a 5 mN trigger force and test speed of 0.3 mm/sec. Using the software from Exponent Stable Micro Systems, it was possible to obtain the Young's modulus (E), from the slope of the initial linear portion of the stress-strain curve; the toughness (T), by calculating the area underneath the stress strain curve; the tension-at-break (σ_{break}), by dividing the load at break by the original cross-sectional area. Measurements were done at least in triplicate, before and after the different sterilization methods.

2.3.4. In vitro biological reactivity

Cytotoxicity assays were carried out following the procedures described in the United States Pharmacopeia ⟨87⟩, concerning determination of the in vitro biological reactivity by Agar Diffusion Testing. A monolayer of L929 cells (NCTC clone 929 (CCIAL 020) cell line from ATCC – CCL-1) was overlaid with agar stained with a vital dye (neutral red, National Aniline Division). Each plate (60 mm of diameter) contained 7 mL of cells suspension with a concentration of 3.5×10^5 cells/mL). Small sections of the hydrogel samples, with a surface area of ~0.25 cm^2 were placed on the top of solidified agar. The cells were in contact with the samples for 24 h, at 37 ± 1 °C, in a humidified incubator containing 5% of carbon dioxide. Latex fragments and non-toxic filter paper discs were used as positive and negative controls, respectively. After the incubation period, the plates were analyzed macroscopically and microscopically (using an optical microscope). According to the zone extending from the samples, the biological reactivity (N) was rated using the scale of grade described on USP ⟨87⟩, were (N = 0) corresponds to no reactivity, (N = 1) to slight reactivity, (N = 2) mild reactivity, (N = 3) moderate reactivity and (N = 4) severe reactivity. All tests were performed in triplicate.

2.4. Sterilization

2.4.1. Ozone gas

The ozonation process was carried out using a previously described ozone gas prototype chamber developed by BrasilOzônio® (Sao Paulo, Brazil) [31]. The hydrogel samples were placed in open plastic containers (to facilitate diffusion of the gas), containing a standard volume of saline solution (NaCl 0.9%) or of the different drug solutions that keeps the ratio sample area/solution volume 0.13. Samples were exposed to 8 pulses of ozonation. Each pulse was composed of four stages: vacuum, chamber filling, plateau (20 min), and vacuum. Inside the chamber the humidity was kept between 80 and 95%, with positive internal pressure ranging from 0.04 to 0.08 MPa during chamber filling and negative internal pressure ranging from − 0.08 to − 0.04 MPa during vacuum. Ozone concentration at the plateau stage was kept at 35–36 ppb, and temperature process between 30 and 35 °C. At the end of the ozonation process, the caps were placed back on each container, with utmost care, in order to avoid possible contamination.

2.4.2. Gamma irradiation

The hydrogel samples were placed in closed test tubes containing the standard volume (see previous section) of NaCl 0.9% solution or drug solution. Just before closing, nitrogen was poured into the tube, in order to reduce free oxygen. Samples were irradiated at a dose rate of 5 kGy h^{-1}, using a ^{60}Co gamma source with 57 kCi activity, until a final dose of 10 kGy was achieved. Perspex dosimeters (Harwell, range 5–50 kGy) were used to monitor and confirm the samples absorbed dose.

2.4.3. Steam heat

Hydrogel samples were conditioned in closed test tubes containing a standard volume of NaCl 0.9% solution or drug solution. Steam sterilization was performed at 121 °C for 1 h in an autoclave Sercon.

2.4.4. Sterility tests

To avoid the possible interference of the drug contained in the loaded samples, a placebo system, constituted by SiHy samples hydrated only in saline solution, was used. The placebo samples were purposely contaminated with different loads of spores (10^2, 10^3 and 10^4 colony forming units per milliliter, CFU/mL) of the biological indicator *Geobacillus stearothermophilus*, a microorganism known to be resistant to steam heat and oxidative processes such as ozonation, or of *Bacillus pumillus*, the biological indicator for gamma irradiation processes, according to USP ⟨1035⟩ [32].

After the sterilization process, samples were transferred in a laminar flow chamber, to test tubes containing tryptone soya broth medium (TSB, Bacto), prepared according to manufacture indications, and then left to incubate for 14 days: at 56 °C for samples contaminated with *Geobacillus stearothermophilus* (ATCC 7953 from 3 M) or at 37 °C for samples contaminated with *Bacillus pumillus* (from NAMSA STP-06). During this period the turbidity of the growing medium was monitored to identify eventual bacterial growth. In all assays, positive and negative controls were used (unsterilized contaminated TSB and autoclaved TSB, respectively). All tests were carried out at least in triplicate.

2.5. Drug release experiments

In vitro drug release experiments were carried out by immersing the loaded discs in vessels containing 6 mL of saline solution (NaCl 0.9%) for 96 h. The experiments were performed at 35 ± 1 °C with constant stirring (150 rpm). At predetermined time intervals, 0.65 mL aliquots of the supernatant were collected and replaced by the same volume of fresh saline solution.

The drug concentration in the collected supernatant samples was determined by high performance liquid chromatography (HPLC) using an Accella Thermo Scientific equipment with an UV–vis detector and a BDS Hypersil C-18 column (12.5 cm × 4 mm, pore size 5 μm). Different mobile phases were used for each drug: water, acetonitrile (Fisher-Scientific), orthophosphoric acid (Fisher-Scientific) and trimethylamine (Sigma-Aldrich) (86/14/0.6/0.3 in volume) for LVF (adapted from the method described by Wong et al. [33]), acetonitrile, orthophosphoric acid (0.05 M) and methanol (Fisher-Scientific) (48/40/12 in volume) for DCF (adapted from the method described by Shaalan et al. [34]), phosphate buffer solution (pH 3.5, Sigma-Aldrich)) and methanol (60/40 in volume) for TML (adapted from the method described by Laddha et al. [35]) and acetonitrile and potassium phosphate monobasic (Sigma-Aldrich, 0.02 mg/mL at pH 2.5) (40/60 in volume) for CHX (adapted from the method described by Zhu et al. [36]). In each case, the mobile phase was introduced into the column at a flow rate of 1 mL/min. Readings were carried out at a wavelength/retention time of 295 nm/6.7 min for LVF, 275 nm/8.0 min for DCF, 300 nm/3.6 min for TML and 255 nm/3.2 min for CHX, respectively.

All release studies were carried out in triplicate and an average value of the measurements was calculated. The results are presented in terms of cumulative release as a function of time.

2.6. Statistics

R Project software v.3.2.0. was used to perform statistical analysis. ANOVA test was used to determine if the different group means are significantly different. Bonferroni's test was done for multiple

comparisons. When data do not follow a normal distribution (evaluated using Shapiro-Wilk test), Kruskall-Wallis and Wilcoxon tests were used to decide whether the population distributions were identical. The level of significance chosen was 0.05. Measures are presented as mean ± standard deviation, unless otherwise specified.

3. Results and discussion

3.1. Swelling capacity

Fig. 1 shows the swelling capacity of the hydrogel samples in water and in drug solution. It can be observed that, for conventional CLs, the water content of the hydrogel has a direct relationship with oxygen permeability, i.e. the higher the water content, the higher the oxygen permeability, since the oxygen transmission is made through the aqueous phase [37]. However, for SiHy CLs with equilibrium water content (EWC)[38] lower than 70%, the silicone component is the primary factor governing oxygen transport, because oxygen permeation takes place mainly through the siloxane-rich phase. Thus, for these hydrogels, the higher the water content, the greater the difficulty for oxygen to move through the material, being observed an inverse relationship between oxygen permeability and water content [39]. Swelling capacity of the unloaded samples remained practically the same after sterilization, exception made to those subjected to steam heat, which presented an increase of ~9% (p-value = 0.0078). Similar results were obtained for the samples soaked in NaCl 0.9% solution. Upon drug loading, significant changes could be observed between the samples swelled in water and those swelled in drug solution. For DCF containing samples, a substantial 41% increase in swelling capacity was observed (p-value = 0.00183) (from 73 to 114%, when compared to unloaded hydrated samples). Unsterilized LVF, CHX and TML containing samples did not show statistical significant differences, regarding this parameter. As for the effect of the different sterilization methods: LVF-SiHy showed no statistical significant differences within the group (p-value = 0.09832), however a slight tendency to swelling decrease upon SH. Considering DCF containing SiHy, although the data points to a tendency of decrease of the swelling capacity upon sterilization, there are no significant statistical differences (p-value = 0.052) due to the high scattering of the results. Samples loaded with TML where only affect (p-value = 0.023) by ozonation, presenting a 13% decrease in swelling capacity (73-60%, when compared to control). CHX-SiHy seemed to be the least affect system, since no statistical difference was observed regarding swelling capacity.

3.2. Optical transparency

It can be observed in Fig. 2, that all unsterilized drug loaded samples presented acceptable values of transparency in the visible region (> 90% is the minimum recommended for CLs applications [38]). This means that the presence of the drugs in the polymeric matrix does not affect significantly the transparency of the hydrogel.

After sterilizing with steam heat, the transparency values remained almost unaltered for all studied samples. Nevertheless, DCF loaded samples appeared to be somewhat more sensitive to steam heat, revealing a slight decrease in the transmittance (%T), from 350 to 420 nm, reaching a minimum of ~80%. In turn, gamma irradiation triggered the most evident changes, leading to a severe decrease in the values for LVF, DCF and CHX loaded samples, which was expected, since a darkening of the drug solution was visually perceptible. However, TML loaded samples did not reveal significant alterations in %T values as a result of radiation exposure. As for samples subjected to ozone treatment, the transmittances were not affected in the visible region, in all studied cases. In the UV region, CHX and DCF containing samples presented the least altered spectrums, whereas LVF-SiHy and TML-SiHy showed an increase in %T values from 250 to 350 nm which shall be attributed to drug degradation.

3.3. Mechanical properties

The results concerning the mechanical properties are presented in Fig. 3. The loading of LVF, CHX and TML in the unsterilized samples origins in the three cases a significant increase in the Young's modulus values relatively to the unloaded samples (p-value < 0.0005) (Fig. 3A). This could indicate the occurrence of drug/polymer interactions.

Steam heat led to a significant increase of Young's modulus for DCF (p-value = 0.007) and TML (p-value = 0.012). Contrarily, samples loaded with LVF and CHX did not show accentuated variations after autoclaving. Tension at break decreased for LVF DCF and CHX (p-values < 0.0001), whereas TML suffered no alteration. Steam heat also decreased toughness for LVF (p-value = 2.1e–05) and CHX samples (p-value = 3.9e–05). Concerning Young's modulus, gamma irradiation only led to statistical significant alteration for DCF loaded samples, where an increase was observed (p-value = 0.016). However, tension at break and toughness significantly decreased for all samples (p-values < 0.03), with exception of the DCF-SiHy's toughness, which although lower in average, is not statistically different. Once more, when comparing these alterations with those observed in the irradiated unloaded samples, it is evident that the drugs affect the behaviour of the material.

Ozonation lead to a significant increase of Young's modulus for LVF loaded samples (p-value = 6.7e–05), while the opposite effect was observed for DCF, which in turn suffered a significant decrease (p-value = 3.6e–10). This could indicate that the predominant effect of ozonation shall be cross-linking for LVF containing samples, whereas for DCF containing samples prevails degradation of the system. As follows, tension at break increased for LVF (p-value = 0.013), whereas DCF and TML showed a decrease. This behaviour was also observed for the values of toughness of DCF-SiHy and TML-SiHy ozonated samples.

Overall, the results indicate that some degradation/alteration in the mechanical properties of the SiHy occurred as a consequence of the sterilizations processes. However, regarding Young's modulus, in all the cases, the obtained values remained within the expected order of magnitude for hydrogels with similar composition (10^6 Pa) [40].

3.4. Drug release profiles

Fig. 4 shows the drug release profiles obtained before and after each sterilization method, and the results are discussed below, separated by studied drug.

3.5. Levofloxacin

Fluoroquinolone eye drops, such as levofloxacin, are commonly used for the treatment of ocular infections, because of their high efficacy and broad spectrum, [41]. This molecule has an intermediate lipophilic character and its zwitterionic form predominates in water at physiological pH [42]. The release of levofloxacin (Fig. 4A) from unsterilized SiHy lasted less than 2 h and showed an initial burst which is typical of drug accumulation near the surface of the sample. Being the release profile a cumulative curve, it only could increase or keep constant. The slight decrease observed between the 2nd and the 8th hour may be attributed to some degradation of the drug. In fact, it is known that LVF can suffer mild degradation

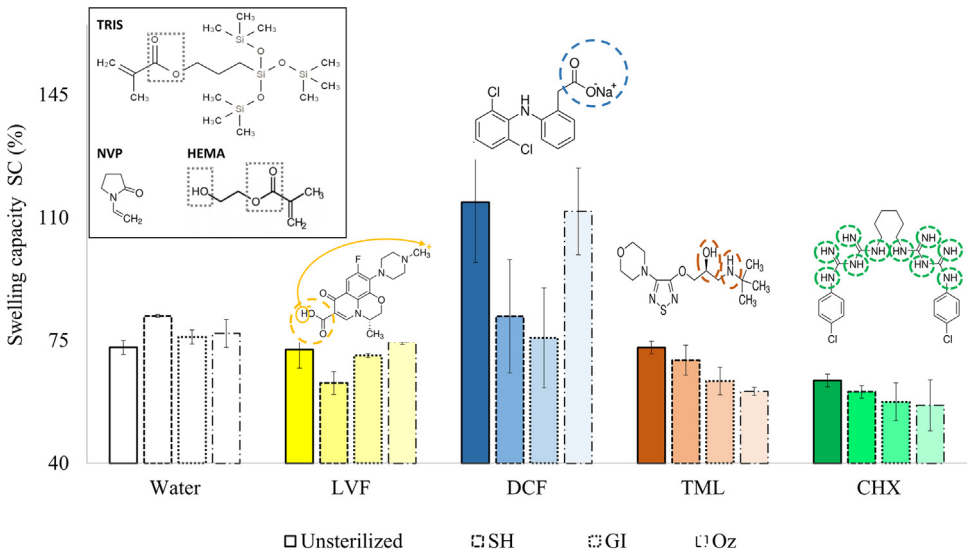

Fig. 1. Swelling capacity of the hydrogel samples in water and in drug solution (levofloxacin, diclofenac, timolol and chlorhexidine), before and after different sterilization processes (SH – steam heat, GI – gamma irradiation, OZ –ozonation). In the figure are also represented the chemical structure of the monomers and drug molecules along with the potential interaction sites.

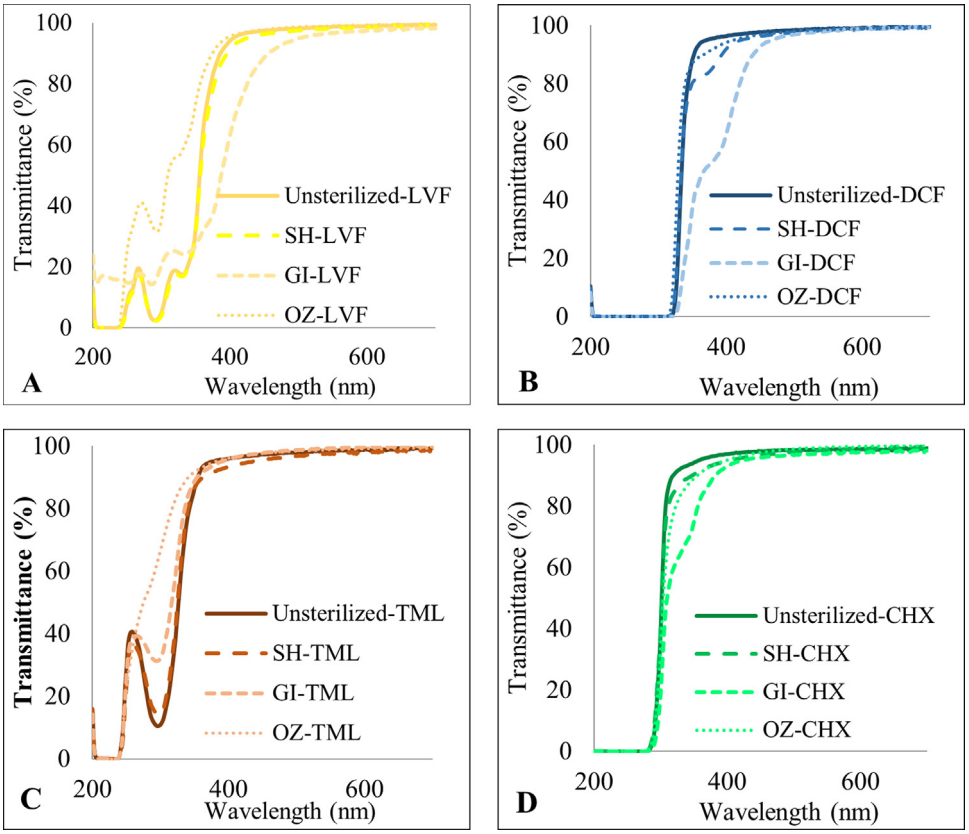

Fig. 2. Optical transparency of the hydrogel samples loaded with (A) levofloxacin, (B) diclofenac, (C) timolol and (D) chlorhexidine before and after different sterilization processes (SH – steam heat; GI – gamma irradiation, OZ – ozonation).

from water hydrolysis [43]. However, it was not detected by HPLC (see supporting files – Fig. S1A), probably due to the low concentration of the eventual degradation products. Steam heat had little influence in the release behaviour besides the slightly decrease in the drug released amount. Nevertheless, again no degradation products were observed in the HPLC chromatograms, which is indicative that the drug can withstand the sterilization conditions without degrading. Yet, the reduction of the amount of drug released suggests that there are drug-matrix interactions occurring. The results mentioned before, concerning the decreased in swelling capacity and alteration in some mechanical properties induced by this sterilization method, can also be indicative of such interactions.

Gamma irradiation led to the complete degradation of LVF (see supporting files – Fig. S1A), hence no release profile could be obtained.

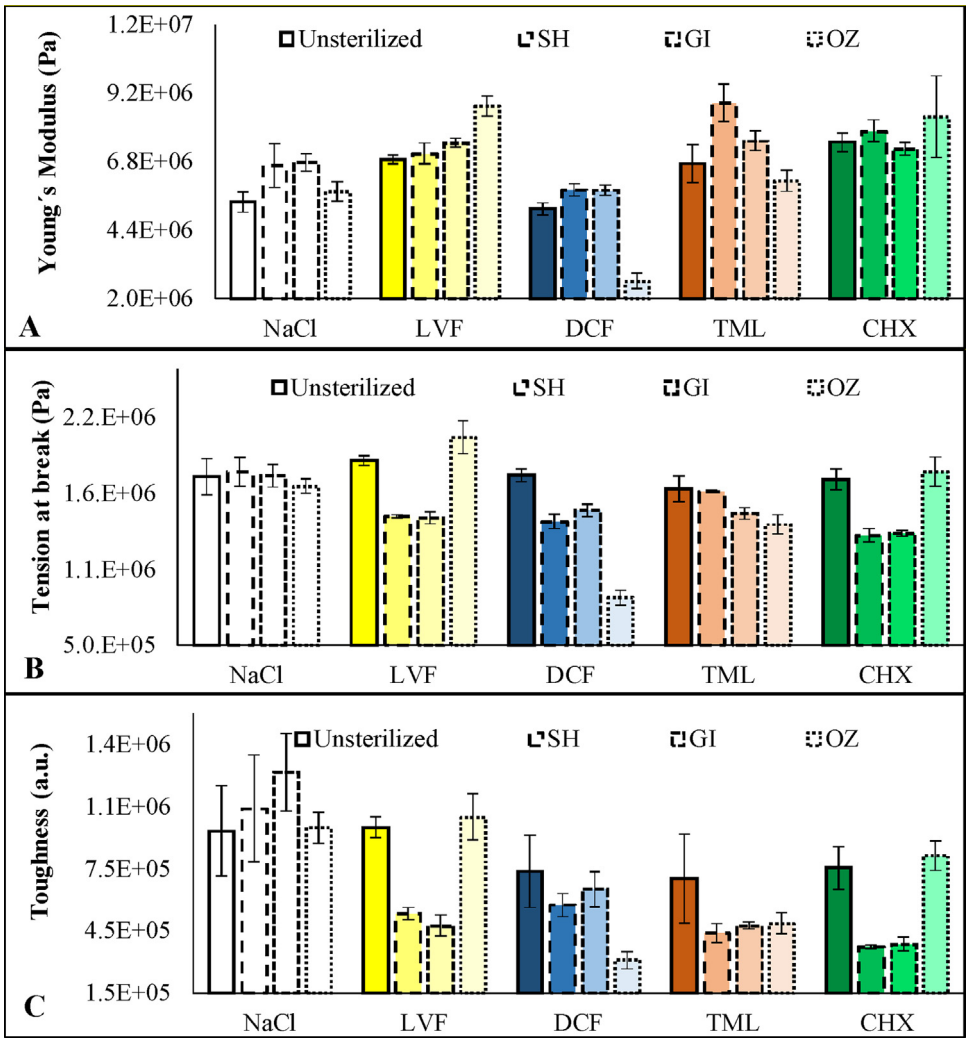

Fig. 3. Mechanical properties data of hydrogel samples unloaded and loaded with levofloxacin, diclofenac, timolol and chlorhexidinee, before and after different sterilization processes (SH – steam heat, GI – gamma irradiation, OZ – ozonation).

As for the ozonated samples, there was a significant reduction of drug amount released (~50%), but in contrast with steam heat results, it was possible to observed the presence of several additional degradation products in the HPLC chromatograms see supporting files – Fig. S1A). These products shall result of the degradation of the drug itself and not of the SiHy. In fact, chromatograms of the supernatant (NaCl solution) of ozonated unloaded lenses did not show signs of any released product, while LVF solutions prepared by drug dissolution and submitted to ozonation showed the appearance of several peaks at different retention times and wavelengths, and the disappearance of the peak characteristic of the drug, indicating its complete degradation. These results suggest that the hydrogel shall offer some protection to the drug. Since the release behaviour profiles obtained from all sterilized LVF loaded samples were not satisfactory (high burst and short release duration), no further characterization and analysis of results was carried out regarding these systems.

3.5.1. Diclofenac

Diclofenac in its anionic form, is a well-known nonsteroidal anti-inflammatory drug with analgesic activity, that is widely used in the treatment of ocular pathologies [44]. The obtained DCF release profiles from SiHy (Fig. 4B) were very different from those of the previous drug (LVF). For the unsterilized samples, the total released amount was much higher (~30 μg/mg dry gel compared to 0.8 μg/mg dry gel for LVF), being released in a more sustained way (>96 h). Previous work showed a similar tendency, when the amount of DCF released from SiHy was compared to other drugs [27].

Steam heat induced a significant reduction of the total amount of drug released to values of the order of the 9 μg/mg dry gel. The presence of a small peak (6.4 min retention time), assumed to be from a degradation product, was observed in the HPLC chromatograms (see supporting files – Fig. S1B), indicating that the drug is somewhat sensitive to this sterilization conditions.

Gamma irradiation and ozonation also led to a significant reduction in the total released amounts, which dropped to 10.6 and 15.5 ug/mg dry gel, respectively, corresponding to ~44 and ~67% drug loss compared to unsterilized samples. For gamma irradiation, several degradation products could be observed in the HPLC chromatograms (see supporting files – Fig. S1B), indicating the unsuitability of this method concerning DCF-SiHy.

As for ozonated samples, no degradation products could be observed, again suggesting that the hydrogel could offer some protection.

3.5.2. Timolol

Timolol is a non-selective β-adrenergic blocking agent that is used to decrease intra-ocular pressure in patients with or without glaucoma. It can act by reducing the aqueous humor production

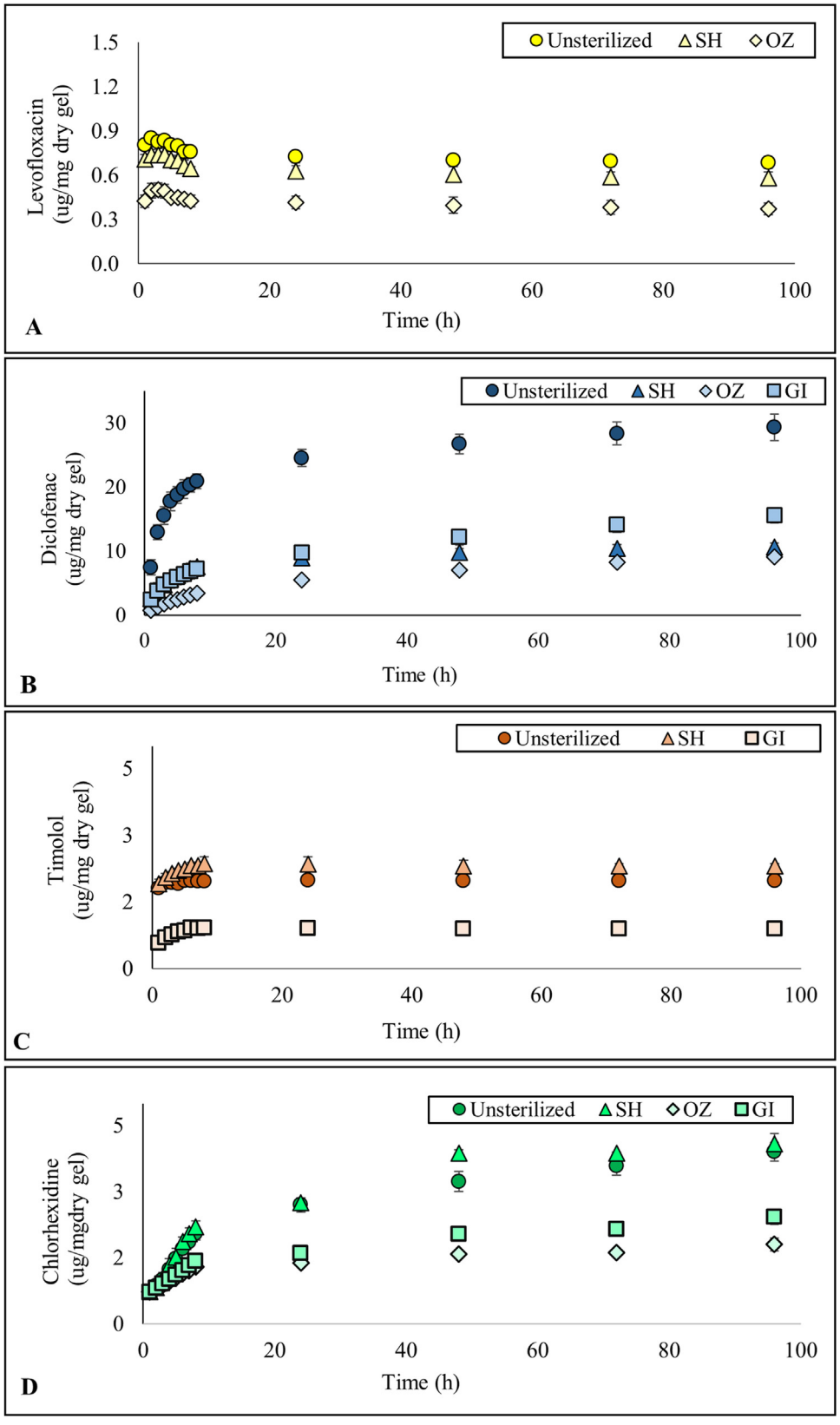

Fig. 4. Cumulative release of (A) levofloxacin, (B) diclofenac, (C) timolol and (D) chlorhexidine before and after different sterilization processes (SH – steam heat, GI – gamma irradiation, OZ – ozonation).

through blocking of the beta receptors in the ciliary body or by reducing its flow [26].

As in the case of LVF, the release of TML (Fig. 4C) from unsterilized SiHy showed an initial burst, occurring the majority of the release in the first hour.

Steam heat sterilization also presented a high burst effect, but with a slightly more controlled release behaviour, along with a minor increase in drug released amount (from 2.0 μg/mg dry gel in the unsterilized samples to 2.4 μg/mg dry gel). Still with regard to steam heat, no degradation products were detected (see supporting files – Fig. S1C), indicating that the TML-SiHy system can withstand the sterilization conditions without suffering major alterations, which is in agreement with the obtained results considering swelling capacity, transparency.

Gamma irradiation showed a significant reduction of the amount of drug released (from 2.0 μg/mg dry gel to 0.9 μg/mg dry gel, equivalent to a ~55% drug loss). However, there were no detectable degradation products in the HPLC chromatograms (see supporting files – Fig. S1C). This could, once again, point to specific drug-matrix interactions as result of the sterilization conditions.

As for ozonation, it was not possible to obtain a release profile since TML was degraded (see supporting files – Fig. S1C) beyond any possible quantification.

3.5.3. Chlorhexidine

Chlorhexidine, in combination with other agents, is used in the treatment of *Acanthamoeba* keratitis [45,46]. It is a cationic molecule believed to bind to negatively charged bacterial cell walls, acting both as a bacteriostatic and a bactericidal agent [47]. The unsterilized and steam heat release profiles were very similar (Fig. 4D), although in the last case two small degradation peaks became clearly visible, at 4.4 and 5.8 min retention time (see supporting files – Fig. S1D). Gamma irradiation and ozonation led to similar release profiles among them. However, in both cases a significant decrease in the drug released amount was observed (to 2.4 μg/mg dry gel for GI and 1.8 μg/mg dry gel for OZ, when compared to 4.1 μg/mg dry gel for unsterilized samples, which corresponds to ~42 and ~56% of drug losses, respectively). In both cases, several degradation peaks were observed (see supporting files – Fig. S1D), indicating the probable unsuitability of theses method regarding the CHX-SiHy system. Zong et al. studied the stability of CHX under different stress conditions, including oxidation[48]. They reported possible degradation pathways along with the formation of several degradation products, which shall be responsible for the degradation peaks observed in the HPLC chromatograms.

At this point, it is interesting to try to relate the chemical structure of the drugs with the results obtained so far. Overall, the presence of LVF, TML and CHX in the hydrogel, tends to increase the Young's modulus of the material (and consequently its stiffness) and to decrease the swelling capacity, independently of the sterilization method. An opposite behaviour was observed for DCF loaded samples. This shall be related with the ability of LVF, TML and CHX to establish interactions with the polymeric matrix and induce eventual crosslinking. In fact, TML is a bifunctional molecule which presents a group amine and a group alcohol (see chemical structure of the molecule in Fig. 1, and the brown circles), which can attack the ester groups of HEMA and TRIS (see upper left corner insert of Fig. 1), interconnecting the chains through covalent bonds. CHX is a polyfunctional molecule with several groups amine and imine (green circles in Fig. 1) able to origin a similar behaviour. Concerning LVF, since the tertiary amines are not reactive, and the molecule is zwitterionic [42] presenting simultaneously negatively and positively charged groups (see full line yellow scheme in Fig. 1), it is plausible that crosslinking may occur only through electrostatic interactions with the polymeric matrix. The carboxylic group of LVF (see yellow dashed circle in Fig. 1) can also react with the hydroxyl group of HEMA (upper left corner insert of Fig. 1) in a reaction of esterification. DCF is a bifunctional agent, presenting a secondary amine and a carboxylate group. The carboxylate group of DCF (blue dashed circle in Fig. 1) can react with the hydroxyl group of HEMA in a condensation reaction (esterification). However, the amine group shall present low reactivity due to the stereochemical hindrance induced by the chlorines in the positions 2,6 of the phenyl group, thus may impair its reaction. The fact that no crosslink occurs, may explain the higher drug release of DCF loaded samples. Additionally, the presence of negative dipoles due to the chlorines shall induce an higher swelling capacity relatively to the other systems, due to electrostatic expansion of the polymeric network and increased affinity to water.

A final remark must be done, concerning the relative importance of the hydrogels water content for their mechanical behaviour, in comparison with the effect of the drug-polymer interactions. It is known that the mechanical properties of hydrophilic materials are extremely sensitive to the degree of water uptake. Generally, it is observed that in this type of systems, the increase in the water content leads to a decrease in mechanical properties like stiffness and toughness [49–51]. However, in the present work, different behaviours were found. Let's consider the following three hydrogel samples: (1) unloaded and unsterilized (control), (2) loaded with diclofenac unsterilized and (3) loaded with diclofenac sterilized with ozone. It can be observed that samples 1 and 2 show similar mechanical behaviour, but quite different swelling capacities: diclofenac loaded samples absorb a significantly higher amount of water. Contrarily, samples 2 and 3 present similar average values of swelling capacity, but distinct mechanical properties: ozone treatment leads to a significant reduction of the Young's modulus, tension at break and toughness. Further examples could also be found among the unloaded samples: steam heat treatment induces an increase in the swelling capacity of the unloaded hydrogel and also an increase of the average value of the Young's modulus. Thus, it may be concluded that besides water content, other factors, like the establishment of specific interactions between drug and polymers and/or the sterilization agents action, shall influence materials properties such as mechanical characteristics.

Table 1
Sterility and biologic reactivity response results before and after different sterilization processes.

Tested condition	Biologic Reactivity				Sterility test (CFU/mL)		
	N						
	NaCl	DCF	TML	CHX	10^2	10^3	10^4
Unsterilized	0	3	0	2	N/A	N/A	N/A
Steam Heat	0	3	0	2	√	√	√
Gamma Irradiation	0	4	0	2	√	√	√
Ozonation	0	3	0	0	√	√	X

(N = 0): No reactivity, (N = 1): Slight reactivity, (N = 2): Mild reactivity; (N = 3): Moderate reactivity, (N = 4): Severe reactivity) [N/A]: Not tested; √: No bacterial growth for 14 days, X:bacterial growth observed.

3.6. In vitro biologic reactivity

Biologic reactivity assays were carried out in order to evaluate the effect of the different sterilization conditions on the studied drug delivery systems. The obtained results are summarized and presented Table 1.

The unloaded SiHy did not show any signs of biologic response when subject to the different sterilization methods, proving that the studied sterilization conditions do not induce adverse biologic effects.

Unsterilized DCF-SiHy showed a moderate biologic reactivity (N = 3), probably due to the amount of DCF that is released from the disc to the cellular medium. However, this reactivity did not change upon steam heat and ozone sterilization methods, denoting that the formed degradation products shall not aggravate the initial biologic response. Contrarily, gamma irradiation led to an intensified severe biologic reactivity (N = 4), which could be attributed to the action of the several degradation products that were formed upon sterilization. This fact points to the unsuitability of this sterilization method for the DCF-SiHy system.

All TML-SiHy samples showed no alterations upon sterilization, demonstrating that all tested methods did not induce biologic reactions upon the studied system.

As for CHX-SiHy, the unsterilized, SH and GI samples demonstrated a slight biologic reaction (N = 2), again probably due to the amount of drug released to the cells. The OZ samples revealed no biologic reactivity, which is in accordance with data from release studies, since this system showed the lower drug released amount. Although all sterilization methods let to the formation of degradation products for the CHX-SiHy system, they did not induce an aggravated biologic reactivity. Lower levels of biological reactivity could be achieved in the case of DCF-SiHy and CHX-SiHy if samples were loaded with lower amounts of drug. However, in vivo tests would be needed to testify the effectiveness of the loaded devices, which is out of scope of the present work.

3.7. Sterility assurance

Finally, regarding the effectiveness of the sterilization methods, no bacterial growth was observed in all samples subjected to steam heat (overkill conditions) and gamma irradiation. As for ozonation, bacterial growth was observed in samples with the higher initial contamination (10^4 CFU/mL), demonstrating that this method is only effective in sterilizing samples carrying a low bioburden of the tested biologic indicator ($\leq 10^3$ CFU/mL). However, it is important to point out that the challenge microorganism used has a high resistance Therefore, it is not ruled out the hypothesis that these conditions could be validated as a suitable method for least resistant bioburdens.

4. Conclusions

In this work, the effect of two conventional methods (steam heat and gamma irradiation) and a new one (ozonation) on drug loaded SiHy samples intended for CLs was investigated. Steam heat sterilization revealed to be the least aggressive method, even though several alterations could be observed, e.g. in the mechanical properties of systems containing DCF and TML. It led to a decrease in the amount of LVF and DCF released, with formation of degradation products in the last case. CHX and TML profiles were not significantly affected. Gamma irradiation led to darkening of the loaded samples and to an increase of Young's modulus for DCF-SiHy. In a general way, it induced the decrease of the amount of drug released and the formation of degradation products. Ozone sterilization affected the swelling capacity of TML-SiHy and the mechanical properties of LVF-SiHy and DCF-SiHy, increasing the Young's modulus in the first case and decreasing it in the last one. It led to drugs degradation (total in the case of TML) and to a significant decrease of the amount of drug released. Overall, there was no aggravation of the cytotoxic response after the different sterilization procedures, except for gamma irradiated DCF-SiHy. While no bacterial growth was observed for steam heated and irradiated samples, ozone was only effective in sterilizing bioburdens $\leq 10^3$ CFU/mL.

In short, the outcome of the sterilization procedures in complex systems like those here studied is difficult to predict. Therefore, each case shall be evaluated *per si* in order to select the method that maintains the essential material properties within the requirements for the specific application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.colsurfb.2017.11.021.

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