



Wear effects on microscopic morphology and hyaluronan uptake in siloxane-hydrogel contact lenses

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3 **Wear effects on microscopic morphology and hyaluronan uptake**
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5 **in siloxane-hydrogel contact lenses**
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56 Running Heads: Wear-dependent morphology of contact lenses
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3 ABSTRACT
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5 The purpose of this study was a comparison between new and worn siloxane-hydrogel contact
6 lenses in terms of microscopic structure, surface morphology, and loading of hyaluronan. The
7 analyses were performed by scanning electron microscopy, with the support of the freeze-drying
8 technique, and by fluorescence confocal microscopy. Along the depth profile of new lenses, a thin
9 porous top layer was observed, which corresponds to the region of hyaluronan penetration inside
10 well defined channels. The time evolution was followed from one day to two weeks of daily wear,
11 when a completely different scenario was found. Clear experimental evidence of a bumpy surface
12 was observed with several crests and regions of swelling, which could be filled by the hyaluronan
13 solution. The modifications are attributed to the progressive relaxation of the structure of the
14 polymeric network.
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33 KEYWORDS:
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- 35 • Contact lens
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- 37 • Siloxane-hydrogel
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- 39 • Freeze-drying
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- 41 • Scanning electron microscopy
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- 43 • Hyaluronan
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Introduction

In recent applications, disposable contact lenses are often made of siloxane-hydrogel materials. Due to the interaction with the tears and with the cornea, their morphological properties, porosity, and hydrophilicity are expected to influence the clinical performances and the comfort during wear. Moreover, in recent years, the lens absorption of various molecules and macromolecules has been taken into consideration. One reason is the presence of molecules and macromolecules both in tears and in maintenance solutions. Another reason is the interest towards contact lenses for uptake and release of drugs and macromolecules of special interest.^[1-3] Hyaluronan (also called hyaluronic acid, HA) is one of these molecular species of special interest for its ophthalmic advantages. It is a polysaccharide present in many tissues of the human body, such as connective, epithelial, and neural tissues, with applications in medicine, pharmacy, biotechnology, and ophthalmology, for example as a remedy for the dry-eye syndrome.^[4] Moreover, the HA uptake in contact lenses plays a role to improve the comfort and also to prevent the adsorption in the polymeric network of the contact lens of other substances, which could possibly determine problems during the wear, such as cytotoxicity or ocular irritation.^[5-12] HA can also be considered a model system to discuss the loading capability of the lenses both before and after wear and to investigate the loading mechanisms in disposable contact lenses of ocular drugs, as potential controlled-release delivery systems.^[13-19]

To determine the amount of HA loaded in one lens after exposure to a water HA solution, some experimental methods were presented based on the use of different dyes, which form complexes with the HA.^[18-21] These methods are indirect because the dye forms complexes also with the polymeric material of the lens itself, so that the content of HA in each lens was deduced from the variation of the HA content in the loading solution before and after the procedure. However, the methods based on dyes are typically time-consuming and they require many steps. Other limitations of these techniques are the lack of information on the HA distribution and penetration in the core of the lens, as well as on the morphology and structure of the HA-loaded lenses, such as the presence

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3 of pores, the capability of the HA to fill them, the possible aggregation of HA in clusters, the size of
4 these clusters, and the compatibility with their internalization in the polymeric network of the lens.
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6 As far as the lens structure is concerned, few studies performed by scanning electron microscopy
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8 are reported in the literature, mainly on unworn contact lenses,^[22-25] but further details deserve to be
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10 investigated, such as the effects of wear and the relationships with solute uptake. Detailed
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12 investigations also require the improvement of the acquisition technique.
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16 The focus of the present work is the comparison between new and worn contact lenses in terms of
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18 microscopic surface morphology, structure, porosity, HA distribution, and penetration depth inside
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20 the lens. To this scope, a combined study was performed by scanning electron microscopy (SEM)
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22 and confocal fluorescence microscopy, the former after freeze-drying the samples to perform SEM
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24 analyses on dehydrated lenses by maintaining the structure of the hydrated lens. A siloxane-
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26 hydrogel contact lens was used as model system.
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33 34 **Experimental**

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36 Fluorescein-labelled HA (F-HA) with molecular weight 1000 kDa was purchased from TdB
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38 Consultancy (Sweden). Aqueous salt solution containing sodium chloride (0.9%) was purchased
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40 from Laboratoires Alcon (France). F-HA was dissolved in the salt solution with F-HA concentration
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42 0.2%^W/v. Siloxane-hydrogel F-HA-free contact lenses of the I FDA group (-3.00 D, thickness at the
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44 centre ~ 90 μm, hydration ~50%) were supplied by Safilens (Italy). They were loaded with F-HA
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46 by immersion at room temperature in the F-HA salt solution.
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50 Both before and after the loading procedure, scanning electron microscopy (SEM) micrographs
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52 were obtained using a LEO 1430 (Carl Zeiss, Oberkochen, Germany) operating at 20 kV and $3 \cdot 10^{-3}$
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54 Torr. Before analyses, samples were firstly freeze dried (-55°C, 0.63 mbar × 24 hours) using an
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56 ALPHA 1-2 LDplus freeze dryer (Martin Christ, Osterode am Harz, Germany). Then, a film of gold
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58 was sputtered on the lens surface with thickness of approximately 10 nm (to avoid charging the
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3 samples) using a Semprep 2 sputter coater (Nanotech Ltd., Prestwick, UK) at 10 mA.

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5 Fluorescence confocal micrographs were obtained using the Leica TCSNT confocal laser scanning
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7 microscope (Leica Microsystems, Wetzlar, Germany). For each lens a Z-stack was acquired (an
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9 image every 0.5 μm) to correctly evaluate the penetration depth of F-HA. The start point of the Z-
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11 stack was fixed at the plane where the green fluorescence appeared and the end point was fixed at
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13 the plane where the green fluorescence disappeared.
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16 17 18 19 20 21 **Results and discussion**

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23 Figure 1 shows two typical SEM micrographs taken from the surface of HA-free contact lenses.
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25 Both empty pores (with diameters as large as several micrometers) and a fibrous texture of the
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27 material are clearly detected in Fig. 1a. Smaller pores with diameters of the order of 1-2 μm in size
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29 are detected in Fig. 1b. These different morphologies at the microscopic scale were observed also
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31 by analyzing different areas of the same lens, thus revealing a heterogeneous pattern attributable to
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33 the manufacturing process. According to the Lowman definition,^[20,26] this siloxane-hydrogel (at
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35 least the volume close to the lens surface observed in Fig. 1) can be reasonably defined as an
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37 intermediate case between the so-called macro-porous hydrogels, with typical pores in the range 0.1
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39 – 1 μm , and the so-called super-porous hydrogels (tens and hundreds of μm).
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44 SEM analyses after the HA loading yielded a different scenario, as confirmed by the representative
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46 micrographs in Fig. 2. Pores, when present, were found to be much less pronounced. The difference
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48 can be attributed to the filling of the pores by HA. Another SEM image of a HA loaded lens is
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50 reported in Fig. 3. The different scale compared to Fig. 2 allows capturing a new branched
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52 morphology and a thickening of the main walls of the pores, which were attributed to the
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54 internalized HA, similarly as the morphological differences between the images of Figs. 2 and 1.
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56 For comparison, we mention that the surface properties of the same type of contact lenses loaded
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58 with 800 kDa HA were also investigated by atomic force microscopy (AFM), as reported
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3 elsewhere.^[27] The AFM micrographs were taken on sample areas of $(10 \times 10) \mu\text{m}^2$ and $(5 \times 5) \mu\text{m}^2$,
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5 which are lower than the areas here investigated. By AFM, a relatively low surface roughness was
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7 measured of approximately 2-3 nm on the area of $(10 \times 10) \mu\text{m}^2$. No depressions of the surface due
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9 to the presence of deep pores were detected on unworn lenses. The lack of pores agrees with the
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11 filling of the pores of the lens with HA, as here observed by SEM. However, since the contact
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13 lenses were not freeze-dried before the AFM analyses, we cannot exclude that the lack of pores was
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15 due to their intrinsic disappearance owing to the water evaporation from the lens during the AFM
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17 analysis.
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21 By SEM analysis, it was also possible investigating the distribution of the pores inside the lens
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23 along its depth profile. Figure 4 shows a SEM micrograph of the depth profile of a contact lens. A
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25 top porous layer can be clearly distinguished from the bulk. A further confirmation of the presence
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27 of a porous thin layer was obtained by fluorescence microscopy. Figure 5 shows two
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29 exemplificative fluorescence micrographs acquired from the surface of F-HA loaded lenses. The
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31 depth profile (i.e. the extent of the penetration of F-HA across the thickness of the lens) is
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33 visualized at the bottom and right sides of the main surface micrographs. Spots of emitted light
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35 from the surface are clearly seen, attributed to the F-HA fluorescein emission. By analyzing many
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37 lenses, emission was typically found to originate from channels with depth of about 5-10 μm , as for
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39 example in the case reported in Fig. 5a. This value is much lower than the thickness of the lens,
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41 which is about 90 μm thick at the centre and thicker in the periphery. This result is attributed to the
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43 reduced depth of the lens pores, which prevents the internalization of F-HA to the core. However, in
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45 few cases emission was also observed from deeper channels (of the order of tens of μm). For
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47 example, in the case reported in Fig. 5b the emission was detected by scanning the sample from the
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49 surface to a depth of about 100 μm , a value which is close to the thickness of the lens in the
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51 peripheral region, where the micrograph was taken. Noteworthy, the emitting spots have
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53 approximately the same size and the same surface density as the structures observed by SEM, which
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3 can thus be definitely attributed to pores filled with F-HA. Based on the fluorescence microscopy
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5 results, this siloxane-hydrogel can be classified as an intermediate case between a macro-porous
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7 hydrogel and a super-porous hydrogel, only as far as the layer close to the lens surface is concerned,
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9 within a variable depth from few micrometers to tens of μm . The bulk of the lens is likely to belong
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11 to the micro-porous or non-porous hydrogel groups, based on the Lowman definition.^[20,26]
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14 For an approximated evaluation of the content of F-HA per lens, the fluorescence micrographs were
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16 analyzed to obtain quantitative information on the emitting volume. Figure 6 shows the statistical
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18 frequency distribution of the area of the fluorescent spots, as deduced from Fig. 5a. Since the
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20 micrograph resolution was $0.3 \mu\text{m}/\text{pixel}$, emission spots with area lower than $0.6 \mu\text{m}^2$ (i.e. with
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22 radius lower than about $0.44 \mu\text{m}$, if assumed circular) were neglected because they were not clearly
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24 distinguishable from image noise. Small emitting spots are more frequent than large ones. The
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26 number of spots is inversely proportional to their area, as indicated by the hyperbolic curve
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28 (continuous line) obtained by the fitting of the data in Fig. 6 ($R = 0.9797$). The total emitting area
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30 was found to be about 3% ($1860 \mu\text{m}^2$) of the total area of the micrograph ($64160 \mu\text{m}^2$). This
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32 percentage and the depth of the emitting channels ($\sim 10 \mu\text{m}$ from the profiles in Fig. 5a) allowed to
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34 deduce the emitting volume and, in turn, the content of F-HA per lens (few tenths of microgram),
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36 taking into consideration the F-HA solution concentration. Therefore, fluorescence confocal
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38 microscopy is found to be very sensitive, down to such a low content of F-HA per lens, which could
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40 not be detected by simple optical absorption measurements performed on the lens. Indeed, the
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42 maximum absorption of a F-HA solution with concentration $0.2\%^{W/V}$ taken with a 10 mm long
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44 cuvette was measured at 496 nm to be approximately 2 absorbance units. The thickness of one lens
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46 (approximated to be $100 \mu\text{m}$) and its hydration ($\sim 50\%$) indicate that the absorbance attributable to
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48 the internalized F-HA solution cannot intrinsically be larger than about $1/200$ of the measured
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50 absorbance of the solution, i.e. 0.01, which is close to the limit of sensitivity of common
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52 spectrophotometers. If 0.01 absorbance is considered to be the experimental sensitivity limit, the
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3 measurement of the absorption spectrum of the lens is likely to be sensitive only to lenses which are
4 completely hydrated by the F-HA solution. However, in our case, this absorbance value is expected
5 to be further reduced by the shape and size of the lens pores, which prevent the F-HA
6 internalization in the core of the lens. For this reasons, optical absorption on the lens was found to
7 be not enough sensitive to provide experimental evidence of the F-HA internalization (the spectra
8 are here omitted).

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16 After wear, the scenario was found to change. After eight hours of wear, some differences were
17 already detectable compared to the unworn lenses, as can be seen in Fig. 7a. The differences
18 became definitively evident after one week of daily wear, when a deep change in morphology was
19 observed, as observed in the SEM micrograph of the lens surface reported in Fig. 7b. The surface is
20 characterized by a granular bumpy morphology. The appearance resembles one of the possible
21 morphologies observed by AFM on worn lenses after one-day wear, as reported elsewhere.^[27,28]

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29 Both by SEM (present work) and by AFM, no large structures deposited on the lens surface were
30 detected after one week. After two weeks, the scenario completely changed and Fig. 8 shows two
31 representative SEM micrographs. A new corrugated morphology appeared, with crests and a more
32 jagged structure. The micrographs suggest the formation of bulges or regions of swelling, which
33 could be attributed to a relaxation of the polymeric network close to the surface. These bulges could
34 be filled by a solution, as can be seen in Fig. 9, where the fluorescence micrograph of a lens after
35 two weeks of wear and after F-HA loading is reported. No defined channels are observed, but large
36 emitting areas. The penetration extent of F-HA across the thickness of the lens is shown at the
37 bottom and right sides and it is comparable to the penetration extent in new lenses (Fig. 5).

54 **Conclusions**

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56 SEM and fluorescence confocal microscopy allowed obtaining information on the structure and
57 loading capability of siloxane-hydrogel contact lenses, in terms of (i) distribution and filling by a
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3 solute of the pores of the lens and (ii) modifications of the lens morphology and uptake as a
4 consequence of wear. The surface of the siloxane-hydrogel material was found to show relatively
5 large pores, with diameters of the order of magnitude of the micrometer or larger. After the F-HA
6 loading, the filling of the pores was found to be almost complete and a new branched morphology
7 was observed with a general thickening of the main walls of the pores. F-HA was found to fill the
8 pores reaching a variable penetration depth from few micrometers to tens of μm , to be compared to
9 the thickness of the lens equal or larger than $90\ \mu\text{m}$. This variation was attributed to differences in
10 the depth of the pores in different portions of the same lens and by a lens to another.
11 Approximately, the content of F-HA per lens was evaluated to be of the order of few tenths of
12 microgram. This value is non negligible, but it is limited by the depth of the siloxane-hydrogel
13 channels, thus being far from the possible F-HA uptake of a lens with a deeper extended porosity.
14 After wear, a different scenario was found. A progressive modification was observed. The longest
15 time of wear of the present study (two weeks) produced a bumpy morphology with regions of
16 swelling on the surface, which could be filled by a solute. The modification was attributed to the
17 relaxation of the polymeric network close to the surface. Preliminary results obtained on other
18 siloxane-hydrogel contact lenses both before and after wear indicated similar scenarios (see
19 Supporting Information).

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 **Acknowledgments**

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FIGURE CAPTIONS

Fig. 1. Surface SEM micrographs of a F-HA-free contact lens taken on two different areas (a and b) of the lens.

Fig. 2. Surface SEM micrographs of two F-HA-loaded contact lenses (a and b).

Fig. 3. Surface micrographs of of two F-HA-loaded contact lenses (a and b).

Fig. 4. Depth profile micrograph of a F-HA-loaded contact lens.

Fig. 5. Fluorescence confocal micrographs taken from the surface of two F-HA-loaded contact lenses (main micrographs) and depth profiles along the grey lines (right and bottom).

Fig. 6. Statistical frequency (bars) of the area of the fluorescence spots in the micrograph of Fig. 5a and hyperbolic curve obtained from the data fitting ($R=0.9797$).

Fig. 7. Surface micrographs of (a) a F-HA-loaded contact lens after eight hours of wear and (b) a F-HA-loaded contact lens after one week of daily wear.

Fig. 8. Surface micrographs of two different F-HA-loaded contact lenses (a and b) after two weeks of daily wear.

Fig. 9. Fluorescence confocal micrograph taken from the surface (main micrographs) of a F-HA-loaded contact lens after two weeks of daily wear and depth profiles along the grey lines (right and bottom).

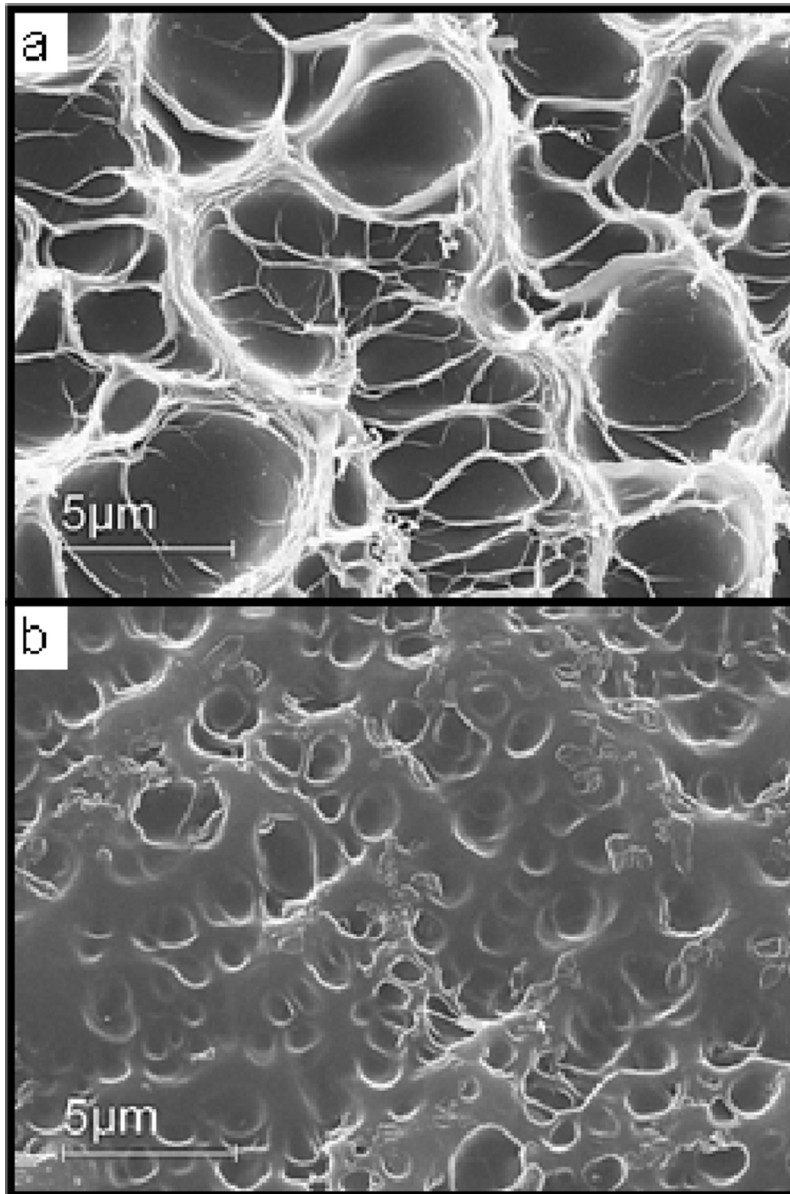


Fig. 1. Surface SEM micrographs of a F-HA-free contact lens taken on two different areas (a and b) of the lens.

296x443mm (96 x 96 DPI)

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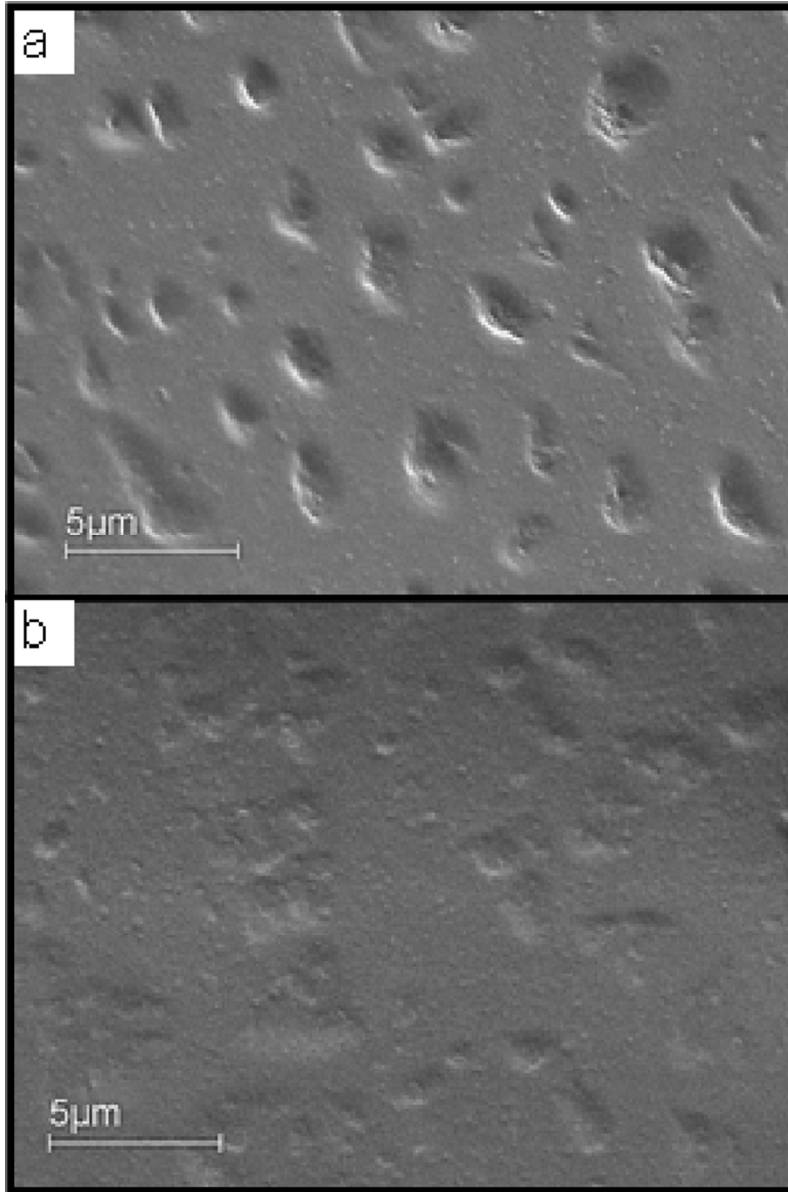


Fig. 2. Surface SEM micrographs of two F-HA-loaded contact lenses (a and b).
294x439mm (96 x 96 DPI)

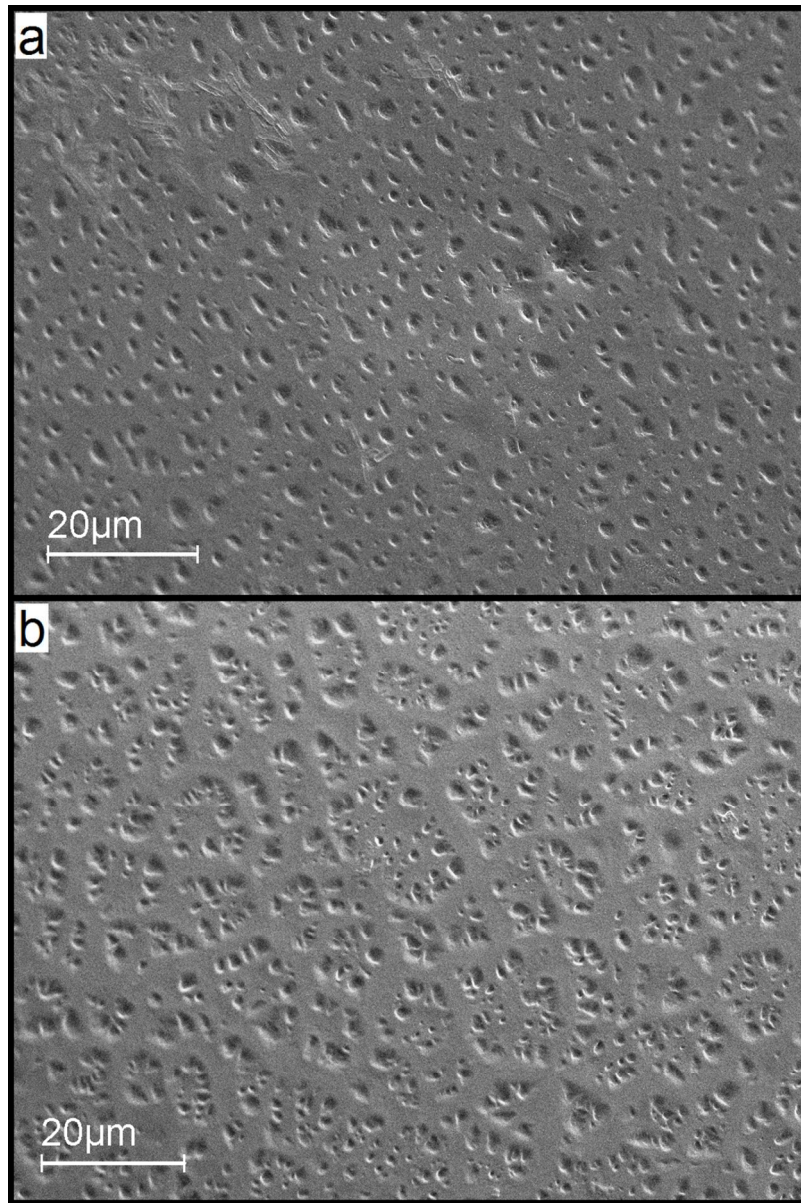


Fig. 3. Surface micrographs of of two F-HA-loaded contact lenses (a and b).
297x443mm (96 x 96 DPI)

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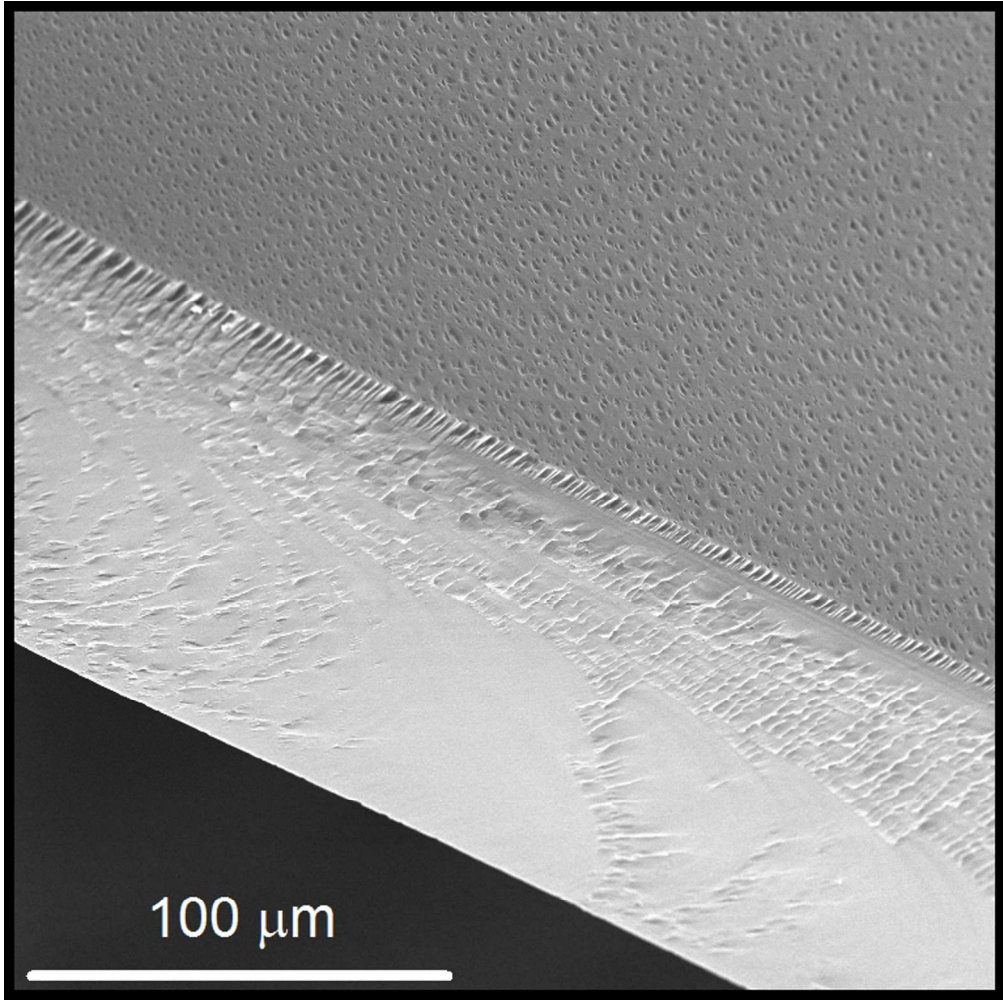


Fig. 4. Depth profile micrograph of a F-HA-loaded contact lens.
394x393mm (96 x 96 DPI)



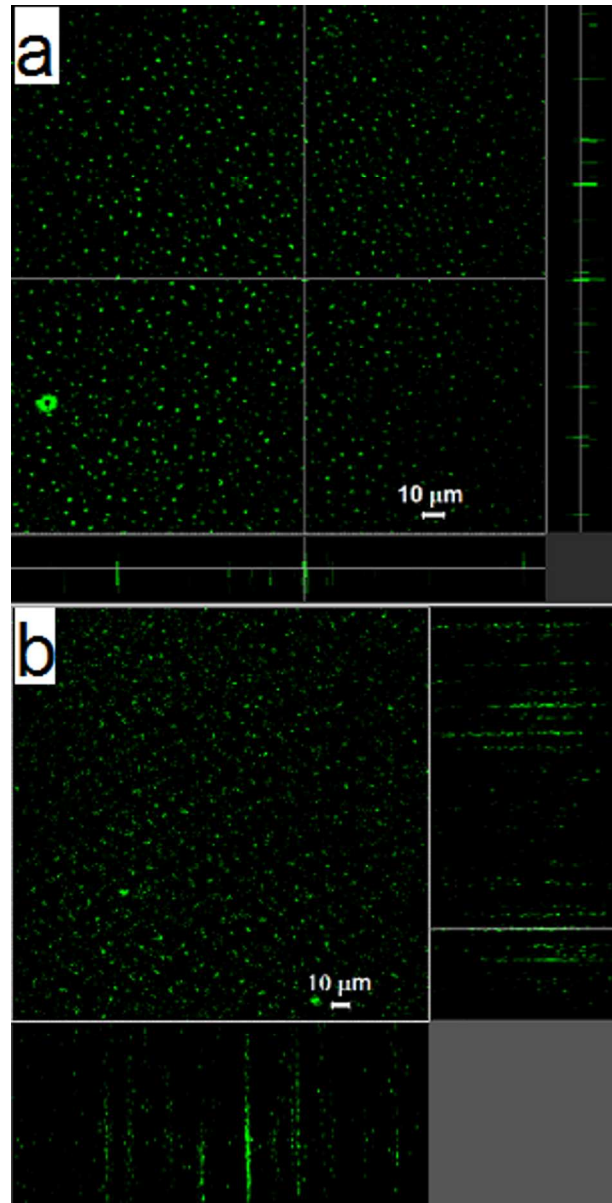


Fig. 5. Fluorescence confocal micrographs taken from the surface of two F-HA-loaded contact lenses (main micrographs) and depth profiles along the grey lines (right and bottom).
273x535mm (96 x 96 DPI)

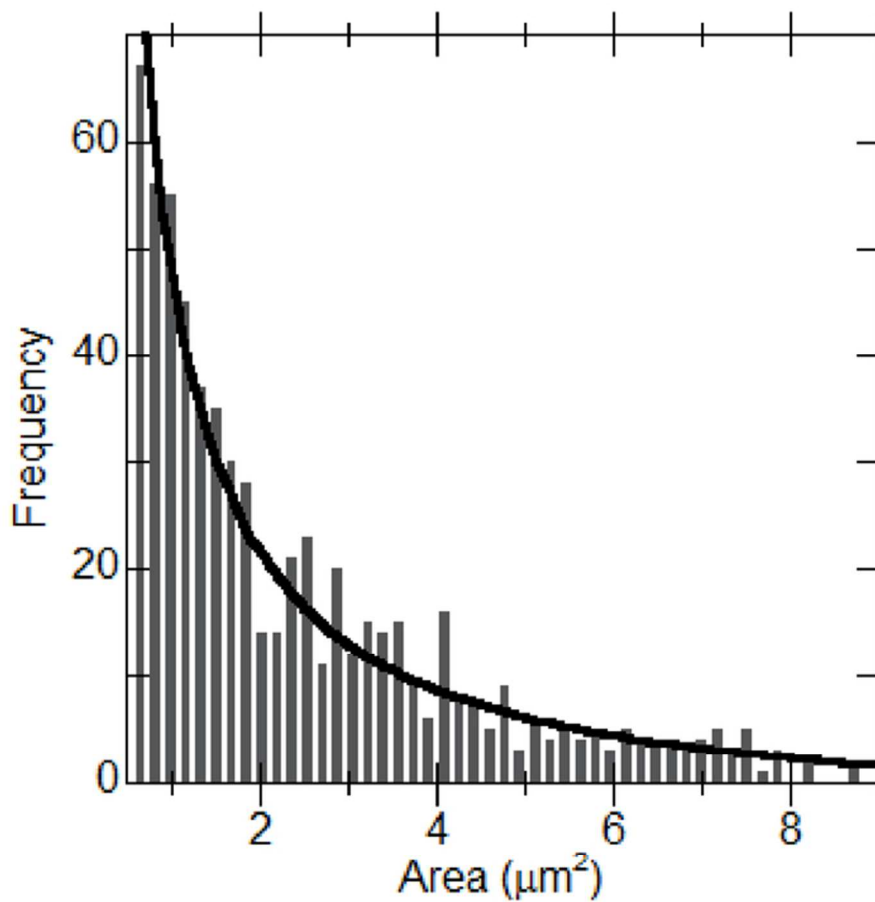


Fig. 6. Statistical frequency (bars) of the area of the fluorescence spots in the micrograph of Fig. 5a and hyperbolic curve obtained from the data fitting (R=0.9797).
67x67mm (300 x 300 DPI)

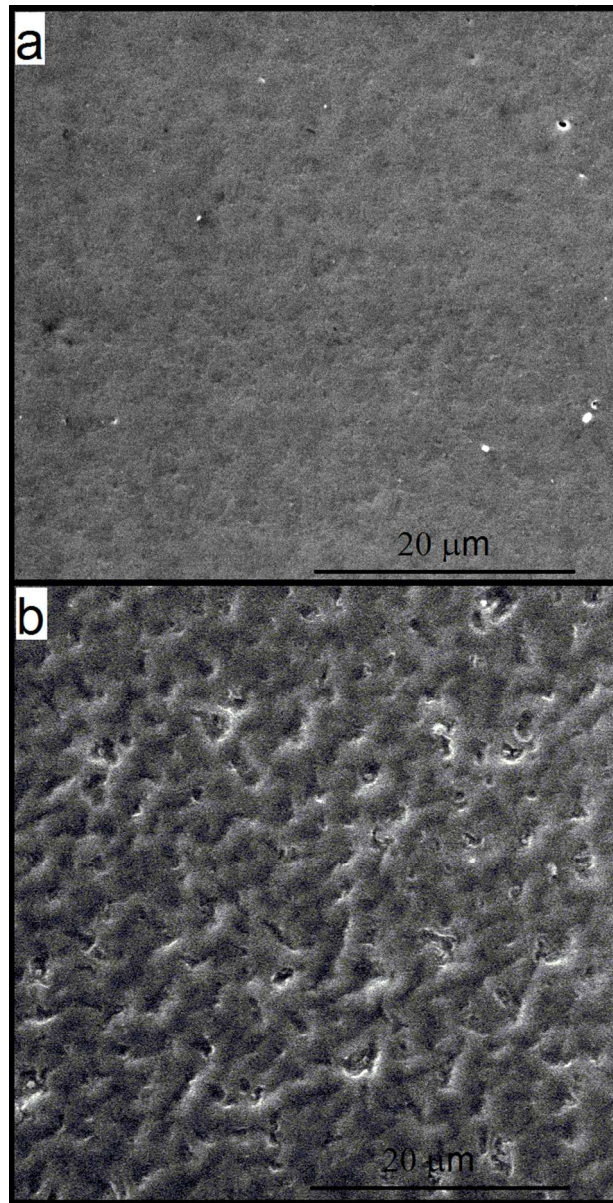


Fig. 7. Surface micrographs of (a) a F-HA-loaded contact lens after eight hours of wear and (b) a F-HA-loaded contact lens after one week of daily wear.
260x508mm (96 x 96 DPI)

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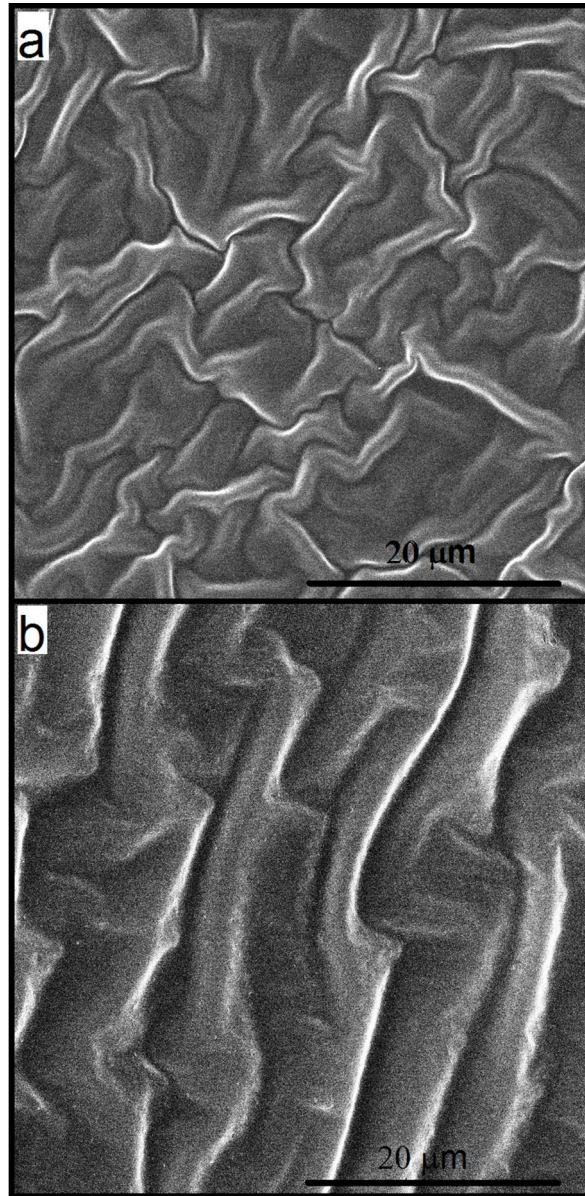


Fig. 8. Surface micrographs of two different F-HA-loaded contact lenses (a and b) after two weeks of daily wear.
250x510mm (96 x 96 DPI)

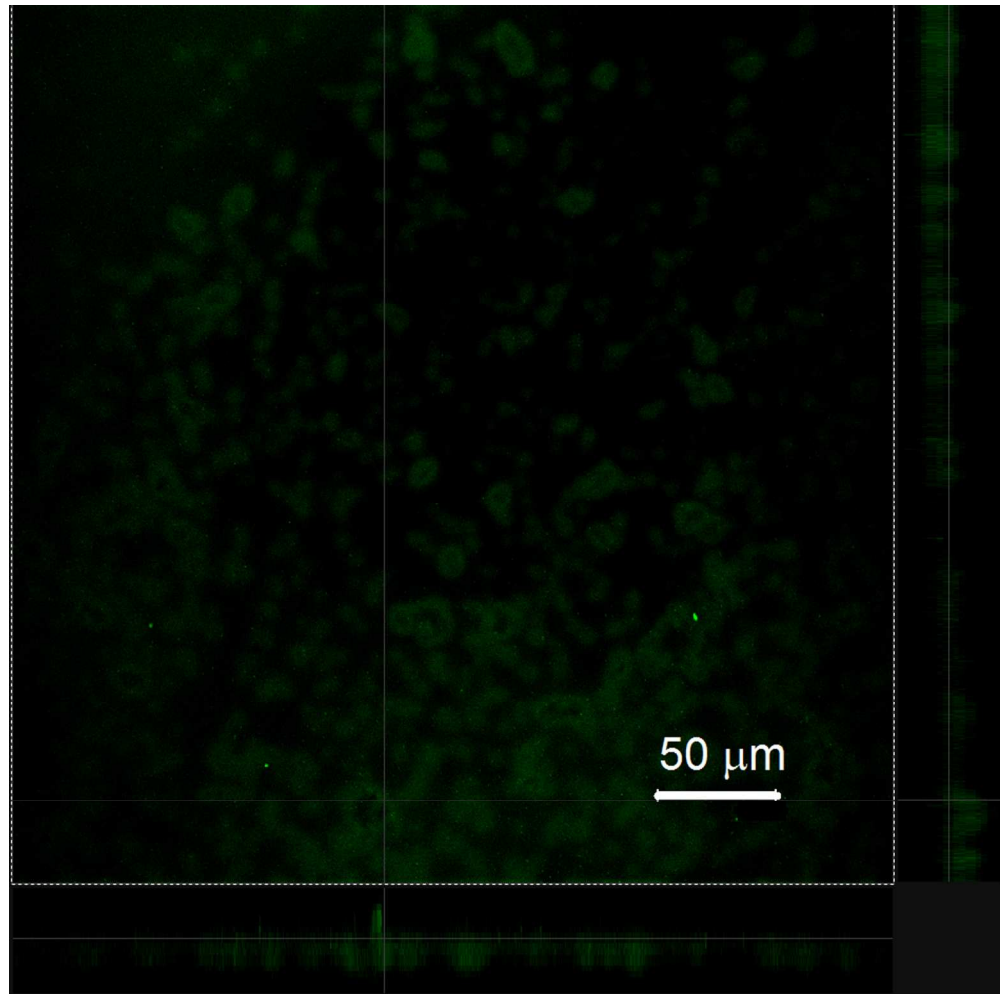


Fig. 9. Fluorescence confocal micrograph taken from the surface (main micrographs) of a F-HA-loaded contact lens after two weeks of daily wear and depth profiles along the grey lines (right and bottom). 439x436mm (96 x 96 DPI)