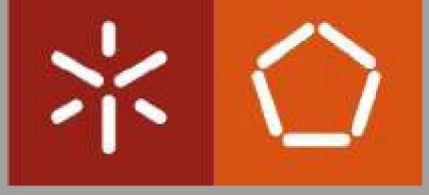

Universidade do Minho
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Lívia Joana Rocha dos Santos

Microbial colonization of contact lenses, tear film deposition, bacterial adhesion and disinfection

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Microbial colonization of contact lenses, tear film deposition, bacterial adhesion and disinfection

Tese de Doutoramento em Engenharia Química e Biológica
Ramo de Conhecimento em Tecnologia Microbiana

Trabalho efectuado sob a orientação da
Professora Doutora Joana Azeredo
Professora Doutora Maria Elisabete Cunha Dias Real Oliveira

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"To suppose that the eye with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection, seems, I freely confess, absurd in the highest degree."

Charles Darwin in "The origin of species"

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Abstract

Biomedical devices are susceptible to microbial contamination. Adhering bacteria to contact lenses (CLs) may induce ocular infections, being microbial keratitis (MK) the most sight threatening. The work presented in this Thesis aimed at investigating the role of surface properties and conditioning film on microbial colonization, bacterial adhesion, detachment, viability and disinfection of silicone hydrogel CLs.

The results herein presented confirmed that the physico-chemical properties of the material influenced the composition of the adsorbed tear film, which in turn affected the hydrophobicity, roughness and the extent of colonising or adhering microbes to CLs. In silicone hydrogel CLs, the presence of adsorbed tear film tended to reduce their hydrophobicity whereas in a conventional hydrogel it increased. This fact might be explained by the amphipilic nature of proteins and lipids that resulted in a decrease of hydrophobicity in silicone hydrogel materials and an increase in the convencional hydrogel lens. Protein adsorption was assessed under "in vivo" conditions. It was found that silicone hydrogel CLs are less susceptible to protein adsorption than conventional hydrogel lenses. The electrophoresis analysis demonstrated that lenses with different chemical composition exhibited distinct protein profiles. The presence of oxidised lipids on CLs was estimated under "in vitro" conditions. The results showed that lenses incorporating N-Vinyl pyrrolidone presented greater amounts of such molecules.

One of the great concerns of silicone-hydrogel material regards its high hydrophobicity and consequently higher propensity for microbial colonisation and/or adhesion. In fact, worn silicone hydrogel CLs, especially balafilcon A exhibited a greater number of colonizing microbes comparing with the other materials. However, adhesion studies of Staphylococcus epidermidis to worn and unworn CLs revealed that all worn silicone hydrogel materials are equally susceptible to bacterial adhesion, and less prone than worn conventional hydrogel CLs. This result can be explained by the decrease of surface hydrophobicity of silicone-hydrogel CLs due to tear film adsorption during wear, already discussed above.

Daily maintenance of CLs is a procedure of outermost importance, conceived to ensure CL hygiene and a safe wear. In this Thesis, the efficacy of a commercially available multipurpose solution (MPS) in the removal and viability of adhered S. epidermidis is described. The results suggest that bacterial removal is material-dependent, since etafilcon A and galyfilcon A lenses exhibited a significant percentage of cell removal regardless being unworn or worn. Conversely, the viability of remained adhered bacteria seems to be influenced by the presence of tear film in particular by the presence of bacteriolytic proteins, given that the number of non-viable cells on worn CL was greater. The disinfection efficacy of several MPS against adhered bacteria to CLs was also assessed using three bacterial strains, four CL materials and three MPS. The protocol was based on the guidelines proposed by the international standard guidelines (ISG) ISO 14729:2001 for ophthalmic disinfecting products. The results revealed that all MPS were capable of accomplishing the goals proposed by stand-alone test, however when challenged against adhered bacteria to CLs, their efficacy was highly affected by the lens material composition. Finally, the use of the surfactants octylglucoside and sodium cholate as quoting agents to enhance MPS efficacy was tested. The results revealed that octylglucoside enabled the reduction of lens surface hydrophobicity and inhibited adhesion of Pseudomonas aeruginosa and Staphylococcus epidermidis both to silicone-based lenses and to conventional hydrogel materials.

Sumário

Os dispositivos biomédicos são susceptíveis à contaminação microbiana. Quando aderidas a lentes de contacto (LC), as bactérias podem induzir infecções oculares, sendo a queratite microbiana (QM) a mais lesiva. O trabalho descrito na presente Tese teve como objectivo investigar a contribuição das propriedades superficiais e do filme lacrimal na colonização microbiana, adesão, remoção e viabilidade bacteriana e desinfecção em LC.

Os resultados obtidos confirmam que as propriedades físico-químicas dos materiais controlam a composição do filme lacrimal depositado, o que por sua vez poderá afectar a hidrofobicidade, rugosidade e o grau de colonização ou adesão microbiana a LC. Quando uma LC é colocada no olho, adsorve de imediato proteínas provenientes do filme lacrimal. A presença de filme lacrimal adsorvido nas LC de silicone hidrogel, tende a reduzir a sua hidrofobicidade devido às propriedades anfifílicas das proteínas e lípidos. A análise electroforética das proteínas em lentes usadas revelou que os perfis diferem em função da composição química do material. A presença de lípidos oxidados nas LC foi determinada através de ensaios de adsorção "in vitro". Os resultados demonstraram que as lentes que incorporavam N-vinil pirrolidona apresentavam uma maior quantidade destas moléculas.

Uma das maiores preocupações em relação aos materiais de silicone hidrogel reside na sua elevada hidrofobicidade e subsequentemente maior propensão para a adesão microbiana. De facto, as LC usadas de silicone hidrogel, em particular a lente balafilcon A exibiu um elevado grau de microrganismos colonizadores quando comparada com os outros materiais. No entanto, estudos de adesão realizados "in vitro" com Staphylococcus epidermidis em lentes novas e usadas revelaram que a adesão bacteriana é sensivelmente a mesma entre lentes usadas de silicone hidrogel e inferior à observada na lente usada de hidrogel convencional. Este resultado foi discutido previamente em relação à adsorção de proteínas devendo-se à diminuição da hidrofobicidade superficial das LC de silicone hidrogel durante o uso.

A manutenção diária das LC com soluções multiuso (SMU) é um procedimento importante a ter no cuidado das lentes tendo sido concebida para promover uma utilização segura das mesmas. Nesta Tese, a eficácia de SMU disponíveis no mercado na remoção e morte de bactérias

aderidas, bem como a adequabilidade da norma internacional (NI) para testar produtos oftalmológicos para desinfecção de LC foram discutidas. Para além disso, foram realizados ensaios com o objectivo de aumentar a capacidade das SMU de inibirem a adesão bacteriana a LC, através da incorporação de tensioactivos naturais.

Os resultados aqui descritos sugerem que a remoção bacteriana induzida pela SMU depende do material da lente e não é afectada pelo respectivo uso, uma vez que as lentes etafilcon A galyfilcon A apresentaram uma percentagem de remoção bacteriana significativa. Pelo contrário, a eficácia da desinfecção parece aumentar devido à presença de moléculas do filme lacrimal em lentes de contacto usadas, uma vez que se verificou que o número de bactérias viáveis que permaneceram aderidas era inferior nas LC usadas. Foram realizados estudos de desinfecção de acordo com a NI para produtos oftalmológicos de desinfecção e os resultados revelaram que apesar de as SMU cumprirem os objectivos delineados pelo critério primário, não alcançaram os objectivos do critério secundário em algumas combinações estirpe bacteriana/LC. Para finalizar, o uso de tensioactivos como agentes potenciadores da eficácia de SMU foi testada e os resultados demonstraram que o octilglucosídeo promoveu a redução da hidrofobicidade superficial das lentes e inibiu a adesão de Pseudomonas aeruginosa e Staphylococcus epidermidis tanto em lentes de silicone como de hydrogel convencional.

Outline of this Thesis

The present Thesis is organised into nine chapters.

Chapter 1 provides an overview of the aspects involved in tear film deposition, bacterial adhesion, detachment and disinfection on silicone hydrogel and conventional hydrogel CLs.

The experimental methods with more relevance under the scope of this work are described in Chapter 2.

Chapter 3 focuses the influence of surface treatment on hydrophobicity, protein adsorption and microbial colonization of CLs.

Chapter 4 examines the surface properties in particular hydrophobicity, roughness and topography of unworn and worn CLs and their susceptibility to Staphylococcus epidermidis adhesion.

Chapter 5 describes the susceptibility of silicone hydrogel and conventional hydrogel lenses to encompass oxidized lipids.

Chapter 6 describes the performance of the surfactants octylglucoside and sodium cholate and a MPS as inhibiting agents of bacterial adhesion to CLs.

Chapter 7 examines the detachment capability of a multipurpose solution (MPS, ReNu Multiplus) against adhered Staphylococcus epidermidis cells. Viability of remaining adhered bacteria was assessed as well.

In Chapter 8, the disinfection capabilities of several MPS were tested against suspended and adhered bacteria to CL.

Chapter 9 provides the general conclusions of the present Thesis and propose recomendations for future work.

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Glossary of Terms and Abbreviations

CL (contact lens)

HEMA (hydroxyethylmethacrylate)

PMMA (polymethylmethacrylate)

RGP (rigid gas permeable)

USAN (United States adopted name)

MA (methacrylic acid)

MMA (methyl methacrylate)

NVP (N-vinyl pyrrolidone)

PVP (polyvinyl pyrrolidone)

MK (microbial keratitis)

MPS (multipurpose solution)

PQ-1 (polyquaternarium-1)

PHBM (polyhexamethylene biguanide)

MAPD (myristamidopropyl dimethylamine)

PNAG (poly-N-acetylglucosamine)

pHEMA (poly(2-hydroxyethyl methacrylate))

CLSM (confocal laser scanning microscopy)

AFM (atomic force microscopy)

EDTA (ethylene diamine tetracetic acid)

PDMS (polydimethylsiloxane)

EPS (extracellular polysaccharides)

LPS (lipopolyssacharides)

Tris ((trimethylsiloxy)-silypropyl methacrylate)

EGDMA (ethyleneglycol dimethacrylate)

ΔG^{adh} (free energy of adhesion per unity of area)

γ_{BS} (free energy of interaction bacterium-substratum)

γ_{BL} (free energy of interaction bacterium-liquid)

γ_{SL} (free energy of interaction substratum-liquid)

XDLVO (extended Derjaguin, Landau, Verwey and Overbeck)

LW (lifshitz-van der Waals)

EL (electrostatic)

AB (lewis acid-base)

LS (liquid-solid interface)

LA (liquid-air interface)

AS (air-solid interface)

γ^{LW} (lifshitz-van der Waals component of the surface free energy)

γ^{+} (electron-donating parameter of the acid-base component)

γ^{-} (electron-accepting parameter of the acid-base component)

ΔG_{sws}^{tot} (hydrophobicity express as $mJ.m^{-2}$)

MW (Molecular weight)

kDA (kilo Daltons)

R_a (average roughness)

SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis)

NaCl (sodium chloride)

TSA (tryptic soy agar)

TSB (tryptic soy broth)

CFU (colony forming units)

EDTA (edetate disodium)

List of Publications related with this Thesis

Santos L, Rodrigues D, Lira M, Real Oliveira MECD, Oliveira R, Y-P Vilar Eva, Azeredo J. The effect of octylglucoside and sodium cholate in Staphylococcus epidermidis and Pseudomonas aeruginosa to soft contact lenses. Optom Vis Sci 2008;84:429-434.

Santos L, Rodrigues D, Lira M, Real Oliveira MECD, Oliveira R, Y-P Vilar Eva, Azeredo J. The influence of surface treatment, hydrophobicity, protein adsorption of silicone hydrogel contact lenses. Contact Lens and Anterior Eye 2007;30:183-188.

Santos L, Rodrigues D, Lira M, Real Oliveira MECD, Oliveira R, Y-P Vilar Eva, Azeredo J. The influence of lens material and lens wear on the removal and viability of Staphylococcus epidermidis. Contact Lens and Anterior Eye 2008;31;126-130.

Lira M, Santos L, Azeredo J, Y-P Vilar Eva, MECD Real Oliveira. The effect of lens wear on refractive index of conventional and silicone hydrogel contact lenses: A comparative study. Contact Lens and Anterior Eye 2008;31:89-94.

Lira M, Santos L, Azeredo J, Y-P Vilar Eva, Real Oliveira MECD. Comparative study of silicone-hydrogel contact lenses surfaces before and after lens wear using atomic force microscopy. J Biomed Mater Res Part B: Appl Biomater 2008;85:361-7.

Santos L, Rodrigues D, Lira M, Real Oliveira MECD, Oliveira R, Y-P Vilar Eva, Azeredo J. Bacterial adhesion of worn silicone hydrogel contact lenses. Optom Vis Sci 2008;85:520-525.

Lira M, Castanheira EMS, Santos L, Azeredo J, Y-P Vilar Eva, Real Oliveira MECD. Changes in IV-Visible transmittance induced by wear on Si-Hi contact lenses. Optom Vis Sci. Accepted.

Santos L, Oliveira R, Real Oliveira MECDA, Azeredo J. Contact lens material influences the disinfection efficacy of multipurpose solutions. Submitted.

Chapter 1 Introduction

The study of CL is a challenging task, as it requires a comprehensive and multidisciplinary approach. The present Chapter reviews some important aspects in particular the surface properties of CLs, microbial colonization, tear film deposition, bacterial adhesion, bacterial detachment, and disinfection.

1.1 Aim of this Thesis

Silicone hydrogel CLs became commercially available in the late 1990s. This type of material supplies upper levels of oxygen to the cornea than conventional ones; thus reducing episodes of hypoxia and overnight oedema.

CL associated microbial keratitis (MK) is a severe eye infection that might lead to vision impairment. Previous studies revealed that such event is less likely to occur in people sleeping with silicone hydrogel CLs than those sleeping with conventional hydrogel CLs(1;2). Also, it was reported that the incidence of MK in people wearing silicone hydrogel CLs as many as 30 nights is the same for those who sleep with conventional hydrogel CL during less consecutive nights(3).

Although important advances have been accomplished, cases of MK are still reported among silicone hydrogel CL wearers. In theory, silicone hydrogel CLs are more susceptible to microbial adhesion because of attractive hydrophobic interactions established between the lens surface and the microbial cell. This means a higher concentration of inoculums transferred to the cornea, thus increasing the chances of developing MK. Other hypothesis concerns the disinfection efficacy of multipurpose solutions (MPS) commercially available. Most contemporary MPS were designed to disinfect conventional hydrogel CLs and not silicone hydrogel materials. Thus, the performance of such products on silicone hydrogel materials is unknown. A poor disinfection efficacy might contribute to the prevalence of MK among silicone hydrogel CL wearers. It must be stressed that some silicone hydrogel CLs are recommended for daily wear and others for continuous wear excluding any disinfection procedure. However, even these last ones can be wearing according to a daily wear schedule. Therefore, disinfection of silicone-hydrogel CL is surely a matter of concern for all kinds of materials. The objectives of the work developed and described in the present Thesis is to investigate the role of surface properties and conditioning film on microbial colonization, bacterial adhesion, detachment and viability and disinfection of silicone hydrogel CLs. Hopefully, the results obtained will contribute for a better understanding of MK prevalence among silicone hydrogel CL wearers.

1.2 Historical overview

The history of CL conception is rather interesting. It begun in 1508 when Leonardo da Vinci suggested the modification of the corneal power through the immersion of the eyes into a recipient filled with water(4;5). In the 1940s, Kevin Touhey developed the first hard lens using the resin PMMA (Polymethylmethacrylate) and in the 1960s, Otto Wichterle and Drahoslav Lím created the material, pHEMA (poly(2-hydroxyethyl methacrylate)), giving birth to soft CLs(4-6). More recently, the combination of conventional hydrogel monomers with silicone elastomers gave origin to a new kind of soft lenses, silicone hydrogel CLs(4;5;7). Currently, CLs are used as a successful form of vision correction by approximately 125 million people worldwide(8).

1.3 Bacterial adhesion: general considerations

Adhesion and biofilm formation are two different concepts. While microbial adhesion is simply defined as adhered cells to a substratum, biofilms are micro-colonies embedded within an EPS (extracellular polysaccharides) matrix, tenaciously adhered to a substratum, and surrounded by channels filled with liquid allowing the inflow of nutrients and the outflow of wastes(9;10).

It is assumed that when a biofilm develops in a CL surface, the wearer loses visual acuity and comfort leading to lens removal or unemployment. Biofilms are found with more frequency and with more density in a lens case than in a CL surface(11). For these reasons, the present Thesis will address bacterial adhesion rather than biofilm formation.

Bacterial adhesion is mediated by the physico-chemical interactions between the microbial cells and the substratum. This process can be interpreted according to the thermodynamic approach and the extended DLVO (approach of Derjaguin, Landau, Verwey and Overbeck, XDLVO) theory(12).

In short, the thermodynamic approach considers the existence of three interfacial free energies: bacterium-substratum (BS), bacterium-liquid (BL) and substratum-liquid (SL)(12). In nature, all systems strive to reach equilibrium, a state of minimum free energy. Microbial adhesion to biotic or non-biotic substrates is not an exception, and this approach allows to predict if adhesion is

favourable or not from a thermodynamic point of view. This can be done using the Dupré's equation(12):

$\Delta G^{adh} = \gamma_{BS}-\gamma_{BL}-\gamma_{SL}$

Where ΔG^{adh} is the free energy of adhesion per unit of surface area, and γ_{BS}, γ_{BL} and γ_{SL} represent the interfacial energy between bacterium-substratum, bacterium-liquid and substratum-liquid, respectively. Accordingly, microbial adhesion is considered thermodynamically favourable if $\Delta G_{adh} < 0$ and unfavourable if $\Delta G_{adh} > 0$.

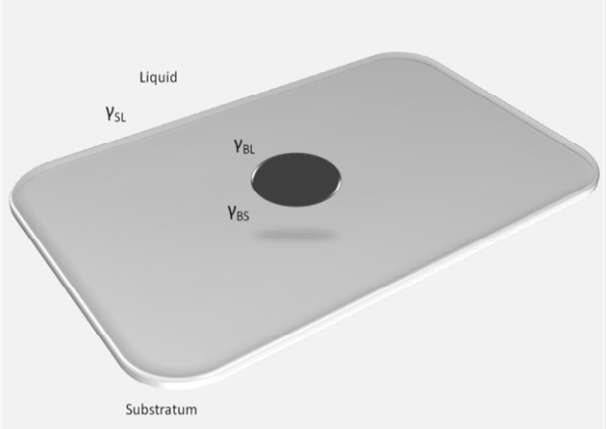

Figure 1.1 Representative scheme of a bacterium adhering to a surface and the interactions involved

The XDLVO theory considers three non-covalent forces acting in aqueous media: Lifshitz-van der Waals (LW), electrostatic interactions (EL) and Lewis acid-base (AB) interactions. AB forces are electron-donating/electron-accepting interactions between polar moieties in aqueous solutions. This type of interaction can be either attractive or repulsive. However, to be effective the range of action must be less than 5 nm. Still, in polar mediums such as water, AB forces are far predominant(12;13). LW is a non-polar force established between non-polar molecules of certain surfaces. This type of interaction is normally attractive and has the longest range of action. EL can be either repulsive or attractive; however, since microbial cells and substrata are usually negatively charged, this force is generally repulsive(13;14).

Although not considered by the previous theories, bacterial adhesion can be assisted by extracellular appendages such as flagella and fimbriae(9). Flagella is a motility device that allows

cell locomotion and approaching to the substratum, while fimbriae are hair-like structures more directly involved in adhesion as they anchorage the cell to the substratum.

1.3.1 Bacterial adhesion to contact lenses

When a CL is placed in the eye, it becomes rapidly coated with proteins and lipids of the tear film. The presence of tear film on the lens surface has been a matter of debate. While some authors suggested that tear film enhance microbial adhesion(15), others show otherwise(16;17). One fact seems certain, lens wear and inherent tear film adsorption modify the surface hydrophobicity(18-20), which in turn may affect the extent of microbial adhesion(16-19).

The presence of tear film on silicone hydrogel CLs, seems to reduce their hydrophobicity, and in several cases the number of cells adhered decreased as well(16;17). Conversely, the presence of tear film on conventional hydrogel materials, appears to increase their hydrophobicity(20), potentially leading for the augmentation of the number of adhered bacteria.

1.4 Contact lens associated microbial keratitis and bacterial virulence

CL associated MK is a severe eye infection arising from the presence of adhered microorganisms on the lens surface. It affects 1 in 10,000 individuals using rigid gas permeable lenses in a daily basis, 3 to 4 in 10,000 individuals using soft CL in a daily basis and 10 to 20 in 10,000 individuals using soft CL according to an extended wearing basis. The chances of vision loss range between 0.3 and 3.6 in 10,000 individuals(21). The following chart (Figure 1.2) illustrates the cascade of steps involved in MK.

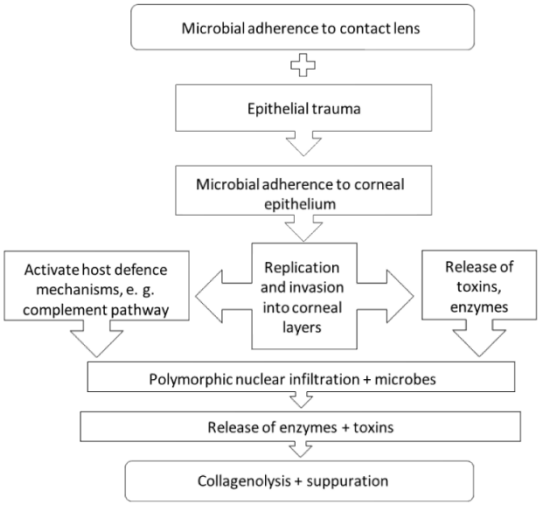

Figure 1.2 Steps involved in microbial keratitis

Adapted from Sankaridurg et al.(22)

MK normally occurs in patients suffering from ocular injury(23), however continuous and overnight wear(24;25), contaminated lens care solutions and lens cases(22;26) are predisposing factors as well. P. aeruginosa and Staphylococci sp. are common causative pathogens; but fungi and acanthamoeba can also cause this infection(22;27-29). P. aeruginosa and Staphylococci sp. exhibit several virulence mechanisms, but only the most directly related with MK will be address herein.

P. aeruginosa is a Gram-negative bacterium. It is considered an opportunistic pathogen and has multiple virulent factors including lipopolysaccharides (LPS) flagella, fimbriae and outer membrane proteins. LPS presents a double function: while protecting the microbial cell against defensive proteins of the tear film it mediates adhesion between the CL and the corneal epithelium(30). Fimbriae are used to anchorage either to the CL surface as to the corneal epithelium. In some strains it was identified the presence of proteases, capable of depleting the protective mucous layer that covers the ocular surface(31).

A photograph of a central corneal ulcer induced by P. aeruginosa is presented in Figure 1.3. Rapid infiltration associated with necrosis and mucopurulent discharge is typically caused by this species.

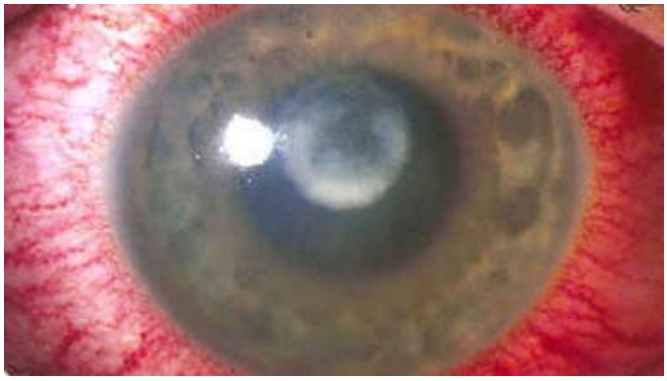

Figure 1.3 Central corneal ulcer in an eye with microbial keratitis

Source: Online Journal of ophthalmology (http://www.onjoph.com/portal/index.php)

S. aureus and S. epidermidis are both Gram-positive staphylococcal species. S. epidermidis is a commensal bacterium of the human skin and mucosa, however under certain circumstances is opportunist and can induce ocular infections(22). Some strains excrete a slime layer, normally of poly-N-acetylglucosamine (PNAG) which mediates bacterial adhesion to the lens surface and is further involved in biofilm formation(32;33). It appears that this layer is also implicated in the severity of MK occurrence(34).

S. aureus produces alpha-toxin and protein A that are believed to damage corneal tissue during a MK episode(35;36). These bacteria also have a collagen-binding adhesin, which interacts with the collagen present in the cornea. It was previously suggested that the collagen-binding adhesin and alpha-toxin influences the severity in which MK occurs(37;38).

1.5 Tear film

The tear film is a complex fluid that covers the ocular surface, delimited by the eyelids. In 1946, Wolff proposed a 3-layered model which is still the most widely accepted(39).

The external layer is composed by lipids secreted by the Meibomian gland and Zeis accessory sebaceous gland(40). It's mainly composed by wax esters, sterol esters and triglycerides(41). Their main function consists in reducing the rate of evaporation of the open eye, lubricating the interface existing between the eye and the eyelids. It also exhibits optical properties.

The main lachrymal gland, and Krause and Wolfring accessory glands produce the middle aqueous layer. This layer has several proteins some of them with bactericidal and bacteriostatic properties, protecting the eye against pathogens(42).

The inner layer is secreted by conjunctival globlet cells possessing high molecular weight glycoproteins called mucins. These molecules help to stabilize and spread the tear film, keep the ocular surface moist, and prevent desiccation and microbial invasion(43;44).

Table 1.1 shows the concentrations of the principal components of the tear film.

Table 1.1 Composition of the tear film

Component	Concentration	Concentration
Mucus		
Glycoproteins	85% of dry weight	
Main proteins	($g.l^{-1}$) *	($g.l^{-1}$) ‡
Lysozyme	2.39	2.07
Lactoferrin	1.51	1.65
Lipocalin	-	1.55
SIgA	-	1.93
IgA	0.411	0.876
IgG	0.032	0.004
Albumin	0.054	0.042
Lipids	(%) †	
Wax esters	32.32-34.96	
Sterol (mainly cholesterol) esters	27.28-29.50	
Polar lipids	14.83-16.04	

Hydrocarbons	0-7.54
Diesters	7.74-8.37
Triglycerides	3.70-4.0
Free sterols	1.63-1.77
Free fatty acids	1.98-2.14

*Data from Gachon et al.(45); †Data from Nicolaides et al.(46). Secretions from the Meibomian gland;‡Data from Fullard et al.(47)

As observed so far, the tear film plays important physiological functions in the eye. However the excessive adsorption of proteins and lipids to CLs may lead to build-up of deposits, reduced visual acuity and development of inflammatory events(48).

1.6 Lipids and proteins

Proteins

Proteins are biomolecules of amino acid units bonded by peptides. These molecules possess aromatic rings called of fluorophores or amino acid residues(Figure 1.4). The fluorophores such as tryptophan and tyrosine exhibit intrinsic fluorescence when excited at 280 nm(49).

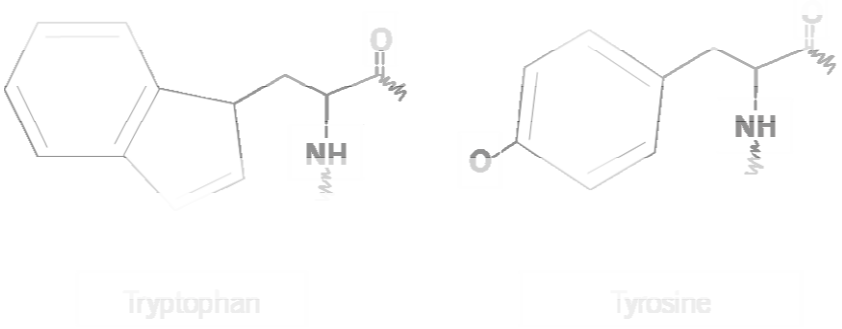

Figure 1.4 Representative scheme of tryptophan and tyrosine molecules

According to Teale spectral classification, proteins are divided into three classes: class A, proteins containing tyrosine residues, but not containing tryptofan residues; class B, proteins containing

both tyrosine and tryptophan residues; and class C, proteins containing only phenylalanine residues(50). Lysozyme, which the most abundant protein of the tear film (
Table 1.1 has 6 tryptophan residues per molecule(51). The fluorescence quantum yields of such residues are distinct. For tryptophan ranges near 0 to 0.35 and for this reason is often used as fluorescence probe(52). Although lipids and proteins seem to be separated within the tear film layers, they interact with each other and contribute to the human tear viscosity(53).

Lipids

Lipids are molecules soluble in non-polar organic liquids. They can be divided into several classes: phospholipids, sphingolipids triglycerides, wax esters, cholesterol esters, free fatty and hydrocarbons(54). Figure 1.5 shows a scheme of the cholesterol molecule.

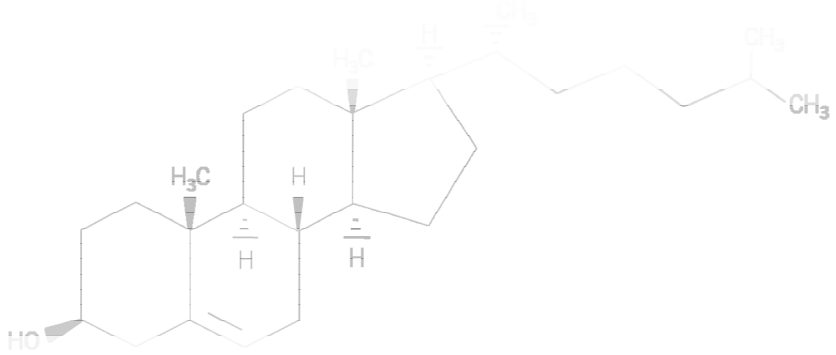

Figure 1.5 Representative scheme of the cholesterol molecule

Lipids oxidation is a phenomenon involved in several pathologies(55-58). The formation of lipids deposits on CLs is recognised as harmful to the lens wearer, however the oxidation of such molecules on the polymeric matrix has not been studied yet and the clinical implications to the lens wearer are unknown.

Lipid oxidation can arise from external factors, or by the presence of free radicals or peroxides present in the eye or in the eye fluids(59). Amino acid residues such as tryptophan and tyrosine exhibit pro-oxidative activity and readily crosslink with lipids metabolites, producing conjugated Schiff-bases. Conjugated Schiff-bases presents the following chemical structure R-N=C-C=C-N-R, where R represents the protein fluorophore to which cross-linked. These molecules exhibits

fluorescence with typical excitation/emission wavelengths of 360nm and 440nm, respectively(60-65).

1.7 Contact lens materials and lens care solutions

1.7.1 Contact lenses

Considering their modulus of elasticity, CLs can be classified as either hard or soft. Soft CLs are divided according to a classification of FDA (Food and Drug Administration) into 4 groups:

Group I - low water content and non-ionic

Group II - high water content and non-ionic

Group III - low water content and ionic

Group IV - high water content and ionic

Soft CL materials are composed of high molecular weight monomers organised according to a 3-dimensional structure. The most common ones are described as follows:

- Hydroxyethylmethacrylate (HEMA) is a widespread monomer and possesses OH radicals. This radical contributes to an increase of lens hydration and therefore to the wearers' comfort. It exhibits 38% of water content, which may differ upon copolymerization with other monomers.
- Methacrylic acid (MA) is a very hydrophilic monomer and the radicals COOH attract water to the polymer surface at physiological pH.
- N-Vinyl pyrrolidone (NVP) is also very hydrophilic and hygroscopic as well. Both MA and NVP can be use to increase the water content of HEMA. These monomers also confer an anionic character to the lens materials.
- Ethyleneglycol dimethacrylate (EGDMA) is a cross-linking agent mainly used to provide structure and stability to the lens material.
- Methyl methacrylate (MMA) is a hydrophobic monomer used in hard lenses manufacturing; however it is very useful in giving stability and some stiffness to soft CLs(66;67).

- Polydimethylsiloxane (PDMS) is a silicone elastomer used in silicone hydrogel CLs.
- (Trimethylsiloxy)-silypropyl methacrylate (TRIS), TRIS-like monomers and fluoracrylates were primarily used in RGP fabrication. Currently are incorporated in silicone hydrogel CLs(68-70). Figure 1.6 shows a few monomers used in silicone hydrogel CL fabrication.

Figure 1.6 Chemical structure of some monomers used in soft contact lenses fabrication

1.7.2 Multipurpose lens care solutions

CLs disinfection is an important strategy to prevent MK as well as other complications associated with lens wear. Multipurpose solutions (MPS) are single solutions which allow to clean, disinfect

and store CLs. Data from Europe, Australia and Canada indicate that 80% of CL wearers use MPS to disinfect their lenses instead of other type of solutions(71).

1.7.2.1 Surfactants

Surfactants are surface-active molecules, which simultaneously possess hydrophobic and hydrophilic domains (amphiphilic). MPS have small concentrations of surfactants, being poloxamine and poloxamer the most common ones. Their major function is to clean the lens surface particularly from debris and deposits (may include microorganisms). To achieve such cleaning, surfactant molecules form micelles around the debris or deposits, being removed during rinsing. Figure 1.7 shows a representative scheme of surfactant molecules adsorbing on hydrophobic and hydrophilic surfaces.

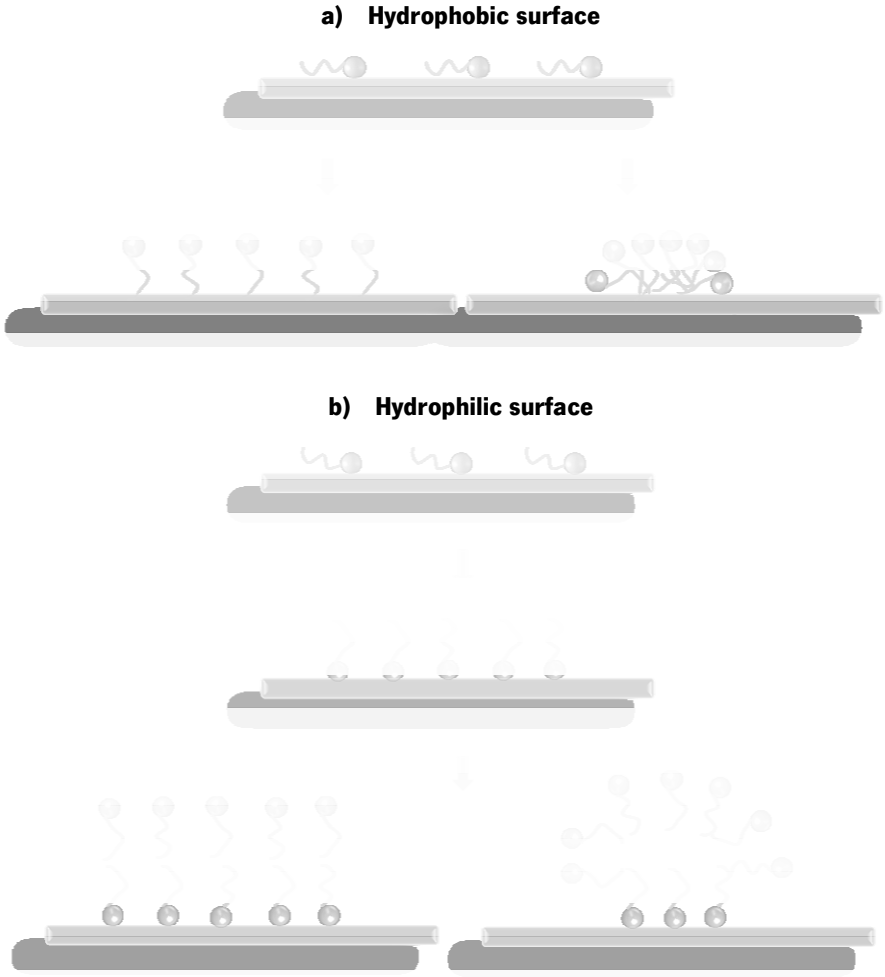

Figure 1.7 Representative scheme of surfactant molecules on a) hydrophobic and b) hydrophilic surfaces

Adapted from Holmberg et al.(72)

When incorporated on the MPS formulation, surfactants can eliminate between 10 to 50% of deposited proteins(73), still dedicated surfactant solutions can express higher levels of efficiency(74). A comparative study revealed that Opti-Free Express and Opti-Free Express No Rub Lasting Comfort formula seems to remove more proteins than ReNu Multiplus or SOLO Care Plus(75). Other study but comparing ReNu Multiplus and Complete Comfort PLUS, found that the last one is more effective in protein removal(76).

Surfactants can also improve lens wettability, leading not only to the augment of wearer comfort(77) but also to a possible reduction of the levels of protein adsorption(78;79) and bacterial adhesion(80). Hydrophilicity and wettabilty should not be mismatched. The first term mainly refers to the affinity between a solid surface and water molecules, whereas the second term is more general and expresses the contact between a liquid and solid surface. If the wetting is favourable the contact angle is low, conversely if it is high the wetting is unfavourable. The only situation where they can be synonymous is in the context of water. In this case, a wettable surface may be also termed as hydrophilic.

1.7.2.2 Biocides and lens disinfection

Small concentrations of biocides are present in MPS formulation. Their major role is to kill pathogens, which may threaten the eye integrity. One of the most common biocides is polyhexamethylene biguanide (PHMB). PHMB belongs to the biguanide family, is cationic and exhibits a high molecular weight. Being cationic, PHBM tends to create an electrostatic attraction between microorganisms (microorganisms usually carry negative charge). Because of the electrostatic attraction, the biocide is pull towards the inner membrane disrupting it. Microbial death occurs when the membrane function is lost and the intracellular components precipitate(81).

Polyquartenarium-1 (PQ-1) belongs to the polymeric quaternary ammonium compounds family and is a cationic high molecular weight biocide as well. Due to the electrostatic attraction, PQ-1 penetrates the cell wall towards the cytoplasmatic membrane opening a pathway for progressive

leakage of the intracellular material(82). This disinfectant presents poor activity against fungi and acanthamoeba(83).

Myristamidopropyl dimethylamine (MAPD) is a biocide composed by a fatty acid coupled to a cationic group. Their molecular size and weight are smaller than PHBM and PQ-1, but it has demonstrated effectiveness against fungi, and trophozoide and cystic form of Acanthamoeba(83;84).

In accordance to Food and Drugs Administration (FDA), a MPS should have a biocide concentration capable of destroying microorganisms in a significant way. These requirements are described in the standard ISO 14729 Ophthalmic optics - Contact lens care products - Microbiological requirements and test methods for products and regimens for hygienic management of contact lenses(85). This standard describes two tests: the primary (stand-alone test) and the secondary (regimen test). According to stand-alone test, products should kill 99.9% of suspended bacteria and 90% of yeast. This corresponds approximately to 3-log and 1-log reduction, respectively. If it fails, the product must be tested according to the regimen test. In this case, a 4 to 5-log reduction of adhered bacteria to CL must be accomplished. For both tests, the panel of microorganisms is the same and includes P. aeruginosa, S. aureus, S. marcescens, C. albicans and F. solani, which can be purchased from culture collections.

One critic that can be addressed to this standard lye in the inability of stand-alone test to provide a reliable assessment of disinfection. Under real conditions, disinfection is performed against adhered microbes to CLs and not against suspended ones. In addition when microbes are adhered to a substratum or within a biofilm, they exhibit higher patterns of resistance to disinfection than suspended ones(86). Although most studies were made according to stand-alone test(87-90), there is one that shows that some MPS meeting stand-alone test, fail the regimen test for some bacteria/lens material(91) supporting the hypothesis that stand-alone test is inappropriate to asses CL disinfection.

Other critic that can be point out to this standard concerns the type of microorganisms used in the disinfection assessment. Clinical isolates are often more tolerant to biocides than microorganisms from collection cultures(92) and the biocide activity may vary upon different species, and even between strains of the same species(93). Additionally, some biocides may lose their activity during lens storage(94-96).

Besides disinfection, microbial detachment is also an important issue. If a MPS fail to induce detachment, the non-viable microbes may remain adhered to the lens surface being attractive to living ones(97). In RGP CLs, two MPS stimulated minor cell removal; however in in soft CLs ReNu® Multiplus™ was able to remove P. aeruginosa in a significantly way(98).

1.7.2.3 Resistance of microorganisms to biocides

Microorganisms exhibit innate and acquired resistance mechanisms against biocides(83;99-102). Innate mechanisms include bacterial adhesion (adhered cells are 500 times less susceptible to biocides) and the presence of impermeable barriers(92;103-105). EPS accumulate outside the cell may protect the bacteria against the biocide activity(93). Efflux pumps are other defence mechanisms and can be either considered intrinsic or acquired. Roughly an efflux pump is a system that removes toxic compounds from the microbial cell surface(92;103-106). Mutations, adaptations or the acquisition of plasmids, transposons or other genetic features are considered extrinsic resistance mechanisms(92;103-105).

It is generally accepted that Gram-negative bacteria are less susceptible to antimicrobial agents, since they possess an additional membrane (outer membrane), which reduces cell permeability and therefore the biocide uptake. The same outer membrane can also carry proteins implicated in multiple drug resistance(107;108) and the composition of the membrane fatty acids play a role in the resistance of this type of bacteria(109). It has been shown that the most cytotoxic strains of P. aeruginosa are also the most resistant to the biocide activity(110). Other study regarding biocide resistance, revealed that some strains of P. aeruginosa were insusceptible to PQ-1(111). The presence of cationic molecules on the surface of such strains repealed the biocide. In other studies, imaging techniques were used to visualise damages provoked by PQ-1 and MAPD on several bacterial species(102). According to that, PQ-1 caused significant damages in the cytoplasmatic membrane of P. aeruginosa and S. marcescens, and in less extent to S. aureus. Indeed, S. aureus can remove PQ components from the membrane core using efflux pumps and that might be related with such result(112). Other study confirmed that S. marcescens is susceptible to PQ-1 as their cytoplasmatic membrane was easily disrupted(113). MAPD also induce damages in the cytoplasmatic membrane; however, since no potassium release was observed, it appears that damages were not so extensive.

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Chapter 2 Methods and rationale for their utilization

The methods and techniques used in the present work are described in this Chapter. The rationale beyond its employment is also discussed.

Most studies described in this Thesis were conducted with worn CLs. Some lens materials are deteriorated during wear(1). In view of that, it is expected that wear modify the lens surface properties as well, which in turn might influence microbial adhesion and detachment. Therefore, the assessment of worn CLs was found important since the results obtained are surely more realistic than if performed with new ones.

Lens fitting and follow-up consultations were carried out in the Laboratories of Clinical Optometry of the Physics Department of the University of Minho. Volunteers were neophytes from the University of Minho comprising students, workers and professors. A contra-lateral eye model was employed. Accordingly, each volunteer used a silicone hydrogel CL in one eye and a conventional one as contra-lateral pair. The volunteers were subject to follow-up consultations in the first, third and sixth month. The conventional hydrogel lens was replaced every two weeks and the silicone hydrogel ones in a monthly basis. Figure 2.1 gives the age distribution of the volunteers enrolled in this study.

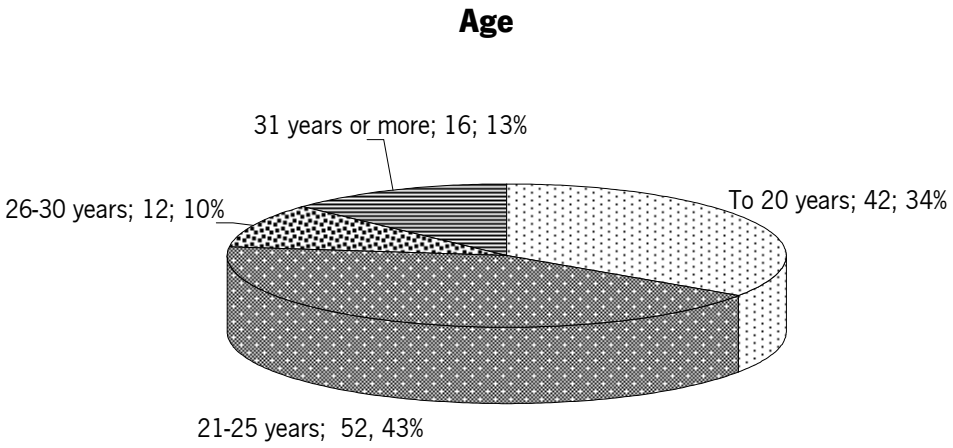

Figure 2.1 Age distribution of the volunteers enrolled in this study

2.1 Hydrophobicity

It is generally recognized that hydrophobicity plays an important role in microbial adhesion to biomedical implants and implant-related infections(2-10). Hence, the assessment of this property was considered essential to provide a better understanding of microbial adhesion results.

Hydrophobicity was calculated as the free energy of interaction between two identical surfaces (s) immersed in water (w)(11):

$$\Delta G_{sws}^{tot} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 - 4\left(\sqrt{\gamma_s^+\gamma_s^-} + \sqrt{\gamma_w^+\gamma_w^-} - \sqrt{\gamma_s^+\gamma_w^-} - \sqrt{\gamma_s^-\gamma_w^+}\right)$$

ΔG_{sws}^{tot} expresses the degree to which the attraction of the surface (s) towards water (w) is greater (hydrophilicity) or smaller (hydrophobicity) than the attraction between two moieties of that surface. Thus, when the global free energy of interaction between two identical surfaces (s) immersed in water is repulsive (has a positive value) the surface (s) is considered hydrophilic. On the other hand, the more negative ΔG_{sws}^{tot} is, the higher the solid surface hydrophobicity is.

γ_S^{LW} is the non-polar component of the surface tension of the solid surface and γ_S^+, γ_S^- the electron-acceptor and electron-donor parameters of the polar component. These parameters can be estimated through contact angle measurements using liquids of different polarities. The polarity values of some reference liquids, including water are detail in Table 2.1. Water and formamide are polar, while α-bromonaphthalene is non-polar.

Table 2.1 Polarity values of bromonaphthalene, water and formamide

Reference liquid	γ_l^{TOT} (mJ.m^{-2})	γ_l^{LW} (mJ.m^{-2})	γ_l^+ (mJ.m^{-2})	γ_l^- (mJ.m^{-2})
α-Bromonaphthalene	44.4	44.4	0.00	0.0
Water	72.8	21.8	25.50	25.5
Formamide	58.0	39.0	2.28	39.6

Figure 2.2 shows a representative scheme of a contact angle established between a drop of liquid and a CL surface, where ls represents the liquid-solid interface, la the liquid-air interface and as the air-solid interface.

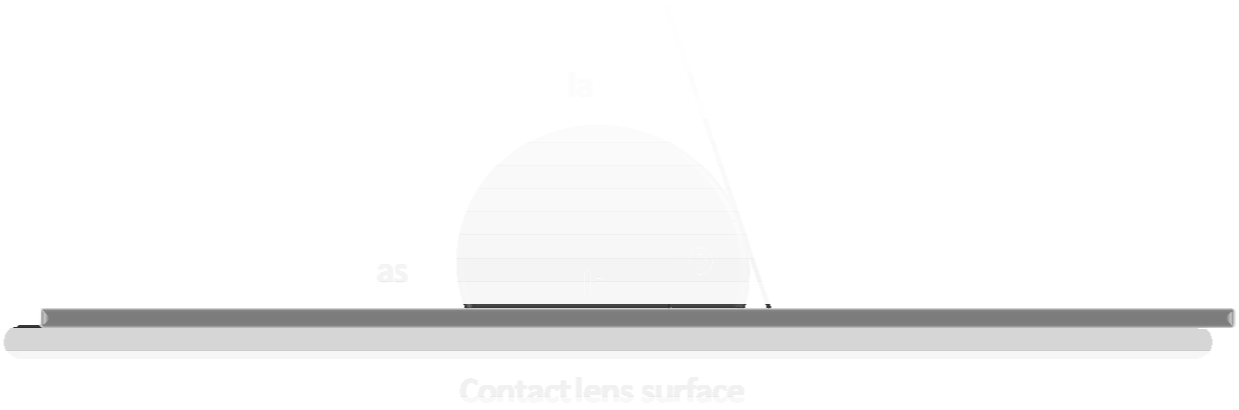

Figure 2.2 Contact angle established by a drop of liquid in contact with a contact lens

Contact angle measurements were performed through the advancing type technique on air. Other standard technique is the sessile drop technique on air. However, advancing type was found simpler to be employed instead of sessile drop, as in very hydrophilic materials a single droplet of liquid rapidly spreads.

The equation of Young-Dupré establishes the relation between contact angles and the surface tension components of the reference liquid.

$$(1+\cos\theta)\cdot\gamma_l^{TOT} = 2\cdot\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^{+}\gamma_l^{-}} + \sqrt{\gamma_s^{-}\gamma_l^{+}}$$

Where Θ is the measured (advancing) contact angle, γ_l^{TOT}, is the surface tension of the liquid with which the contact lens is measured, γ_S^{LW}, γ_S^{+}, γ_S^{-} are the non-polar component of the surface tension of the solid surface, the electron-acceptor and electron-donor parameters of the polar component, respectively.

The non-polar component of the lens surface is calculated as follows:

$$\Delta G_{sls}^{LW} = -2\cdot\left[\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_l^{LW}}\right)\right]$$

While the polar component is calculated through the equation:

$$\Delta G_{sls}^{AB} = -4 \cdot \left(\sqrt{\gamma_s^{LW} \gamma_s^{LW}} + \sqrt{\gamma_l^- \gamma_l^-} + \sqrt{\gamma_s^+ \gamma_l^-} - \sqrt{\gamma_s^- \gamma_l^+} \right)$$

The unknown solid surface tension components can be calculated with a 3-equation system derived from this equation and the surface tension components of the reference liquids.

2.2 Roughness and topography

The atomic force microscope is a high resolution scanning probe microscope. This apparatus permits to obtain topographic images and roughness values within the nanometer range. Atomic force microscopy (AFM) measurements comprise scanning surfaces with a sharp probe attach to a cantilever, usually made of silicon or silicon nitride. Among other parameters, AFM allows the determination of surface roughness and topography. These parameters may affect CL microbial adhesion and thus their assessment was undertaken(10;12;13).

2.2.1 Tapping Mode™ AFM

Tapping Mode™ AFM was the technique used to estimate roughness and topography of CL surfaces. This technique has been previously employed on CLs since the scanning process does not damage their delicate surface. As the name suggests in Tapping Mode™ AFM, the probe intermittently taps the surface instead of continuously contacting with it. This fact allows eliminating drag forces, hence maintaining the lens surface integrity. When the probe is in close proximity to the lens surface, forces such as van der Waals or electrostatic forces drive the probe away. These forces create an amplitude decrease; however, when the probe is far enough to suffer their influence, tends to approach to the surface again giving origin to the oscillatory movement typically termed as tapping mode. The amplitude is maintained constant by this feedback(14).

2.3 Protein adsorption

2.3.1 SDS-PAGE

Medical devices in contact with body fluids readily adsorb proteins(15). Adsorbed proteins on CLs can be estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)(4;10). This technique allows protein separation according to their molecular weight within a polyacrylamide gel. The passage of an electrical current through the gel is the driving force of protein migration. Prior to electrophoresis, proteins must be denaturated with SDS. Proteins may exhibit secondary and tertiary structures and denaturation makes them loosing these structures acquiring a primary one. The possession of a primary structure is important because in this state proteins exhibit a typical molecular weight (MW) which is the characteristic that allows estimating the type of protein. In Figure 2.3 a polyacrylamidade gel electrophoresis can be visualised. The visible spots are proteins. The ones closer to the bottom exhibit low MW, while the ones near the top have high MW. A 10% gradient enables the retention of proteins with a molecular weight between 14 kDA and 205 kDA. This gradient was found appropriate since the most representative proteins in tears have a molecular weight within this range of values.

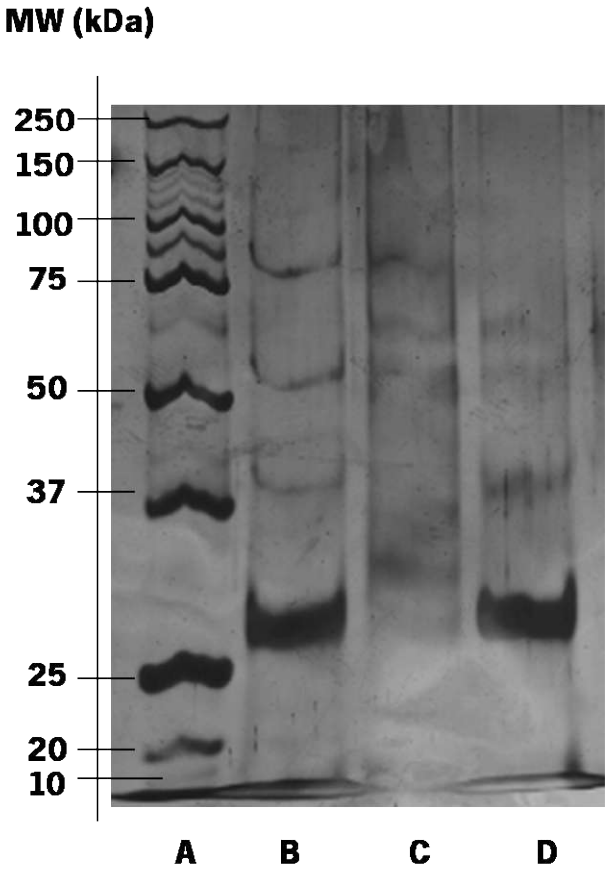

Figure 2.3 Section of a polyacrylamide gel stained with silver nitrate

Beside denaturation, SDS plays another important role. This surfactant is anionic and while it binds to proteins grants them a negative charge, permitting them to migrate during electrophoresis.

2.3.2 Fluorescence spectroscopy

Fluorescence is a photon emission process that occurs during molecular relaxation from excited electronic states. This process involves transitions between electronic and vibrational states of polyatomic fluorescence molecules (fluorophores)(16;17). The Jablonski diagram represents the excited state and relevant transitions (Figure 2.4).

Figure 2.4 Jablonski diagram of fluorophore excitation, radiative decay and nonradiative decay pathways

Absorption of a light quantum, induce molecule transference from the ground state (S0) to one of the vibrational levels of the excited states, S1, S2, and so on. Fluorescence emission occurs when the molecule transit from S1 to S0 state. The number of quanta emitted per unit time is proportional to the number of quanta adsorbed per unit time and fluorescence quantum yield(16;17):

$N_f = I_0(1-10^{-D}).q_f$

Where I_0 is the intensity of the incident light, D is the absorbance of the solution and q_f is the fluorescence quantum yield. The fluorescence quantum yield is the ratio of the number of quanta emitted from an excited state to the number of quanta absorbed during the transitions from the ground to the excited state per time unit. Several factors may affect the fluorescence quantum yield such as radiative and nonradioactive processes, coalitional quenching among others(16;17).

Quantification of adsorbed proteins

Fluorescence spectroscopy is a widespread method and allows estimating the amount of proteins adsorbed to a CL(18-21). As mentioned in the introduction section, protein exhibits intrinsic

fluorescence because of the presence of fluorophores such as tyrosine and tryptophan(18). Although each protein species may possess different numbers of these fluorophores, is assumed that the fluorescence intensity is proportional to the protein quantity.

Adsorbed proteins were extracted from the lens matrix using a solution of trifluoracetic acid and acetonitrile, as described by Keith et al.(19). The fluorescence intensity of the extracted portion was measured in the fluorescence spectroscope using a xenon lamp. Solutions with different concentrations of bovine serum albumin (BSA) were prepared to verify the linearity of the apparatus (Figure 2.5) and the wavelength according to which emitted the highest fluorescence intensity value. When excited at 280 nm, BSA emited a maximum of fluorescence intensity at 360 nm. Changes of the medium pH, provokes a reversible conformation of this protein. BSA can exhibit conformation E expanded (E) fast, (F) (normal) (N), Basic (B) and aged (A) is observed for pHs ranging from 2.9 to 9.0(21). Tryptophan was used as fluorescence probe once the fluorescence quantum yield of tryptophan (0.20 at pH 7.0 and 20 °C) is superior to tyrosine (0.14 at pH 7.0 and 20 °C). Although both residues absorb at 280 nm, they emit at different wavelengths. Under neutral conditions (pH 7 in water), the maximum emission wavelength of tryptophan is 348 nm whereas tyrosine is 303 nm.

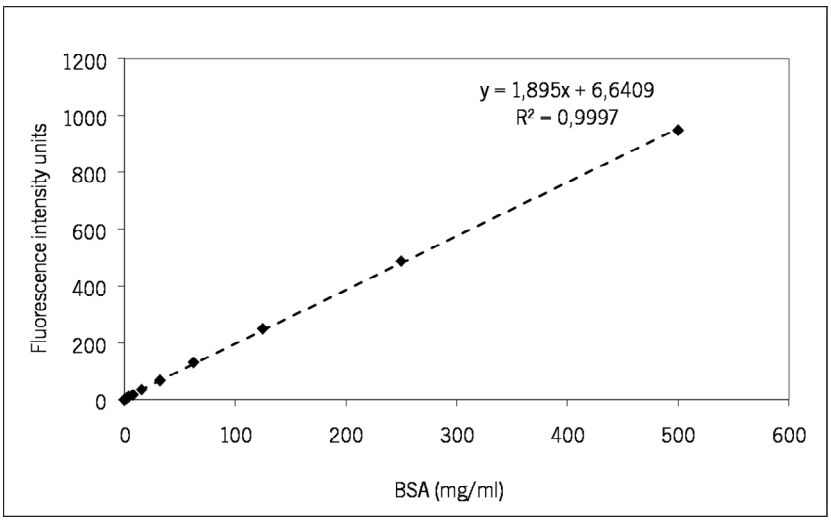

Figure 2.5 Linearity of BSA fluorescence intensity obtained with solutions at different concentrations

Tryptophan absorption is caused by π→π* electron-vibrational transition in the indole chromophore, consisting of aromatic π-system of which formed by ten π-electrons(20). Tryptophan residues and tyrosine residues absorb in the same wavelength region and at neutral pH, when the excitation wavelength used is 280 nm this could result in some energy transfer from tyrosine to tryptophan and because of that the fluorescence of tryptophan could be augmented by this process(22). To overcome the problem an acidic solution was used to extract the adsorbed proteins since under acid conditions, this process is eliminated due to the configuration of the protein (expanded configuration). Figure 2.6 shows the typical absorption and emission spectra of tryptophan.

Figure 2.6 Absorption and emission spectra of tryptophan

Quantification of oxidized lipids

Atmospheric oxygen and photo-oxidation were the pathways used to promote lipid oxidation. For the assays, a 24-well culture plate was employed (Figure 2.7). Lenses were individually inserted into each well containing an artificial tear solution made with proteins and lipids. Proteins were part of the artificial tear solution not only to mimic natural tear film, but also to promote the formation of conjugated Schiff-bases as explained in Chapter 1.

To improve their solubility, lipids were firstly dissolved in foetal calf serum. After dissolving completely, proteins were added to the formula followed by phosphate buffer saline solution.

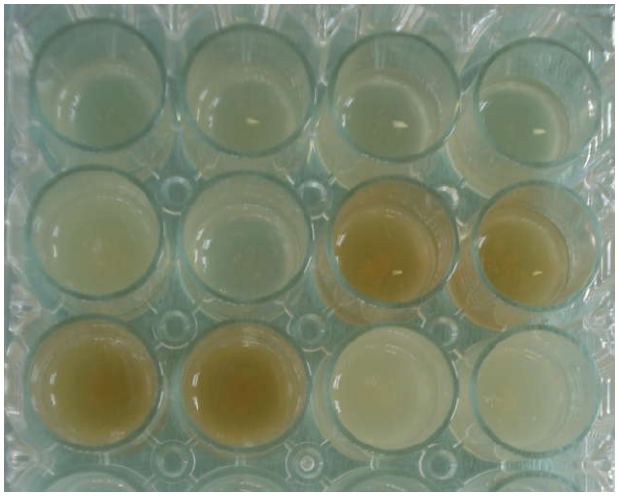

Figure 2.7 Contact lenses incubated in artificial tear solution

2.5 Bacterial adhesion and detachment

Microbial adhesion to medical devices can be studied under static and dynamic conditions(6;8;34;35).

A parallel plate flow chamber (dynamic) was previously used to asses bacterial adhesion and detachment on CLs(4;5;10). In the present work, this system was used to study the initial adhesion of bacteria to CLs under a laminar regimen as well as to promote cell removal(36). Static assays were employed to study the capability of surfactants and a MPS in inhibiting bacterial adhesion to CLs.

2.5.1 Parallel plate flow chamber

The external dimensions of the chamber used in this study are 16x8x1.6 cm. The chamber is composed of a bottom plate made of Perspex and a top plate made of glass. The Perspex/glass dimensions are both 7.8x4.8 cm. In the Perspex plate, two circular sections were removed in order to fit the CLs (Figure 2.8). In between the plates, a Teflon 0.0.6 cm spacer is inserted.

The chamber core is transparent so that adhesion can be imaged through phase contrast microscopy and the number of adhered cells can be quantified. Therefore, the flow chamber can be placed on the microscope. The overall system also includes two flasks, one containing a

saline buffer and the other a microbial suspension connected to the flow chamber. The flow is regulated by a peristaltic pump connected to the chamber.

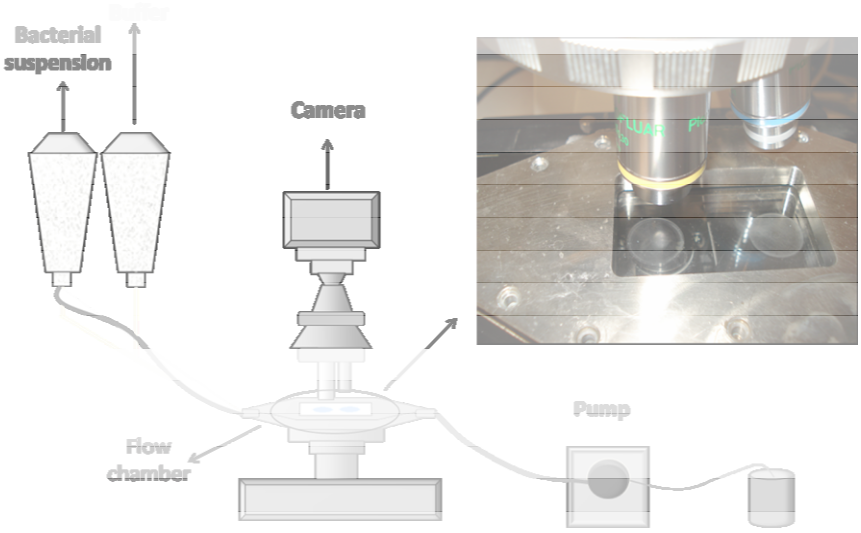

Figure 2.8 Representative scheme of the parallel plate flow chamber system

A CCD camera coupled to the microscope allows the capture of pictures of adhered microorganisms to CLs. Phase contrast and a long-range objective is required. The camera should be set to 512x512 pixels frame (corresponds to 0.0041 mm^2). Although flatten, CLs can still exhibit some curvature, so, to diminish this problem a small frame is preferred.

As mentioned above, bacterial detachment can also be evaluated using a parallel plate flow chamber. After adhesion occur, it is possible to dispense a solution (surfactant or MPS) throughout the system to induce such detachment.

2.5.2 Static adhesion

Static adhesion assays were performed to assess the ability of certain surfactants to inhibit bacterial adhesion to CLs. Surfactants are capable of adsorbing to CLs, and the interaction between the chemical structure and the lens chemical composition rules the orientation according to which is adsorbed. Lens coating with surfactants inhibits bacterial adhesion by exposing their hydrophilic groups to the surrounding environment. Hidrophilicity is a known

repulsive force, and might act to reduce adhesion(37). A 24-well tissue culture plate was used to promote lens coating and bacterial adhesion either to uncoated and coated materials.

2.6 Cell viability

Viable bacteria are metabolic active microorganisms. However, several factors may contribute to the loss of their viability, namely the contact with MPS and bacteriolytic proteins. Viability assessment of adhered bacteria is important because viable bacteria are potentially harmful to the lens wearer. On the other hand, non-viable bacteria that remain adhered to lens surface may attract living ones.

In the present work the fluorochrome propidium iodide (PI) was use to evaluate bacterial viability. This dye stains non-viable cells through bonding to DNA molecules. This is possible because the cellular membrane is damage and thus permeable to this dye(26).

To obtain the number of viable cells, the number of non-viable bacteria should be subtract to the number of total adhered cells. The number of total adhered bacteria can be estimate through phase contrast microscopy followed by enumeration using adequate software. The number of non-viable bacteria can be estimate by epifluorescence microscopy followed by enumeration using proper software. The excitation and emission wavelengths are respectively 530 nm and 615 nm(26).

Figure 2.9 shows bacteria adhered to CLs observed under phase contrast microscopy and fluorescence microscopy. Cells stained with PI exhibit red colour.

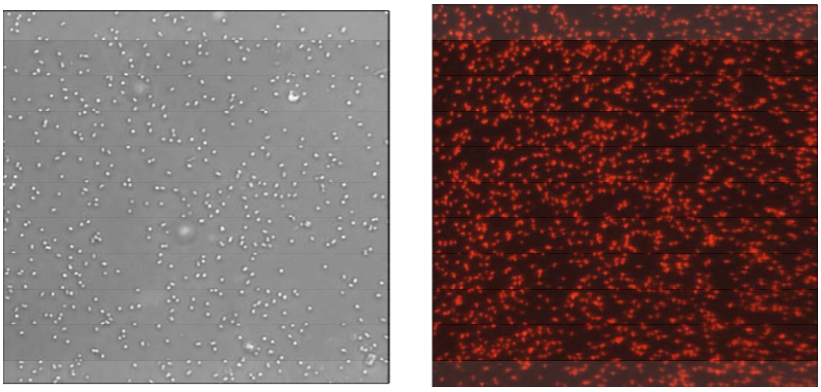

Figure 2.9 Bacterial cells observed under phase contrast microscopy (left) and epifluorescence microscopy (right)

2.7 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a valuable research instrument of biology and medicine. The confocal microscope produces high-resolution optical images even from thick samples. This is possible due to a process known as optical sectioning. With adequate software, images can be stack and form a 3-dimensional image. Like in epifluorescence microscopy, samples have to be previous stained with an adequate fluorocrome(38).

In this Thesis, CLSM was use to visualise adhered bacteria to worn lenses. Prior to image processing, cells were killed by exposing to a MPS, and stained with PI. Several scan where made above the lens surface. Afterwards, the scans were gathering through proper software creating a 3-dimensional image as can be seen in Figure 2.10.

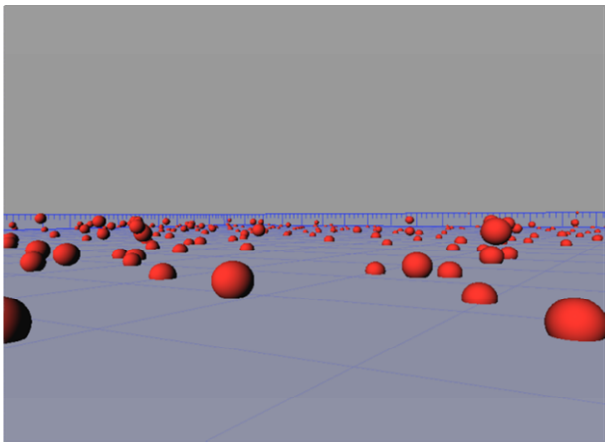

Figure 2.10 CLSM observation of non-viable Staphylococcus epidermidis cells adhered to a worn contact lens.

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Chapter 3 The influence of surface treatment on hydrophobicity, protein adsorption and microbial colonization of silicone hydrogel contact lenses

Published on Contact Lens and Anterior Eye 2007;30:183-188

3.1 Abstract

Purpose: To evaluate the influence of surface treatment of silicone hydrogel contact lenses (CLs) on lens hydrophobicity and "in vivo" protein adsorption and microbial colonization on several worn silicone hydrogel CLs with and without surface treatment. The lenses used in this study were balafilcon A, lotrafilcon A, lotrafilcon B and galyfilcon A. A conventional hydrogel CL (etafilcon A) was also tested.

Methods: Hydrophobicity was determined through contact angle measurement using the advancing type technique on air. The type and quantity of proteins adsorbed were assessed through SDS-PAGE and fluorescence spectroscopy, respectively. Microbial colonization was studied by removing the microbes from the lenses through sonication, and counting the colony-forming units on agar plates.

Results: Regarding hydrophobicity, both surface and non-surface-treated silicone hydrogel CLs were found to be hydrophobic, and the conventional hydrogel CL was found to be hydrophilic. Concerning protein adsorption, different protein profiles were observed on the several lenses tested. Nevertheless, the presence of proteins with the same molecular weight as lysozyme and lactoferrin was common to all lenses, which is probably related to their abundance in tears. In terms of total protein adsorption, silicone hydrogel CLs did not exhibit any differences between themselves. However, the conventional hydrogel etafilcon A adsorbed a larger amount of proteins. Regarding microbial colonization, balafilcon A exhibited the greatest amount of colonising microbes, which can be due to its superior hydrophobicity and higher electron acceptor capacity.

Conclusion: This study suggests that silicone hydrogel lenses adsorb a lower amount of proteins than the conventional hydrogel lenses and that this phenomenon is independent of the presence of surface treatment. Concerning microbial colonization, the surface treated balafilcon A, exhibited a greater propensity, a fact that may compromise the lens wearer's ocular health.

3.2 Introduction

Silicone hydrogel contact lenses (CLs) are the latest kind of soft lenses commercially available. This type of lens provides excellent oxygen transmissibility to the cornea on account of silicone's high oxygen transmissibility when compared to the conventional hydrogel CLs(1,2). Silicone is a hydrophobic polymer, and for this reason most of the silicone-based CLs possesses surface treatment, which decreases the surface hydrophobicity. The reduction in hydrophobicity gives a greater comfort to the wearer and additionally prevents the formation of deposits such as lipids and proteins, as well as microbial colonization(3-6). The reduction in the lens surface hydrophobicity can be obtained through two methods. The first one consists in performing a treatment on the lens surface, which can be achieved in a gas plasma reactive chamber by creating an ultra-thin permanent coating in the cases of lotrafilcon A and lotrafilcon B (Ciba Vision), or by plasma oxidation, transforming the silicone into silicate compounds, in the case of balafilcon A (Bausch & Lomb, Inc.) (Table 3.1). The second method consists in the incorporation of a wetting agent such as Polyvinyl Pyrrolidone (PVP), which is the case of galyfilcon A (Johnson & Johnson Vision Care) (Table 3.1).

Silicone hydrogel CL, despite the advantages they offer due to their high oxygen transmissibility, also present some pitfalls, which are related to the migration of the silicone hydrophobic moieties to the lens surface(7). As mentioned above, less hydrophobic surfaces are advantageous, since they prevent protein adsorption and microbial colonization.

The main goal of this study is to evaluate the influence of surface treatment on CL hydrophobicity, protein adsorption, and microbial colonization, since lenses with surface treatment are expected to exhibit different behaviours to the non-surface-treated lenses. For that, a group of human volunteers wore four types of silicone hydrogel lenses: three surface-treated CLs and one non-surface-treated CL. A conventional hydrogel CL (etafilcon A, Johnson & Johnson Vision Care) was also tested. The implications of protein adsorption and microbial adhesion have already been established through "in vitro" studies(8-13). However, due to the complexity of the ocular environment, it is difficult to mimic all the conditions affecting protein adsorption and microbial adhesion. Therefore, "in vivo" experiments offer potentially more reliable and conclusive results. Moreover, the degree of protein adsorption and microbial colonization in lotrafilcon B has never been reported before.

3.3 Materials and methods

Contact lenses and subjects

The properties of the CLs used in this study are detailed in Table 3.1. The experiments were performed on CLs previously worn by a group of 31 subjects from both sexes. The group of subjects was selected according to several criteria: the subjects had never worn CLs before, were not taking any medication during the trial, did not suffer from any kind of ocular allergy, and had no predisposition to dry eye syndrome. Each type of silicone hydrogel CL was worn by approximately 8 subjects and the conventional hydrogel CL was worn by all the 31 patients, since each individual wore a certain type of silicone hydrogel CL in one eye and the conventional hydrogel CL in the other one. The subjects were instructed to wear their lenses on a daily wear schedule for 12 and 14 h, and to clean and disinfected the CLs overnight with a multipurpose lens care solution (ReNu MultiPlus®, Bausch & Lomb, Inc.). The patients were not informed about the brand or type of lenses they were using. Silicone hydrogel CLs were replaced every 30 days and the conventional hydrogel CL was replaced every 15 days during the 6-month period. At the end of each wearing period, the CLs were collected, placed in sterile saline solutions, and preserved at 4 °C until further analysis. According to the manufacturer's instructions, galyfilcon A should only be worn for a 15-day period. However, in this study, patients wore this type of lens for 30 days, in order to make possible the comparison with the other silicone hydrogel CLs tested. It must be stressed that full ethics approval was obtained, and clinical cover was provided during the trials.

Table 3.1 Contact lens properties

Commercial name	Manufacturer	Material	FDA group	Water content	Surface treatment
Acuvue®	Johnson & Johnson Vision Care	Etafilcon A	IV	58%	No
Acuvue®Advance™	Johnson & Johnson Vision Care	Galyfilcon A	I	47%	No
Purevision™	Bausch & Lomb, Inc.	Balafilcon A	III	36%	Plasma oxidation
Focus® Night & Day™	CIBA Vision	Lotrafilcon A	I	24%	25nm plasma
O_2Optix™	CIBA Vision	Lotrafilcon B	I	33%	25 nm plasma

Culture medium and solutions

The culture medium used in this work was tryptic soy agar (Merck, Germany). This medium was prepared according to the manufacturer's instructions, sterilised and transferred to Petri dishes. A solution of NaCl (0.9 %, Sigma-Aldrich, Germany) was also prepared and sterilized.

Contact angle measurements

CL hydrophobicity was determined through the measurement of the advancing contact angle on air with a measurement apparatus. The measurements were performed at room temperature using three standard liquids of different polarities – Millipore water, formamide and 1-bromonaphtalene. Water and formamide are polar liquids, whereas 1-bromonaphtalene is non-polar. For the measurements, unworn CLs were removed from their original blisters and cut into quarters. Each quarter was flattened onto a microscope slide and the excess water was gently removed with a tissue paper. The measurements were immediately performed, in order to avoid lens dehydration, using the apparatus OCA 20 (DataPhysics, Germany). For each standard liquid, 4 CLs from each type were tested and 4 measurements per lens were performed.

Polyacrylamide gel electrophoresis (SDS-PAGE)

The types of proteins adsorbed onto worn CLs were determined through SDS-PAGE, with a 10% gradient gel. For protein extraction, lenses were cut into quarters and incubated in 100 µl of electrophoresis buffer (1mM EDTA, 10 mM Tris-HCL pH 8.0, 2.5% SDS and 5% ß-mercaptoethanol). After boiling for 15 min, the CLs were centrifuged for 10 minutes at 9000 rpm. The supernatant was removed and applied to a 10% gradient gel. Electrophoresis was performed in the MINI-PROTEAN® 3 Cell (BIO-RAD, USA) using 60 volts. Gels were stained with silver nitrate.

Fluorescence spectroscopy

The total amount of proteins adsorbed onto worn CLs was estimated by fluorescence spectroscopy. When excited at 280 nm, proteins emit fluorescence due to the presence of

fluorophore tryptophan(14). Since there is no standard solution of tear-film proteins, the method was calibrated with different concentrations of bovine serum albumin (BSA) (Sigma-Aldrich, Germany). An excellent linearity between the BSA concentration and fluorescence intensity was found ($Y = 1.895\,x + 6.6409$; $R = 0.9998$). Protein extraction was performed as described by Keith et al.(15). According to the authors' procedures, CLs were soaked in extraction buffer (acetonitrile and 0.2% trifluoroacetic acid (50:50)) and incubated in an orbital shaker (140 rpm) overnight. After this period, lenses were centrifuged for 10 minutes at 9000 rpm. Samples were analysed at an excitation wavelength of 280 nm and an emission of 360 nm (Spectrofluorimeter Jasco FP-6200, Japan). The measurements were performed in a quartz cell (Hellma, Germany).

Colony-forming units

Microbial colonization was evaluated through the enumeration of colony-forming units (CFU). After wear, each CL was aseptically removed from the eye of the volunteer and placed in 1 ml of sterile saline solution (0.9% NaCl). The lenses were sonicated (450W Ultrasonic Processor, Cole & Parmer, USA) for 1 minute at an amplitude of 20 with a 1/8 inch probe. The suspension was spread onto a TSA plate and the CFU were enumerated after 24 and 48 h of incubation at 37 °C. The sonication time and power were optimised in order to detach the maximum number of adhered cells without cell disruption (assessed by plating the final suspension onto TSA plates).

Statistical analysis

The total amounts of proteins adsorbed onto the different types of CLs was compared through one-way ANOVA, and the amounts of microbial cells colonising each CL were compared using the non-parametric Mann-Whitney U test at a 95% confidence level. The statistical analysis was performed using the statistical program SPSS (Statistical Package for the Social Sciences).

3.4 Results

Hydrophobicity

The values of the surface tension components and hydrophobicity are detailed in Table 3.2. From the results obtained it can be concluded that silicone hydrogel CLs are hydrophobic, since ΔG_{sws}^{tot} is <0, and the conventional hydrogel CL is hydrophilic. Balafilcon A surface presents the greatest surface hydrophobicity and can be considered a great electron acceptor (high value of γ^+).

Table 3.2 Apolar component (γLW), electron donnator (γ-) and electron acceptor parameter (γ+) of the lens surface tension and hydrophobicity expressed in mJ/m2

Material	□LW	□+	□-	ΔG_{sws}^{tot}
Etafilcon A	28.34	0.83	7.40	23.14
Galyfilcon A	3.59	1.63	13.50	-36.17
Balafilcon A	5.53	11.50	7.41	-39.40
Lotrafilcon A	39.40	2.16	12.37	-27.10
Lotrafilcon B	35.60	3.00	7.40	-34.24

Surface tension components and lens surface hydrophobicity (ΔG_{sws}^{tot})

Types of proteins adsorbed

The adsorbed proteins' molecular weights are detailed in Table 3.3. According to the results obtained, every lens material exhibited a specific protein profile, galyfilcon A being the lens presenting a greater variety of molecular weights. Proteins with molecular weights similar to those of lactoferrin and lysozyme were the most frequently found in the lenses tested.

Table 3.3 Proteins of different molecular weights adsorbed onto different worn contact lenses (%)

Adsorbed proteins (Mw-kDa)	Etafilcon A	Galyfilcon A	Balafilcon A	Lotrafilcon A	Lotrafilcon B
14.4 (lysozyme)	29.27	5.70	11.76	14.28	0.00
21.0 (lipocalin)	7.31	5.70	0.00	0.00	25.00
80.0 (lactoferrin)	12.20	17.14	11.76	14.28	50.00
37.0 (igA)	4.88	8.60	5.88	0.00	0.00
66.2 (human serum albumin)	4.88	11.40	0.00	14.28	0.00
Other proteins	14.46	51.56	70.60	57.16	25.00

Total amount of proteins

The estimated amounts of proteins adsorbed are present in Table 3.4. It is possible to conclude that all silicone hydrogel CLs exhibit lower levels of protein adsorption (p=0.000) when compared with the conventional hydrogel CL (etafilcon A). Despite the diversity of proteins observed in galyfilcon A using SDS-Page, this lens is not more prone to protein adsorption than the other silicone hydrogel CLs. It must be stressed that the amounts of proteins were estimated using BSA as standard - therefore, the values presented can not be seen as absolute amounts of proteins.

Table 3.4 Fluorescence intensity at 360 nm of the contact lens extract

Etafilcon A	Galyfilcon A	Balafilcon A	Lotrafilcon A	Lotrafilcon A
472.97±196.95*	9.19±5.53	44.64±15.24	20.45±10.85	35.34±31.96

* Statistically different (ANOVA performed with 95% of confidence level)

Microbial colonization

The values of CFU/ml present in Table 3.5 indicate the amount of microbial cells able to grow on TSA plates at 37 °C, and are estimates of the extent of the CL's colonization with viable microbial cells. The results show that balafilcon A seems to be more prone to microbial colonization than the other CLs, exhibiting an average value of 2.32xE6 CFU/ml, which is statistically significant ($p<0.005$). Microbial colinisation in galyfilcon A, despite the absence of surface treatment, was similar to those of lotrafilcon A ($p=0.231$) and lotrafilcon B ($p=0.817$). Concerning the conventional hydrogel CL (etafilcon A), this lens exhibited a greater amount of viable cells than galyfilcon A ($p=0.017$) and lotrafilcon A ($p=0.000$).

Table 3.5 Colony forming units of worn conventional and silicone hydrogel contact lenses

Etafilcon A	Galyfilcon A	Balafilcon A	Lotrafilcon A	Lotrafilcon A
9.30E5±3.49E5●	4.08E5±2.0E5	2.32E6±1.45E6·	2.30E5±1.17E5	8.83E5±7.84E5

* Statistically different from all tested lenses; ● Statistically different from Galyfilcon A and Lotrafilcon A
Mann-Whitney U performed with 95% of confidence level

3.5 Discussion

The present study focuses on the effect of silicone hydrogel CL's surface treatment on hydrophobicity, protein adsorption, and microbial colonization. Due to the important role of hydrophobicity in protein adsorption and microbial colonization, this property was evaluated in a quantitative way, contrarily to most of the studies(17-19).

In the present study, it was found that silicone hydrogel CLs with surface treatments (Table 3.2) and the non-surface-treated CL (galyfilcon A) present similar degrees of hydrophobicity, meaning that the wetting agent and the application of a surface treatment have a similar effect on the lens hydrophobicity. The conventional hydrogel CL is hydrophilic, as reported in other studies(17).

SDS-PAGE analysis (Table 3.3) revealed different protein profiles on the several lens materials. Proteins with molecular weights equivalent to the molecular weights of lactoferrin and lysozyme were the most frequently extracted from all lens materials, probably on account of their abundance in the tear film(20). It seems that 14.4 kDa proteins (probably lysozyme)

preferentially adsorb onto etafilcon A CL. This is probably due to the electrostatic attraction between these two entities, since this protein is positively charged at physiological pH, while etafilcon A is negatively charged. It is well reported that protein adsorption is a phenomenon determined by the lens hydrophobicity and, in lesser extent, by the hydrophobicity of the proteins' amino acid residues. This interaction is also influenced by the electrostatic attraction between the lens surface and proteins with opposite charges(21). Galyfilcon A adsorbed a greater diversity of proteins when compared with all the other lenses, which may be related to the absence of surface treatment and also to its chemical composition. It must be stressed that this type of lens was worn for a longer period than that recommended by the manufacturer (30 days). Nevertheless, neither signs of material degradation nor wearer discomfort were observed (data not shown) during the wearing period.

The fluorescence data (Table 3.4) revealed that etafilcon A adsorbs a greater amount of proteins than silicone hydrogel CLs. This result has been previously reported by several authors(9,12,13), while specifically studying "in vitro" lysozyme adsorption. Hydrophilic polymers such as etafilcon A naturally hinder protein adsorption because the water must be displaced for protein adsorption to occur, and this process is energetically unfavourable. However, the accentuated dehydration of this lens(22) could allow the interaction with other molecules - in this case, proteins. "In vitro" dehydration studies have revealed that conventional hydrogel CLs are more prone to dehydration than silicone hydrogel CLs(23), so, regardless of their hydrophilicity, conventional hydrogel CL adsorb more proteins than the other lenses, even if worn for a shorter period of time (15 days). Concerning galyfilcon A, it was interesting to conclude that the absence of surface treatment did not lead to an increase in the amount of proteins adsorbed, despite the great diversity of proteins adsorbed. It should be considered the possibility that the variety and amount of proteins adsorbed onto the different CLs could have been influenced by the lens material or by the lens care solution used. According to Pritchard et al.(24), the use of ReNu Multiplus has been associated to higher levels of corneal staining when compared to ReNu Multipurpose Solution and Opti-Free Express. The measurement of the corneal staining is a useful tool to determine the impact of a multipurpose system or the impact of their interaction with the lens material on cornea. Despite being out of the aim of the present study, this evaluation was performed. The main finding is that all CL wearers exhibited corneal staining with the exception of one silicone hydrogel lens. As different levels of corneal abrasion may induce different levels in irritation and

protein secretion, we believe that the protein levels and profiles observed for each CL may have been influenced either by the multipurpose lens care solution as by their interaction with lens material.

Regarding CFU's analysis, the CLs presented different levels of microbial colonization (Table 3.5), balafilcon A being the lens more prone to microbial colonization. This fact may be related to its hydrophobicity, as it is already well established that microbial adhesion is determined by lens surface hydrophobicity(25), as well as microorganisms, are usually negatively charged. However, hydrophobic interactions are stronger than repulsive forces and tend to attract bacteria near to the surface, leading to their adhesion(26). Moreover, this lens presents a high electron acceptor capacity, which may possibly enhance adhesion on account of the increase in Lewis acid base interactions with the microbial cells. Henriques et al. reported the results of "in vitro" adhesion study of S. epidermidis and Pseudomonas aeruginosa to several silicone hydrogel CLs and they found that balafilcon A was also more prone to adhesion than the others lenses(25). In that study, the adhesion assays were performed on unworn CLs. However, "in vivo" CLs are subjected to the adsorption of tear-film molecules that may influence lens surface's properties(19) and adhesion propensity. Nevertheless, both "in vivo" and "in vitro" studies led to the same conclusion concerning the high susceptibility to microbial colonization of balafilcon A. One other factor that may bear influence on this lens's susceptibility to microbial colonization is its high roughness. Balafilcon A is rougher than both lotrafilcon A and galyfilcon A due to its surface treatment, which presents silicate islands(27). The surface treatment of this lens based on plasma oxidation is different from those of lotrafilcon A and lotrafilcon B, which are made through plasma coating, resulting in a smoother surface with a high refractive index. It was previously demonstrated that microbial adhesion may increase by enhancing roughness(28,29), and for this reason we believe that this surface property may have played an important role in microbial colonization.

Summarising, all silicone hydrogel CLs are hydrophobic and adsorb smaller amounts of proteins than the conventional hydrogel CL, regardless of the presence of surface treatment. Nonetheless, all lenses exhibited the presence of different protein profiles. The possibility of the multipurpose lens care solution or the lens material having an impact on this result should not be excluded, since shifts on ocular irritation may induce different protein secretion. The surface-treated balafilcon A seems to be more prone to microbial colonization, which might be relate to its

greater hydrophobicity and higher electron acceptor capacity. In terms of clinical implications, there are apparently no differences between surface-treated and untreated CLs, except for balafilcon A, since it exhibited a higher amount of colonising microbes.

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Chapter 4 Bacterial adhesion to worn silicone hydrogel contact lenses

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4.1 Abstract

Purpose: The aim of this study was to, firstly, investigate whether silicone-hydrogel contact lenses (CLs) are more or less susceptible to bacterial adhesion than conventional ones and, secondly, assess the influence of lens wear in the extent of "in vitro" bacterial adhesion. Four silicone-hydrogel CLs (galyfilcon A, balafilcon A, lotrafilcon A and lotrafilcon B) and one conventional hydrogel (etafilcon A) CL were tested.

Methods: Bacterial adhesion experiments were performed on unworn and worn CL using the strain Staphylococcus epidermidis 9142. Worn lenses were obtained from a group of 31 subjects fitted with a silicone-hydrogel CL in one eye and a conventional hydrogel CL as contralateral pair. These lenses were used on a daily basis in combination with a multipurpose lens care solution (MPS). Adhesion assays were carried out in a parallel plate flow chamber, followed by image analysis. Hydrophobicity, roughness and topography of the lenses surfaces were assessed through contact angle measurements and atomic force microscopy (AFM).

Results: Unworn conventional and silicone-hydrogel CLs were equally susceptible to bacterial adhesion of S. epidermidis. Conversely, worn conventional hydrogel (etafilcon A) were more prone to bacterial adhesion than worn silicone-hydrogel materials, which exhibited similar adhesion extents among them. The results also showed that the lens surface properties such as hydrophobicity, roughness and surface topography changed during wear. The alteration of surface hydrophobicity of silicone and conventional hydrogel CLs during wear had a great impact on their bacterial adhesion susceptibility. Accordingly, balafilcon A becomes significantly less hydrophobic and less prone to bacterial adhesion after lens wear, whereas etafilcon A becomes more hydrophobic and also more susceptible to bacterial adhesion ($p<0.05$).

4.2 Introduction

Microbial keratitis is a rare but serious ocular infection, which can lead to permanent vision loss. Extended wear(1), ocular trauma(2;3), hypoxia(3) and lack of compliance(3), among other factors, are predisposing issues for its occurrence.

With the introduction of silicone hydrogel CLs several improvements were achieved. Overnight oedema was found to be similar to those found on non-CL wearers and much smaller than observed on conventional hydrogel CL wearers as a result of their high oxygen transmissibility(4). It was also found that the incidence of microbial keratitis was five times smaller than with conventional hydrogel lenses, for extended wearing periods(5) This lower incidence seems to be associated with their higher oxygen transmissibility. However, some authors have also suggested that it could be related with less bacterial binding to the lens surface as well(6).

The first aim of this study was to verify whether silicone-hydrogel CL bind fewer bacteria than HEMA-based conventional ones, which might serve to reduce the incidence of microbial keratitis. The second aim involved assessing the influence of lens wear in the extent of adhesion. For this purpose, microbial adhesion assays onto four unworn and worn silicone-hydrogel CLs and one conventional hydrogel CL were performed. It is well established that an increase in surface hydrophobicity or roughness can lead to an increase of bacterial adhesion susceptibility(7;8). Therefore, these properties were also assessed in this work. Microbial adhesion studies to silicone-hydrogel have been previously performed by several authors(9-11). Nevertheless, the present work covers a wider range of CL materials with different surface treatments, some of them never evaluated before with respect to roughness and bacterial adhesion(9-11).

4.3 Materials and methods

Contact lenses

The properties of the CL used in this study are detailed in Table 4.1. Four silicone (galyfilcon A, balafilcon A, lotrafilcon A and lotrafilcon B) and one conventional hydrogel (etafilcon A) CL were tested in their unworn and worn states. The silicone-hydrogel lenses are surface treated by gas

plasma during manufacturing, except galyfilcon that has an incorporated wetting agent (Hydraclear®).

Table 4.1 Contact lens properties

Commercial name	Manufacturer	Material	FDA group	Water content	Surface treatment
Acuvue®	Johnson & Johnson Vision Care	Etafilcon A	IV	58%	No
Acuvue®Advance™	Johnson & Johnson Vision Care	Galyfilcon A	I	47%	No
Purevision™	Bausch & Lomb, Inc.	Balafilcon A	III	36%	Plasma oxidation
Focus® Night&Day™	CIBA Vision	Lotrafilcon A	I	24%	25 nm plasma coating with high refractive index
O_2Optix™	CIBA Vision	Lotrafilcon B	I	33%	25 nm plasma coating with high refractive index

Experimental design

Thirty one subjects from both sexes were enrolled in this study, excluding any lost to follow-up. The average age of the individuals was 23.6±5.5 years and they were chosen according to the following parameters: they had never worn CL before (neophytes), they were not taking any medications during the trial, they did not suffer from any kind of ocular allergy and they had no tendency for dry eye syndrome.

The subjects were randomly divided into 4 groups. Each group wore a specific type of silicone-hydrogel lens and a conventional one as contralateral pair in a single masked fashion way. The silicone-hydrogel CL were monthly replaced and the conventional hydrogel every 15 days, for 6 months. Apart from physiological changes, no complications occurred during the trial. For the adhesion studies 8 galyfilcon A, balafilcon A, lotrafilcon A and etafilcon A and 7 lotrafilcon B CLs were assayed. CLs were worn on a daily wear schedule of between 12 and 14 hours. The

subjects were instructed to remove the lenses at the end of this period and to soak them overnight in a multipurpose solution (ReNu MultiPlus®, Bausch & Lomb, Inc. polyhexanide 0.0001%, hydranate 0.03% and poloxamine 1%).

At the end of the wearing period, each lens was aseptically removed from the eye and placed in a sterile vial containing sterile saline solution (0.9% NaCl). Vials were labelled with a code and details of the lens material. The CL were stored at 4 °C for no longer than 5 days until analysis.

The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of this study.

According to the manufacturer's recommendation, galyfilcon A and lotrafilcon B should be replace every two weeks. However, in this study they were worn for 30 days in order to establish a comparison with the other lenses which are recommended for use up to 30 days. It must be stressed that no complications resulted from the extension of the wearing period.

Bacterial Strain and Growth Conditions

The bacterium S. epidermidis 9142 was used in the present study. This species is a Gram-positive pathogen commonly involved in extended wear microbial keratitis(12) and was therefore chosen for the in vitro adhesion assays. S. epidermidis 9142 is a well known producer of the polysaccharide that promotes coagulase negative staphylococci adherence and biofilm formation, which is referred to as either polysaccharide intercellular adhesin (PIA) or by its chemical composition, poly-N-acetyl glucosamine (PNAG). This strain was kindly provided by Dr. Gerald B. Pier, Harvard Medical School, Boston, USA and its adhesion and biofilm formation capabilities have been characterized in previous studies(13;14).

A 4 °C culture stock was inoculated into an Erlenmeyer flask containing 10 ml of tryptic soy broth (TSB, Merck, Germany) and incubated at 37° C for 24 h. Following this period, 1 ml of the culture suspension was transferred to a second Erlenmeyer flask containing 30 ml of TSB and incubated at 37 °C for 18 h in order to obtain a mid-exponential growth culture. Cells were harvested by centrifugation (15 min, 4000 rpm) and washed twice with ultrapure water and

finally, the cells were ressuspended in phosphate buffer saline (PBS, 8g l^{-1} NaCl 0.2 g l^{-1} KCl 0.2 g l^{-1} KH_2PO_4 1.15 g l^{-1} Na_2HPO_4 pH 7.4).

Adhesion assays and image analysis

The adhesion assays were performed with unworn and worn CLs in a parallel plate flow chamber. Two opposite edges of each CLs were cut to flatten the surface and the lens mounted on the bottom plate of the flow chamber. All the tubes and the flow chamber were filled with a PBS solution (special care was taken in order to remove all air bubbles from the system) which circulated through the system for 15 minutes. A pulse-free flow was established by hydrostatic pressure, and the suspension was fed using a peristaltic roller pump. Afterwards, the flow was switched to a bacterial suspension (6E10CFU/ml) that circulated throughout the system at room temperature for 120 minutes in laminar regime, at a flow rate of 2E-4 ml/s. After this period, fresh PBS was circulated throughout the system to remove the non-adhered or loosely adhered cells. Cell quantification was performed using a phase contrast microscope (Carl Zeiss, Germany) connected to a CCD video camera (Carl Zeiss, Germany) that acquires images at a magnification of 1622× with a resolution of 1300×1030 pixels. Twenty images were randomly taken from each lens. In order to eliminate image interference, the background was captured and subtracted from the original image. Cells were enumerated using the Sigma Scan Pro program and, with the magnification used, 1 cm^2 was equivalent to 5110 captured images. These measurements were repeated 7 or 8 times for each lens material.

Hydrophobicity

CL hydrophobicity has been assessed in other studies. Some authors(18) have estimated lens surface hydrophobicity using the sessile drop technique while, more recently, other authors(19) have made use of the advancing type technique. In the present study, water contact angle measurements were performed with Millipore ultrapure water using the advancing type technique and an apparatus OCA 20 (Dataphysics, Germany). Each lens was cut into four pieces and placed on a microscope slide. The excess moisture was removed by gentle blotting with

absorbent paper. The measurements were carried out on 4 CL from each type and repeated 4 times on each lens piece.

Roughness

Surface roughness was assessed through atomic force microscopy (AFM) using the Tapping® Mode (PicoScan Controller, Molecular Imaging, USA). Measurements were performed in a liquid cell containing PBS and using a V-shaped Si_3N_4 cantilever with a constant spring of 0.58 N/m. AFM assays were performed on unworn and worn CL. Roughness was expressed as average roughness (Ra) of the surface.

The analysed lenses were balafilcon A, lotrafilcon B and galyfilcon A. Balafilcon A and lotrafilcon B exhibit different types of surface treatment and galyfilcon A incorporates wetting agents as an alternative to surface treatment. The analysed area for balafilcon A was 100 μm^2 due to the fact that some surface details can only be observed with this frame(15), whereas galyfilcon A and lotrafilcon B were analysed within a 25μm^2 frame. The measurements were repeated 3 or 4 times per CL material.

Data analysis

Hydrophobicity data were evaluated by the one-way ANOVA test. The extent of bacterial adhesion and lens roughness was statistically evaluated through the non-parametric Mann-Whitney U test because data were not normally distributed. All tests were performed with a 95% confidence level using the statistical program SPSS (Statistical Package for the Social Sciences). Two distinct comparisons were made: between lens materials and within the same material before and after lens wear.

4.4 Results

Bacterial adhesion of Staphylococcus epidermidis

The number of adhered cell to unworn and worn CL is presented in Figure4.1. No statistical differences were found among unworn CL ($p>0.05$). Comparing worn CL, silicone-hydrogel materials showed to be less prone to bacterial adhesion than the conventional hydrogel lens ($p<0.05$). Lotrafilcon B was the only exception, exhibiting a value ($p=0.055$) that is almost statistically significant. After wear, balafilcon A became less susceptible to bacterial adhesion, but no statistical differences were found among the other silicone-hydrogel materials. Conversely, the conventional hydrogel became more prone to bacterial adhesion.

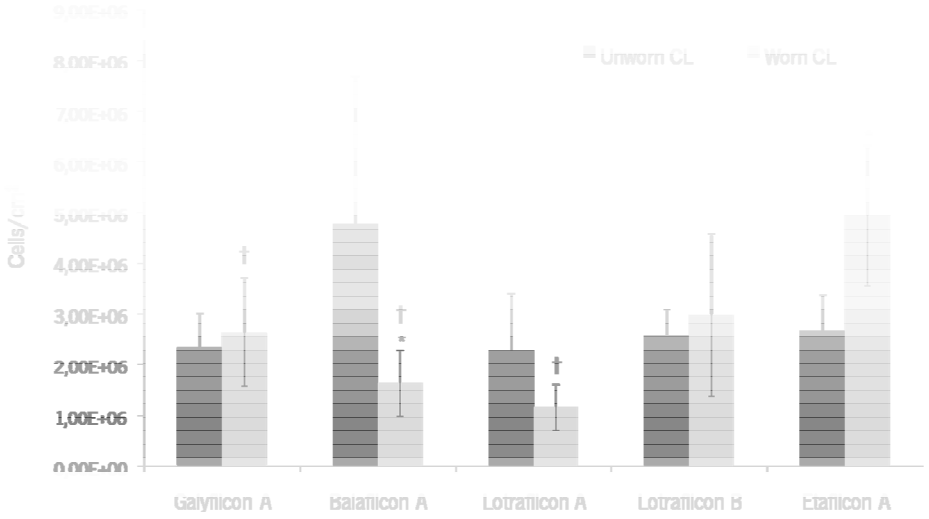

Figure4.1 Number of adhered cells to unworn and worn contact lenses

*Statistically different compared to unworn CL; †Statistically different from worn etafilcon A; Error bars represent standard deviations (n=7) (Mann-Whitney U Test)

Hydrophobicity

Inspection of Figure 4.2 reveals that unworn silicone-hydrogel CL have water contact angles greater than 50° (the breakpoint between hydrophilicity and hydrophobicity) and are relatively more hydrophobic than the conventional hydrogel. Balafilcon A and galyfilcon showed to be the most hydrophobic ones ($p<0.05$). After lens wear, silicone-hydrogel CLs became less hydrophobic ($p<0.05$) and balafilcon A remained relatively more hydrophobic than the other materials ($p<0.05$). Conversely, the conventional hydrogel etafilcon A, after being worn, displayed an increase in the water contact angle values from 44.8°±2.4 to 82.3°±11.6.

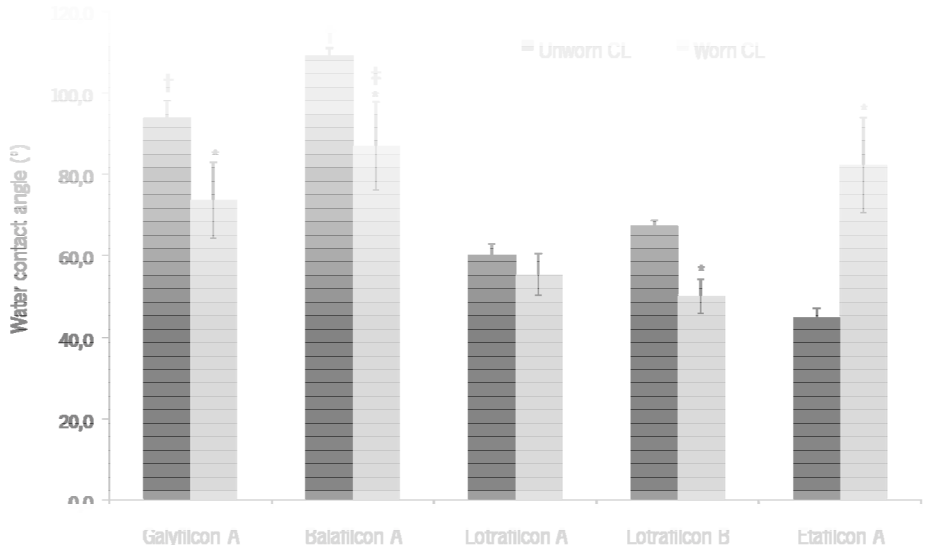

Figure 4.2 Contact angle (advancing) measurements performed with water to unworn and worn CL

*Statistically different from unworn lenses; †Statistically different from lotrafilcon A, lotrafilcon B and unworn etafilcon A‡ Statistically different from worn galyfilcon A, lotrafilcon A and lotrafilcon B; Error bars represent standard deviations (n=16)
(One-way ANOVA performed with 95% of confidence level)

Roughness and topography

The mean roughness (R_a) values are detailed in Table 4.2. Analysis of unworn CL revealed that galyfilcon A seems to exhibit the smoothest surface (p=0.05). After lens wear, galyfilcon A and balafilcon A roughness increased and galyfilcon A became significantly rougher than lotrafilcon B (p=0.05). As can be observed in Figure 4.3, balafilcon A shows the presence of macropores, while lotrafilcon B (Figure 4.4) exhibits grooves. These structures disappeared after lens wear in both cases. Figure 4.5 confirms that galyfilcon A has a very smooth and uniform surface. Although galyfilcon A become rougher after lens wear, maintained the same smooth appearance.

Table 4.2 Mean roughness of unworn and worn CL (nm)

	Galyfilcon A (5×5 µm)	**Balafilcon A (10×10 µm)**	**Lotrafilcon B (5×5 µm)**
Unworn	2.32 ± 0.085†	7.04±0.66	4.51±2.83
Worn	30.09±11.27*‡	17.63±14.78*	4.96±4.12

*Statistically different from unworn CLs; †Statistically different from unworn balafilcon A and lotrafilcon B; ‡ Statistically different from worn lotrafilcon B; (Mann-whitney with 95% confidence level) (n=3)

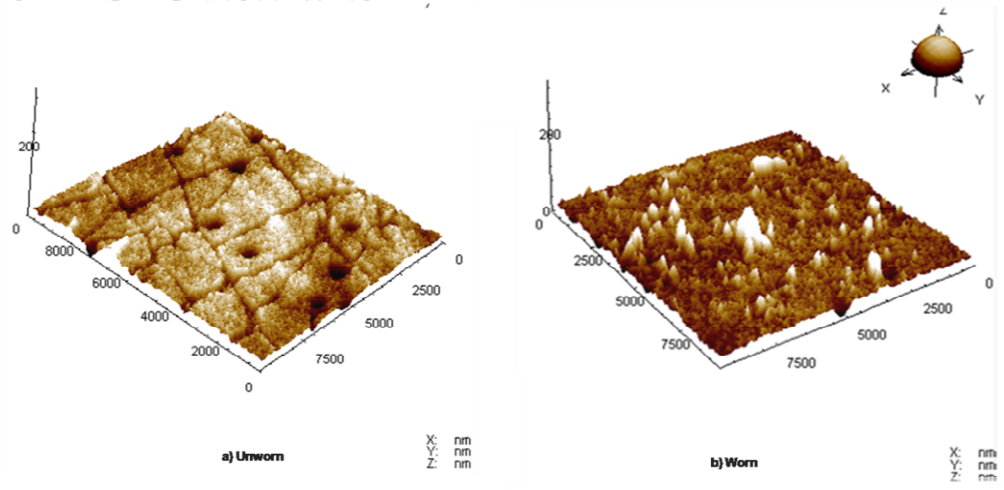

Figure 4.3 Topography of balafilcon A before (a) and after wear (b)

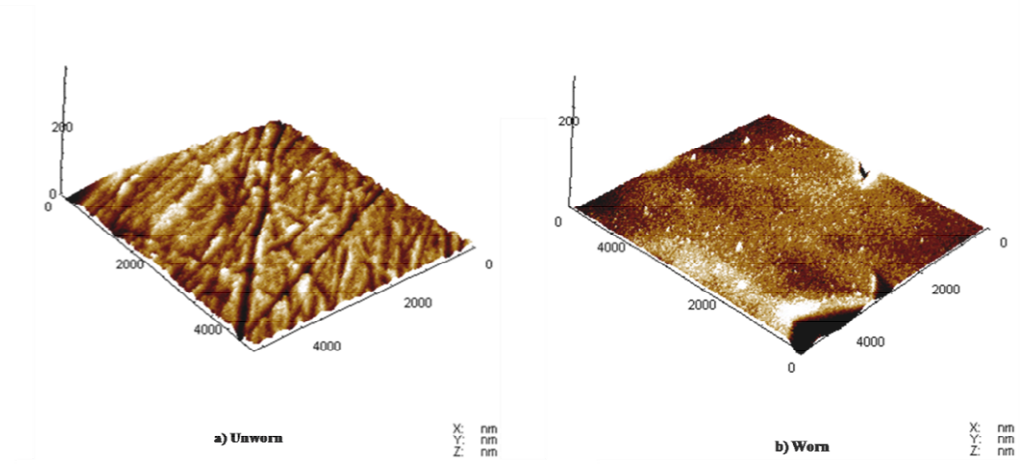

Figure 4.4 Topography of lotrafilcon B before (a) and after wear (b)

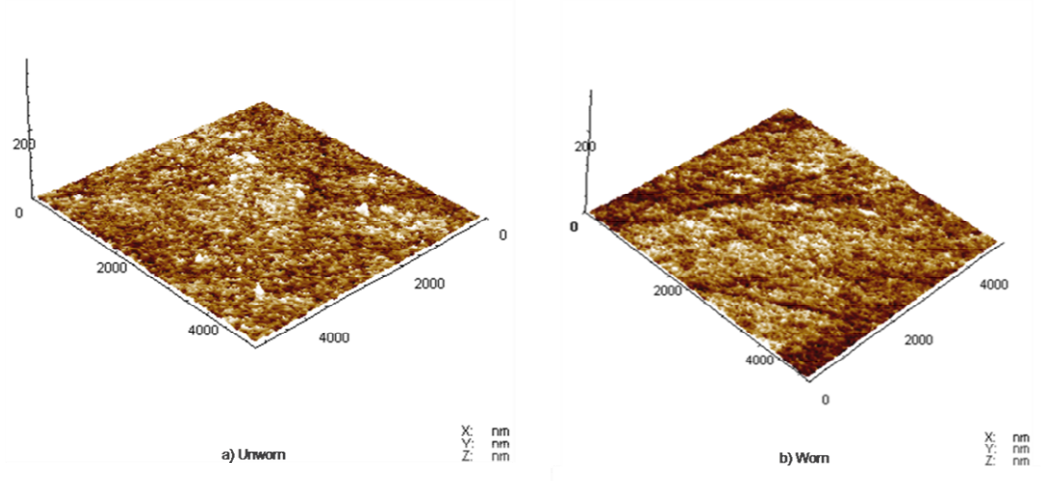

Figure 4.5 Topography of galyfilcon A before (a) and after wear (b)

4.5 Discussion

The present study focuses on bacterial adhesion to four silicone hydrogel CLs and one conventional hydrogel lens. The main purpose was to compare the propensity of silicone-hydrogel CLs for bacterial adhesion and to verify if these materials are more or less prone to bacterial adhesion than a conventional hydrogel material. A similar comparative study was previously performed; however, in that case the adhesion assays were performed only on unworn CL and using static adhesion methods(15). Therefore, in this work the "in vitro" adherence experiments were carried out on both worn and unworn materials in order to address the influence of wear on the susceptibility for adhesion. Moreover, in the present work, adhesion was assessed by a dynamic adhesion method, using a parallel plate flow chamber as this is a more realistic methodology and virtually induces less variability(16).

The results obtained in the present work revealed that worn silicone hydrogel CL exhibited similar levels of adhered bacteria and are thus equally prone to microbial colonization. However, when compared with worn etafilcon A, these materials generally bind fewer cells (Figure4.1). This fact supports the hypothesis that silicone-hydrogels potentially induce fewer cases of microbial keratitis on account of their weaker susceptibility to S. epidermidis binding, although this is only true for worn CLs. In fact, the susceptibility for adhesion of unworn silicone and conventional hydrogels is the same (Figure4.1) or even higher for silicone-hydrogel, as reported in a previous study(15). However, it shall be considered that as soon as a CL is placed in the eye it becomes rapidly conditioned by the tear film molecules that greatly influence bacterial colonization.

This study demonstrates that wear has a great impact on bacterial adhesion to CLs with special relevance for balafilcon A and etafilcon A adhesion susceptibilities. In fact, the extent of S. epidermidis adhesion to the silicone-hydrogel decreased when worn. This evidence corroborates the results of previous studies, in which P. aeruginosa adhered in a slightly lower degree (not statistically significant) to worn balafilcon A(9), and P. aeruginosa adhered in a lesser extent to worn lotrafilcon A and balafilcon A materials(10). The fact that worn conventional hydrogel is more prone to S. epidermidis adhesion than unworn is contradicted by a previous study in which worn etafilcon A lenses became less susceptible to P. aeruginosa adhesion(17). Some authors have suggested that CL wear increases susceptibility to bacterial adhesion while others show the

reverse(9-11). The use of different strains, methods, wear schedules, lens materials, maintenance solutions and subjects are certainly the underlying reasons for this disparity.

The use of only one strain and one conventional hydrogel CL could be considered a limitation of this work since, and as mentioned before, the strain involved and the lens material can influence the adhesion extent. Therefore, the results obtained could not hold true for all bacteria and all conventional hydrogels. However, it has already been demonstrated that S. epidermidis is one of the most frequent pathogens colonising CLs(10,17) and, together with P. aeruginosa, is one of the most common bacteria involved in microbial keratitis(18). Also, etafilcon A is one of the most popular conventional hydrogel CL and thus it can be considered representative in order to establish a comparison with silicone-hydrogel materials.

It is generally recognized that silicone-hydrogel CL are relatively more hydrophobic than conventional hydrogel lenses(18) (Figure 4.2) and therefore potentially bind more microbes(7). Previous reports also found that balafilcon A and galyfilcon A have the most hydrophobic surfaces(19;20). However, this study reveals that their hydrophobicity significantly decreases after wear, which may explain their lower adhesion capability. The adsorption of proteins and lipids from the tear film may have induced these changes, since these molecules are amphiphilic and are therefore capable of modifying the surface hydrophobicity. For silicone-hydrogels, the orientation of the hydrophilic regions of adsorbed molecules to the outer environment may render the lens surface less hydrophobic. Conversely, the conventional hydrogel etafilcon A, which is hydrophilic, must have adsorbed the proteins and lipids through their hydrophilic region thus orienting their hydrophobic region to the outer environment. As the surface becomes less hydrophilic, it attracts more lipids which in turn increase surface hydrophobicity(21).

It is generally accepted that roughness may boost microbial adhesion, but that was not observed in this study. In fact, although surface roughness has increased in most of the worn lens, the adherence capability did not follow the same tendency. It is possible that the decrease of hydrophobicity might have neutralised the roughness impact. Additionally, surface topography may have played an important role in this process. For example, in the case of balafilcon, despite the increase in roughness of this worn lens, the surface crevices and macroporous disappeared after wear and this may have contributed to a decrease in adherence. Still, AFM analysis was a useful tool to study the impact of wear on lens roughness and topography. This aspect is

discussed elsewhere and related with lens clinical performances(22). Nonetheless, in this study we would like to stress the increase in roughness of galyfilcon A and balafilcon A after lens wear. Since these lenses possess polyvinyl pyrrolidone (PVP), a liposoluble monomer, it is possible that lipids from the tear film are responsible for such augmentation. However, an in vitro study assessing lipid deposition (data not shown) revealed that lipid deposition on galyfilcon A was not greater than that observed for other silicone-hydrogel lenses. The total quantity of adsorbed proteins onto this worn lens is similar to that observed in other silicone-hydrogel lenses(20), which also excludes the hypothesis of higher protein adsorption. Therefore, we address the possibility that PVP could have been lost during wear, resulting in such increment in roughness.

To sum up, this study demonstrates that worn silicone-hydrogel galyfilcon A, balafilcon A, lotrafilcon A and lotrafilcon B are equally prone to microbial colonization and generally less susceptible than the conventional hydrogel, suggesting that wearing this type of material does not increase the risk of developing ocular events associated to S. epidermidis colonization.

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Chapter 5 Fluorescence studies of lipids oxidation on conventional and silicone hydrogel contact lenses

5.1 Abstract

Purpose: To verify if oxidation occurs on lipids adsorbed to contact lenses (CLs). To assess the influence of the lens chemical composition on the quantity of adsorbed oxidized lipids was another goal of this study.

Methods: Experiments were carried out under "in vitro" conditions. CLs were incubated in an artificial tear solution during 28 days. The fluorescence intensity of the fluorophore tryptophan and conjugated Schiff-bases were measure in a weekly basis through fluorescence spectroscopy. Such measurements were able to provide an estimation of the quantity of oxidized lipids adsorbed onto the CL material.

Results: The fluorescence intensity of tryptophan and conjugated Schiff bases suggests that the lipids oxidation occurred. The CLs containing N-vinyl pyrrolidone (NVP), vifilcon A and balafilcon A, appear to display a higher amount of oxidized lipids than the others do. This fact probably results from greater amounts of adsorbed lipids once NVP is a liposoluble monomer. Moreover, after 14 days of incubation, the fluorescence intensity of conjugated Schiff-bases dramatically increases up to the end of the incubation period.

Conclusion: CLs incorporating NVP appears to be more susceptible to the presence of adsorbed oxidised lipids. Based on the kinetics of oxidized lipids adsorbed, it is strongly recommended to shorten the length of wear in order to prevent associated ocular complications.

5.2 Introduction

CLs readily adsorb proteins and lipids from the tear film as soon as they are placed in the eye. Previous studies with conventional hydrogel CLs demonstrated that anionic lenses are more disposed to protein adsorption due to the electrostatic attraction established between their negatively charged surface and the positively charged lysozyme, while lipids adsorption tend to occur in greater extent in materials containing NVP since this monomer is lipid soluble (1-4). With respect to silicone hydrogel CLs, protein adsorption is generally smaller on these materials than in conventional hydrogel lenses(5;6). Lipids adsorption take place in greater extent in balafilcon A and lotrafilcon B materials than in the conventional hydrogel, etafilcon A(6).

Lipids are molecules susceptible to oxidizing processes, which can be triggered by environmental factors, or by the presence of peroxides and free radicals present in the eye(7). The eye produce oxidizing agents(7), and for this reason it is believed that adsorbed lipids onto CLs might oxidize. The action of oxidized lipids has been associated with the occurrence of vascular calcification and osteoporosis(8) and with age related macular degeneration(9). For this reason, it is believed that oxidized lipids may be harmful to the lens wearer.

One of the methods employed to estimate the presence of oxidized lipids is the measurement of the intrinsic fluorescence of conjugated-Schiff-bases(10). These molecules result from the cross-link between lipids metabolites and amino acid residues. Amino acid residues exhibit pro-oxidative activity and might react with primary and secondary products of lipids oxidation such as peroxides or aldehydes. When they do, the amino acid residue cross-links with products of the oxidized specie producing conjugated Schiff-bases(11-14). It is assumed that when the fluorescence intensity of conjugated bases increases, lipids oxidation is in process.

As previously addressed, some lenses are more prone to lipid adsorption; thus, it is suspected that might encompass higher levels of oxidation. The aim of this study is to verify if oxidation occurs on adsorbed lipids to CLs, and if it does happen, which materials are more susceptible. For that, the fluorescence intensity of conjugated Schiff-bases and tryptophan will be determined.

5.3 Materials and methods

Contact lenses

The CLs used in this study are detailed in Table 5.1. Five-silicone hydrogel and two conventional hydrogel CLs were tested. The recommended wearing period of etafilcon A is 1 day; galyfilcon A, senofilcon A and lotrafilcon B up to 2 weeks, and lotrafilcon A, balafilcon A and vifilcon A up to 30 days. Protein analysis was not performed on galyfilcon A, senofilcon A and etafilcon A materials, since these lenses present UV blocking.

Table 5.1 Contact lens properties

Brand	CL material	Manufacturer	FDA Group	Water content (%)	Main monomers
1-Day Acuvue®	Etafilcon A	Johnson&Johnson Vision Care	IV	58	HEMA +MA
Focus® Monthly	Vifilvon A	CIBA Vision	IV	55	HEMA+MA+MMA
Acuvue® Advance™ with Hydraclear™	Galyfilcon A	Johnson&Johnson Vision Care	I	47	mPDMS +DMA+HEMA+siloxane macromer+PVP+EGDMA
Acuvue® Oasys™ with Hydraclear™ Plus	Senofilcon A	Johnson&Johnson Vision Care	I	38	mPDMS +DMA+HEMA+siloxane macromer+PVP+TEGDMA
Focus Night and Day®	Lotrafilcon A	CIBA Vision	I	24	DMA+TRIS+siloxane monomer
O_2Optix™	Lotrafilcon B	CIBA Vision	I	38	DMA+TRIS+siloxane monomer
Purevision®	Balafilcon A	Bausch&Lomb	III	36	NVP+TPVC+NCVE+PBVC

HEMA(2-hydroxyethyl methacrylate); MA (methacrylic acid); mPDMS (monofuncional polydimethylmethacrylate); NVP (N-vinyl pirrolidone); EGDMA (ethyleneglycol dimethacrylate); TEGDMA (tetra ethyleneglycol dimethacrylate); TPVC (tris(trimethylsiloxysilyl) propylvinyl carbamate); NCVE (N-carboxyvinyl ester); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); PVP (poly-vinyl pirrolidone); DMA (N,N-dimethylacrylamide)

Artificial tear solution preparation

Lipids adsorption is highly patient depended(1;2). in order to avoid subject variability, an artificial tear solution was prepared to induce protein and lipid adsorption to CLs. This formulation was previously employed in another study(15), and their components are detailed in Table 5.2.

To prepare the artificial tear solution, lipids were firstly dissolved in one volume of foetal calf serum (Sigma-Aldrich) and the solution gently mixed using a magnetic stirrer. After dissolving all the lipids, proteins were added and the solution stirred again. Two volumes of phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 potassium chloride, 0.137 M sodium chloride, pH 7.4 at 25 ° C) were transfer to the previous solution. The artificial tear solution was freeze into aliquots and kept at -20 °C.

Table 5.2 Components of the artificial tear solution

Artificial tear solution	Product reference
Lysozyme (0.002 g/ml)	L6876, Sigma
Lactoferrin (0.001 g/ml)	L4765, Sigma
Albumin (0.0002 g/ml)	A1653, Sigma
Triolein (0.000016 g/ml)	44895-U, Supelco
Linalyl acetate (0.00002 ml/ml)	45980, Fluka
Cholesterol (0.0000016 g/ml)	C-8667, Sigma
Mucin (0.001 g/ml)	M3895, Sigma
Undecyclenic acid (0.00000316 ml/ml)	W324701, Aldrich
Cholesteryl linoleate (0.000024 g/ml)	C0289, Sigma

Adsorption assays

To induce protein and lipid adsorption, each lens was inserted into a well of a 24-tissue culture plate containing 1 ml of artificial tear solution. The plate was incubated at 140 rpm at room temperature for 28 days. The fluorescence intensity was measured after 4 hours of incubation

and then in a weekly basis. The solution was replaced according to this schedule as well. Four CLs from each material were assayed.

Oxidised lipids onto contact lenses

The presence and quantity of oxidised lipids adsorbed onto CLs was estimated through fluorescence spectroscopy (Hitachi F-4500, Japan) using conjugated Schiff-bases and tryptophan as probes. When excited at 360 nm, conjugated Schiff-bases exhibited a maximum of fluorescence intensity at 440 nm whereas tryptophan fluorescence intensity was the highest at 340 nm when excited at 280 nm. For these measurements, each CL was removed from the well and rinsed with ultrapure water in order to eliminate non-bonded or loosely bond proteins or lipids. Afterwards, the lens was placed in the cuvette, also containing ultrapure water and inserted in the apparatus for the measurements. The fluorescence intensity from tryptophan and conjugated Schiff-bases were recorded subsequently for each lens.

Statistical analysis

Data obtain through the fluorescence measurements was statistically analysed. A comparison was established between the values obtained after 4 hours, 14 day and 28 day. The non-parametric Mann-Whitney U test with 95% confidence level was used.

5.4 Results

Fluorescence intensity of conjugated Schiff bases

Inspection of Figure 5.1 reveals that balafilcon A and vifilcon A exhibits the higher fluorescence intensity values, either after 4 hours, 2 or 4 weeks ($p<0.05$). A remarkable deflection was observed on day 14 for both balafilcon A and vifilcon A.

Figure 5.1 Fluorescence levels observed for lenses incubated with artificial tear solution (λ_{ex}=360/λ_{em}=440)

Fluorescence intensity of tryptophan

Examination of Figure5.2 reveals that the fluorescence intensity on lotrafilcon A and vifilcon A is greater than measured in balafilcon A and lotrafilcon B ($p<0.05$).

Figure5.2 Fluorescence levels observed for lenses incubated with the artificial tear solution (λ_{ex}=280/λ_{em}=340)

5.5 Discussion

In this study, the presence of oxidized lipids adsorbed onto CLs was estimated through fluorescence spectroscopy. To promote lipids oxidation, silicone hydrogel and conventional hydrogel CLs were soaked over 28 days in an artificial tear solution. The reaction was induced by photo and atmospheric oxidation. It is acknowledge that the ocular environment has other type of oxidizing agents, (free radicals) and antioxidants as well(7); however "in vitro" experiments allow to avoid the variability induced by the lens wearer.

The fluorescence intensity of conjugated Schiff-bases was far superior on vifilcon A and balafilcon A lenses when compared with the other materials (Figure 5.1). Although these materials possess different chemical compositions, they both incorporate NVP, a monomer known by their liposolubility. As demonstrated in previous studies, the presence of this monomer increase the adsorption of lipids to CLs(1-4), and such propensity might have contributed for greater oxidation levels. On day 14, the fluorescence values suffered a deflection and dramatically increased up to the 28th day. The other lenses revealed very low values of fluorescence intensity suggesting that oxidation was little.

On vifilcon A, tryptophan fluorescence intensity remained constant during the first three weeks increasing on the last week, whereas on balafilcon A it remained relatively low and constant during the 4 weeks (Figure5.2). Based on the results, it seems that tryptophan fluorescence have been quenched by the lipids metabolites. Otherwise, these materials would progressively emit higher values of fluorescence intensity overtime.

Lotrafilcon A should adsorb little amounts of proteins since it has a non-ionic character and proteins are negatively charged. However, the results show (Figure5.2) that the fluorescence intensity significantly increases on the second week of incubation. Several hypotheses can be addressed in order to explain this result. The type, quantity, conformation and orientation of adsorbed proteins is modulated by the lens chemistry(16;17). This material might have adsorbed proteins preferentially containing tryptophan, or in alternative exposed tryptophan molecules to the surrounding environment more than the other materials. Other hypothesis is that tyrosine emission might have transferred energy to tryptophan augmenting the fluorescence intensity of this fluorophore. In this experiments ultrapure water was used as solvent (pH 7.0) and at this pH the proteins should be on normal conformation. On normal conformation, tyrosine and

tryptophan present an overlap between the emission spectra of tyrosine and the absorption spectra of tryptophan, which might triggered direct energy transfer from tyrosine to tryptophan.

Summarizing, CLs incorporating NVP appears to be more susceptible to the presence of adsorbed oxidised lipids. The presence of such lipids on balafilcon A and vifilcon A dramatically increases after 14 days of incubation in an artificial tear solution. This result suggests that the length of wear should be shortened in order to prevent associated ocular complications.

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Chapter 6 The Effect of octylglucoside and sodium cholate in Staphylococcus epidermidis and Pseudomonas aeruginosa adhesion to soft contact lenses

Published on Optometry and Vision Sciences 2007;84:429-434

6.1 Abstract

Purpose: In this study the effect of the natural surfactants octylglucoside and sodium cholate in inhibiting Staphylococcus epidermidis and Pseudomonas aeruginosa adhesion to conventional and silicone-hydrogel contact lenses (CLs) was assessed. Hydrophobicity was also evaluated on conditioned and non-conditioned CLs.

Methods: The inhibiting effect of the tested surfactants was determined through "in vitro" adhesion studies to conditioned and non-conditioned CLs followed by image acquisition and cell enumeration. Hydrophobicity was evaluated through contact angle measurements using the advancing type technique on air.

Results: Sodium cholate exhibits a very low capability to inhibit microbial adhesion. Conversely, octylglucoside effectively inhibited microbial adhesion in both types of lenses. This surfactant exhibited an even greater performance than a multipurpose lens care solution used as control. Octylglucoside was the only tested surfactant able to lower the hydrophobicity of all CLs, which can explain its high performance.

Conclusions: The results obtained in this study point out the potential of octylglucoside as a conditioning agent to prevent microbial colonization.

6.2 Introduction

Over the last few decades, the number of CL wearers has grown rapidly because of the esthetic, therapeutic, visual and comfort reasons. There are several kinds of lenses commercially available; however, soft CLs are the most common. These lenses are composed of hydrophilic monomers such as hydroxyethylmethacrylate (HEMA), N-vinyl pyrrolidone (NVP), methacrylic acid (MA) and polyvinyl alcohol (PVA)(1,2). Recently, the introduction of silicone-containing hydrogel CLs having the same comfort and significantly higher oxygen permeability than conventional-hydrogel has resulted in a new generation of soft CLs. The high oxygen permeability, on account of the siloxane component, makes it possible to wear these lenses on a continuous basis for 30 days(1-3).

The occurrence of CL associated keratitis as well as other ocular complications has been a target of continuous research in several fields. When a CL is placed in the eye, the lachrymal tear components are adsorbed on its surface, building an organic substrate for subsequent microbial adhesion(4). In particular, when the corneal tissues are no longer intact due to hypoxic conditions or mechanical friction, microbes can invade the cornea and induce an ocular infection(4,5). So, the development of strategies such as the improvement of lens materials and lens care systems that avoid or decrease CL associated infections are very important aspects of soft CL research. The incorporation of surfactants in the lens care systems is useful not only to solubilise the organic tear film components adsorbed on lens surface, but also to disrupt microbial membranes(6). Nonetheless, surfactants are also able to modify the CL surface properties and thus may inhibit microbial adhesion(7,8).

Octylglucoside is a non-ionic and non-toxic surfactant which belongs to the alkylglucoside class(9), being frequently used to solubilise membrane bound proteins in their native state. Sodium cholate is a negatively charged (anionic) and non-toxic surfactant that belongs to the bile salts class. The use of sodium cholate has already been tested and when used below 0.5 % (w/v) is harmless to the ocular tissues(10). The aim of this work is to compare the effect of two natural surfactants, octylglucoside and sodium cholate and one commercial multipurpose lens care solution which incorporates the surfactant poloxamine in inhibiting the adhesion of one strain of Staphylococcus epidermidis and one of Pseudomonas aeruginosa to conventional hydrogel and silicone hydrogel CLs. These bacterial species are two of the most frequent pathogens(4,5,11)

involved in the occurrence of microbial keratitis and thus considered representative for this study. The efficacy of the surfactants was tested on CLs belonging to each FDA group.

6.3 Materials and methods

Contact lenses

CLs from each of the four FDA groups were used in this study. Group I materials are non-ionic and posses a water content lower than 50%. Group II materials have water content of 50% or greater and are non-ionic. Group III lenses are made of low-water content ionic materials and, finally, group IV lenses consist of high-water content ionic materials. The properties and commercial designations of the lenses used in this study are detailed inTable 6.1.

Table 6.1 Contact lenses and their properties

Category	Material	Commercial name	Manufacturer	FDA Group	Charge	Water content (%)	Surface treatment
Conventional hydrogel	Nelfilcon A	Focus Dailies	CIBA Vision	II	Non ionic	69.0	No
	Etafilcon A	Acuvue®	Johnson and Johnson	IV	Ionic	58.0	No
Silicone hydrogel	Lotrafilcon B	O_2Optix™	CIBA Vision	I	Non ionic	33.0	25 nm plasma coating
	Balafilcon A	Purevision™	Bausch&Lomb	III	Ionic	36.0	Plasma oxidation

Surfactants and multipurpose solutions

The tested surfactants were n-Octylglucoside [n-Octyl-B-D-glucopyranoside] (Sigma-Aldrich, Germany), a non-ionic surfactant, and sodium cholate (Sigma-Aldrich, Germany), an anionic surfactant. The physical-chemical properties of these surfactants are detailed in Table 6.2 and

their structures represented in Figure6.1 and Figure 6.2. The concentration used for both was half of their respective critical micelle concentration (CMC). Surfactant solutions were prepared with sterile deionised water and used immediately after preparation. The multipurpose lens care solution was Renu Multiplus with Hydranate® (Bausch & Lomb, USA). This solution is composed of 1% poloxamine (non-ionic surfactant), 0.0001 % Dymed® (cationic biocide), 0.03% Hydranate® (protein remover) and ethylene-diamine-tetra-acetic-acid (EDTA).

Table 6.2 Properties of octylglucoside and sodium cholate

Surfactant	CMC (% w/v)	Molecular weight (g/mol)	Chemical formula
Octylglucoside	0.60	292.38	$C_{14}H_{28}O_6$
Sodium cholate	0.73	430.53	$C_{24}H_{39}O_5Na$

CMC = Critical micelle concentration

Figure6.1 Schematic representation of the sodium cholate molecule

Figure 6.2 Schematic representation of the octylglucoside molecule

Bacterial strains and growth conditions

The strains used in this study were the clinical isolate S. epidermidis 9142, and P. aeruginosa ATCC 10145 (American Type Collection Culture). S. epidermidis 9142 is a well known producer of the major surface polysaccharide promoting coagulase negative staphylococci adherence and

biofilm formation, referred to as either polysaccharide intercellular adhesin (PIA) or by its chemical composition, poly-N-acetyl glucosamine (PNAG). This strain was kindly provided by Gerald B. Pier, Harvard Medical School, USA and its adhesion and biofilm formation capabilities were characterised in previous studies(12). P. aeruginosa ATCC 10145 was obtained from the ATCC and was isolated by F. Kavanagh (Merck Sharp and Dohme).

A 4 °C culture stock was inoculated into an Erlenmeyer flask containing 10 ml of tryptic soy broth (TSB, Merck, Germany) and incubated for 24 h at 37 °C. After this period, 1 ml of the culture suspension was transferred to a second Erlenmeyer flask containing 30 ml of TSB and incubated for 18 h at 37 °C in order to obtain a mid-exponential growth culture. Cells were harvested by centrifugation (15 min, 4000 rpm) and washed twice with Millipore water. Finally, the cells were resuspended in phosphate buffered saline (PBS: 8g l^{-1} NaCl, 0.2 g l^{-1} KCl, 0.2 g l^{-1} KH_2PO_4, 1.15 g l^{-1} Na_2HPO_4) and the concentration adjusted to 1◊E10 CFU/ml.

Before to the experiments, cell viability was evaluated through plating and CFU enumeration. The results showed that both strains maintain their viability after 18 h of incubation (data not shown).

Contact angle measurements

The CLs relative hydrophobicity was determined through contact angle measurement with Millipore water using the advancing contact angle as described by Bruinsma et al.(4). Contact angles were measured on non-conditioned and conditioned CL using the apparatus OCA 20 (Dataphysics). The conditioning process was performed by simple immersion of the lenses in the surfactant solution, or in the multipurpose lens care solution for a 16-h period. For the measurements, lenses were cut into four pieces and placed on a microscope slide. The excess of moisture was removed by gentle blotting with absorbent paper. These measurements were repeated 15 times per CL material at room temperature (22 °C) and a humidity of50±3 %.

Adhesion assays and image acquisition

The method used to assess bacterial adhesion to CLs was the static adhesion assay. Each CL was immersed in a well of a 24-well microtiter plate containing 1 ml of a cell suspension (6x1E10

CFU/ml) prepared in PBS. The tissue microtiter plate was then incubated for 2 h at 37 °C and after this period, each CL was removed and washed three times by immersing the lens in clean sterile PBS solution for 10 s. This washing step was carefully performed in order to remove only the cells that were suspended in the liquid interface formed along the lens surface and to minimize adhered cells detachment as described by Cerca et al.(13). The adhesion assays were performed with non-conditioned (control) and conditioned CLs. The lenses were conditioned by simple immersion in the surfactant solutions, or in the multipurpose lens care solution for 16 h followed by the adhesion assay. The adhesion assays were made in triplicate and repeated twice for each CL type and each conditioning agent.

After the adhesion assays, two opposite edges of each CL were cut to flatten the surface and the lens mounted on a microscope slide. Cell quantification was performed using a phase contrast microscope (Carl Zeiss, Germany) coupled to a 3 CCD video camera (Carl Zeiss) that acquires images at a magnification of 1622× with a resolution of 1300×1030 pixels and 20 images were randomly take from each CL. To eliminate image interferences, the background was captured and subtracted from the original image. Cells were enumerated using the Sigma Scan Pro program and for the magnification used 1 cm^2 was equivalent to 3906.25 captured images.

The % of adhesion inhibition by each solution was calculated as follows:

$$\%\text{Inibition} = \left(\frac{\#\text{ cells adhered to non - conditioned CL} \;\; \#\text{ cells adhered to conditioned CL}}{\#\text{ cells adhered to non - conditioned CL}} \right) \diamond 100$$

Statistical analysis

Data analysis was performed using the statistical program, SPSS (Statistical Package for the Social Sciences). After the evaluation of data distribution by K-test, contact angles data were compared using the parametric test analysis of variance (ANOVA) with Tukey's pairwise comparison whereas the extent of adhesion was compared by the non parametric Mann-Whitney U test. All tests were performed with a confidence level of 95%.

6.4 Results

Contact angles

Figure 6.3 presents the values of water contact angle measurements performed on the studied CLs. According to van Oss and Giese(14) a surface can be considered hydrophobic if the water contact angle exceeds 50° and hydrophilic if it is inferior to 50°. Thus, contact angles of non-conditioned CLs showed that silicone hydrogel CLs are hydrophobic whereas the conventional hydrogel CL are hydrophilic, with nelfilcon A being the most hydrophilic one (etafilcon A p=0.023; balafilcon A; p=0.000; lotrafilcon B p=0.000).

After conditioning the CLs with the surfactants or the multipurpose solution, the contact angles generally decreased except for balafilcon A (p=1.000) and etafilcon A conditioned with sodium cholate, which increased (p=0.000). This result indicates that sodium cholate is not such an effective surface agent as octylglucoside or the multipurpose solution.

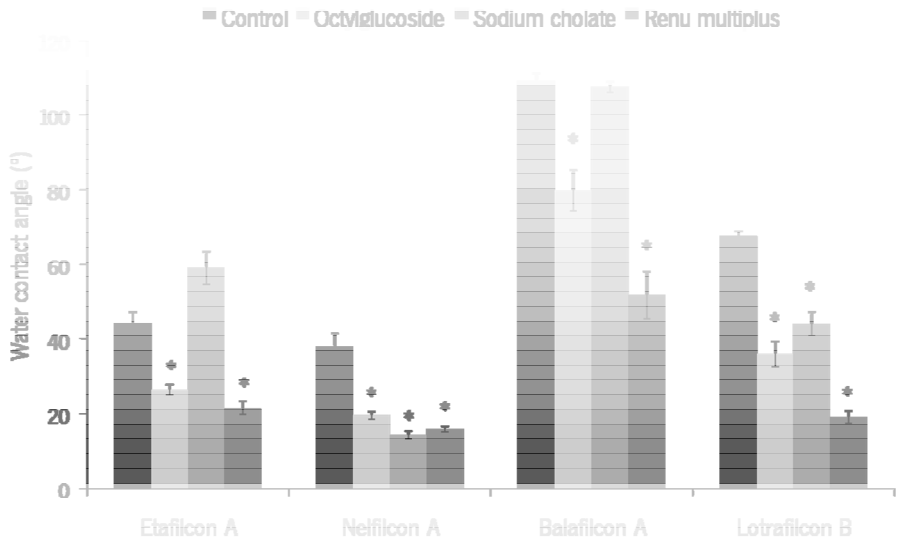

Figure 6.3 Water contact angles of uncoated and coated CL measured at room temperature

*Statistically superior to the control (One-way ANOVA – Tukey's with 95% confidence level);Error bars means standard deviations

Bacterial adhesion to non-conditioned contact Lenses

Static adhesion results can be observed in Figure 6.4 and Figure 6.5. For both tested strains it was observed that bacterial adhesion occurred in larger extent to silicone hydrogel CLs than to

conventional hydrogel CLs. Adhesion of S. epidermidis 9142 and P. aeruginosa ATCC 10145 to balafilcon A and lotrafilcon B was significantly greater than that observed in etafilcon A and nelfilcon A ($p<0.05$).

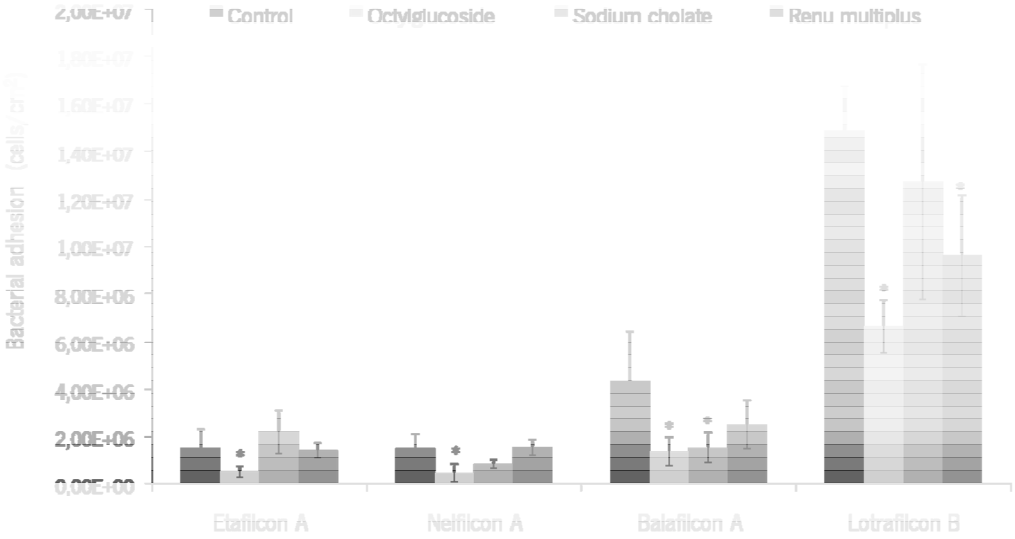

Figure 6.4 Number of cells of Staphylococcus epidermidis adhered to uncoated and coated CL with octylglucoside, sodium cholate, and the multipurpose lens care solution.

*Statistically superior to the control (Mann-Whitney U Test); Error bars means standard deviations

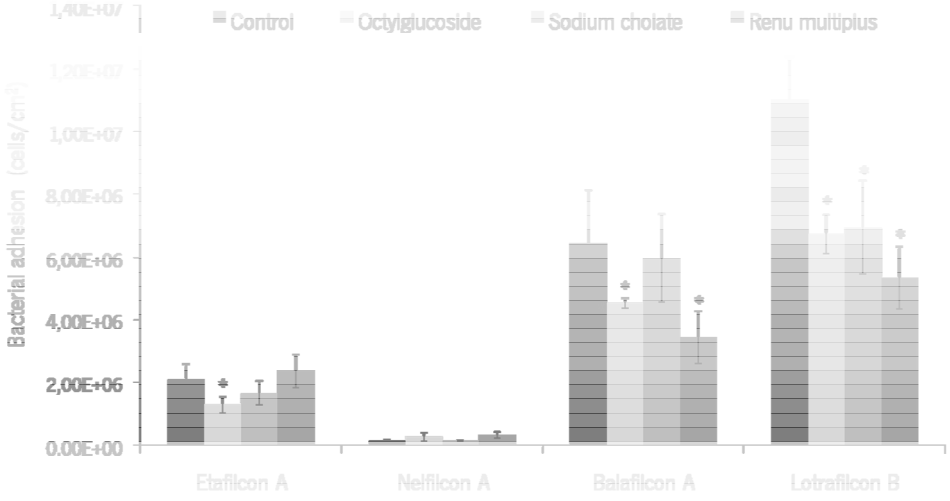

Figure 6.5 Number of cells of Pseudomonas aeruginosa adhered to uncoated and coated CL with octylglucoside, sodium cholate, and the multipurpose lens care solution.

*Statistically different to the control (Mann-Whitney U Test); Error bars means standard deviations

Adhesion to conditioned contact lenses and inhibition of adhesion

Figure 6.4, Figure 6.5 and Table 6.3 present, respectively, the results of microbial adhesion to conditioned CLs and the % of inhibition promoted by the surfactant solutions and the multipurpose solution. Generally, octylglucoside exhibited the best performance for both strains and the tested CLs. Octylglucoside was very effective in inhibiting S. epidermidis adhesion, because all CLs conditioned with this surfactant showed a significant decrease in the number of adhered cells (etafilcon A p=0.021 nelfilcon A p=0.021 balafilcon A p=0.020 and lotrafilcon B p=0.009). This surfactant was also effective against P. aeruginosa in all lenses (etafilcon A p=0.008 balafilcon A p=0.020 and lotrafilcon B p=0.020) except for nelfilcon A, which may be explained through the low levels of bacterial adhesion. Concerning sodium cholate, this surfactant only inhibited the adhesion of the S. epidermidis strain to balafilcon A CL (p=0.021) and of P. aeruginosa to lotrafilcon B. The multipurpose solution, did not demonstrated a significant inhibition effect in S. epidermidis adhesion with the exception of lotrafilcon B, whereas for P. aeruginosa this effect was relevant in balafilcon A (p=0.006) and lotrafilcon B (p=0.014).

Table 6.3 Inhibition of adhesion (average values) promoted by octylglucoside, sodium cholate and the multipurpose solution (% ± standard deviation)

		Etafilcon A	**Nelfilcon A**	**Balafilcon A**	**Lotrafilcon B**
Staphylococcus epidermidis 9142	Octylglucoside	65.5±24.4	68.0±8.6	68.2±23.7	55.3±3.0
	Sodium Cholate	-45.6±3.2	42.8±11.7	64.7±21.8	14.5±3.0
	Multipurpose solution	5.6±1.8	-2.8±0.5	42.2±10.2	35.7±1.5
Pseudomonas aeruginosa ATCC 10145	Octylglucoside	37.6±4.7	-63.6±32.7	30±6.6	39.0±2.6
	Sodium Cholate	20.3±1.3	4.6±0.65	7.8±0.26	37.0±0.5
	Multipurpose solution	-14.1±0.6	-96.6±33.7	47.0±2.8	51.5±0.08

6.5 Discussion

In this work, the pre-conditioning effect of two surfactants and one multipurpose solution on bacterial adhesion to CLs was evaluated. The adhesion assays were performed on unworn CLs, however it must be considered that "in situ" the CLs become rapidly conditioned with adsorbed components of the tear film such as proteins and lipids(15,16,17), which may influence lens surface properties and thus microbial adhesion. Nevertheless, this fact does not invalidate the methodology used because the purpose of this work is to study the CL pre-conditioning as a way to promote the inhibition of adhesion and as a palliative strategy to avoid ocular complications. The modification in CL surface hydrophobicity due to surfactant conditioning was also evaluated.

Contact angle measurements (Figure 6.3) revealed that the silicone hydrogel CLs, balafilcon A and lotrafilcon B are hydrophobic, whereas conventional hydrogel CLs are hydrophilic. Silicone-hydrogel CL hydrophobicity was already demonstrated and explained by the presence of silicone in the lens matrix which is a hydrophobic monomer. Contact angles have also revealed that the surfactants and the multipurpose solutions are capable of modifying the CLs surface properties. Generally, conditioned CLs resulted in a decrease of the water contact angle with the exception of balafilcon A and etafilcon A with sodium cholate. It is commonly accepted that surfactant adsorption depends mainly on the surfactant structure and surfactants with longest alkyl chain usually adsorb the most(9). Sodium cholate exhibits a very different chemical structure from octylglucoside and poloxamine, which may explain its lower performance. In fact, this bile salt is a planar molecule, (Figure6.1) punctuated with hydrophilic groups conversely to octylglucoside or poloxamine, which exhibit well defined hydrophilic and hydrophobic domains. The adsorption of octylglucoside and poloxamine on the lens surface through the hydrophobic moieties, exposing the hydrophilic groups to the aqueous media certainly contributed to the decrease of the hydrophobicity of all CLs.

Regarding bacterial adhesion to non-conditioned lenses, silicone-hydrogel CLs revealed to be more prone to S. epidermidis and P. aeruginosa adhesion than conventional hydrogel CLs. These results seem to be strongly related with the lens surface hydrophobicity. In a previous study we have demonstrated that silicone-hydrogel CLs are more prone to S. epidermidis and P. aeruginosa adhesion than conventional hydrogel(18), corroborating other "in vitro" studies using different microorganisms(19-21).

Concerning the conditioning effect of the surfactants or the multipurpose solution, this study showed that the modification of the surface properties, particularly the decrease of hydrophobicity not always leads to a decrease in bacterial adhesion. It is well established that surfactants are able to modify the surface properties of materials and thus influence adhesion(22-24), however in this study only CLs conditioned with octylglucoside, revealed a significant decrease of hydrophobicity as well as a reduction in the extent of microbial adhesion compared with the control (Figure 6.4, Figure 6.5 and Table 6.3). This result is most probably related with the amphiphilic properties of the surfactant molecules as well as their structure. Accordingly, well defined hydrophilic/hydrophobic regions of both octylglucoside and poloxamine enabled them to coat the lens in a uniform and consistent way, in opposite to sodium cholate. Sodium cholate only inhibited microbial adhesion in balafilcon A, although it did not reduce the lens hydrophobicity. This CL has a non-uniform surface, presenting "silicate islands" and probably sodium cholate molecules were adsorbed between these "islands" building a physical barrier against bacterial adhesion. The multipurpose solution was effective in inhibiting the adhesion of the S. epidermidis strain to lotrafilcon B and the P. aeruginosa strain to balafilcon A and lotrafilcon B. A better performance of this solution was expected since it incorporates the surfactant poloxamine which possesses antimicrobial properties(25,26) and in addition, has a higher surfactant concentration than the tested surfactant solutions. Nevertheless, the presence of other complex components in the multipurpose solution may have contributed for lowering its performance.

This study provides evidence that octylglucoside can effectively inhibit bacterial adhesion either to conventional or to silicone-hydrogel CLs. This finding is most likely related to their amphiphilic properties as their molecular structure. Many other conditioning agents such as poly(ethylene glycol) (PEG)(27), salycilate(28) and heparin(29) have been tested on CLs with the aim of reducing microbial adhesion. Still, octylglucoside has the increased advantage of inhibiting adhesion and being non-toxic and inexpensive. Despite the good results obtained for octylglucoside more experiments must be performed in order to test if the inhibiting capability of octylglucoside is affected by other chemical components that may be present in multipurpose solution such as biocides and preservatives.

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Chapter 7 The influence of lens material and lens wear on the removal and viability of Staphylococcus epidermidis

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7.1 Abstract

Purpose: The aim of this study was to evaluate the influence of lens material and lens wear on the removal capability of Staphylococcus epidermidis. Assessment of viability of remaining adhered bacteria was another goal of this work. Four silicone hydrogel materials (galyfilcon A, balafilcon A, lotrafilcon A, lotrafilcon B) and one conventional hydrogel material (etafilcon A) were assayed.

Methods: Detachment studies on S. epidermidis were carried out in a parallel plate flow chamber. CLs were fitted to the bottom of the flow chamber and a bacterial suspension was perfused into the system, promoting bacterial adhesion. Afterwards, detachment was stimulated using a multipurpose solution (MPS, ReNu Multiplus®) and the percentage of removed bacteria estimated through microscopic observation and enumeration. Remaining adhered bacteria were stained with propidium-iodide (PI) and enumerated in order to assess their viability. Additionally, the worn lenses were observed by confocal laser scanning microscopy (CLSM) to visualize bacterial distribution along the lenses surfaces.

Results: Bacterial removal was significant ($p<0.05$) for both unworn and worn galyfilcon A and etafilcon A. Galyfilcon A exhibited a detachment percentage of 59.1 and 63.5 while etafilcon A of 62.6 and 69.3, both for unworn and worn lenses respectively. As far as bacterial viability is concerned, it was found that worn lenses exhibit a superior amount of non viable bacteria than unworn CLs. Images obtained by CLSM revealed an irregular bacterial distribution for all lens materials.

Conclusions: It appears that surface and/or bulk structure of the lens material affects removal of S. epidermidis while CL wear influences their viability.

7.2 Introduction

CL solutions were first produced in the late 40's and have been developed ever since. These solutions should comprise several functions as to enhance CL wettability, prevent the build-up of deposits, and provide effective disinfection against pathogenic microorganisms(1). Currently, MPS are the most popular CL solutions since they permit in a single step to clean, rinse and disinfect(2). Disinfection is mainly promoted by the presence of biocides and it is essential to prevent ocular infections, which ultimately can lead to vision impairment. This process may be affected by numerous factors which includes the biocide, the challenging microbe, the material and the presence of organic matter(3-6). Due to the presence of surfactants, MPS may also promote bacterial removal; however and according to a previous study it was not significant(7).

Polyhexamethylene biguanide (PHMB) is one of the most popular biocide agents and has been used since the mid 70's in ophthalmic solutions. It is a polymeric cationic surfactant that belongs to the biguanide family and is currently used in several commercially available MPS. PHMB perform by enabling membrane disruption and lysis in bacteria, which results in their death(8,9).

Several studies have investigated disinfection and bacterial detachment to CLs(7,10-17). However, since silicone hydrogel CLs were launched, very few works have been performed with this type of material. The present study aimed to evaluate the influence of lens material and lens wear on the detachment capability of S. epidermidis. Four silicone hydrogel materials (galyfilcon, balafilcon A, lotrafilcon A, lotrafilcon B) and one HEMA material (etafilcon A) were worn daily, for one month with nightly disinfection with a single multipurpose solution (ReNu Multiplus). In addition, viability and distribution of the remained adhered bacteria were analysed through epifluorescence microscopy and CLSM respectively. Matched unworn lenses served as the control.

7.3 Materials and methods

Contact Lenses

The CLs used in this study are detailed in Table 7.1.

Table 7.1 Contact lens properties

Commercial name	Manufacturer	Material	FDA group	Water content	Surface treatment
Acuvue®	Johnson & Johnson Vision Care	Etafilcon A	IV	58%	No
Acuvue®Advance™	Johnson & Johnson Vision Care	Galyfilcon	I	47%	No
Purevision™	Bausch & Lomb, Inc.	Balafilcon A	III	36%	Plasma oxidation
Focus® Night & Day™	CIBA Vision	Lotrafilcon A	I	24%	25 nm plasma coating
O_2Optix™	CIBA Vision	Lotrafilcon B	I	33%	25 nm plasma coating

Clinical Trial

Thirty one subjects from both genders enrolled the present study, excluding any lost to follow up. The volunteers were predominantly from the north of Portugal and the average age was 23.6±5.5 years. These were chosen according to the following parameters: they have never worn CLs before (neophytes), they were not taking any medications during the trial, they did not suffer from any kind of ocular allergy and they had no tendency for dry eye syndrome.

Subjects were divided into 4 groups. Eight individuals were fitted with galyfilcon A, 8 with balafilcon A, 8 with lotrafilcon A and 7 with lotrafilcon B. Etafilcon A was used as contralateral pair into the four groups since a parallel study was ongoing in order to evaluate morphological changes between one eye fitted with a silicone hydrogel and the other fitted with a conventional hydrogel. According to the manufactures' recommendations, one of the silicone hydrogel lenses, galyfilcon A wear is recommended under a two week planned replacement modality. However, in this study, it was worn for 30 days in order to establish a comparison with the other silicone hydrogel lenses. None of the groups was aware of the CL material or brand they were using.

Silicone hydrogel CLs were used during 30 days and etafilcon A for 15 days (replaced at the end of 15 days), according to a daily wear schedule. The subjects were instructed to remove their lenses and place them directly into a solution (ReNu MultiPlus®, Bausch & Lomb, Inc. polyhexanide 0.0001%, hydranate 0.03% and poloxamine 1%) for overnight disinfection, between 12 and 14 hours wear (no rub or rinse). At the end of the wearing period, each lens was aseptically removed from the eye and placed in a sterile vial containing a sterile saline solution (0.9% NaCl). Vials were labelled with a code and details of the lens material. The CLs were stored at 4 °C no longer than 5 days until analysis. Unworn CLs were stored at room temperature (20 °C±2) and managed under sterile conditions until the beginning of experiments.

Each subject signed an informed consent following an explanation related to the nature of the study and its possible risks to the participant. No significant adverse events occurred throughout the course of this study.

Microorganisms and growth conditions

The challenging microorganism was S. epidermidis 9142. This Gram-positive bacterium is a clinical isolate and was kindly provided by Dr. Gerald B. Pier, Harvard Medical School, Boston, USA. Its adhesion and biofilm formation capabilities were characterised in a previous study(18).

A 4 °C culture stock was inoculated into an Erlenmeyer flask containing 10 ml of tryptic soy broth (TSB, Merck, Germany) and incubated for 24 h at 37 °C. After this period, 1 ml of the culture suspension was transferred to a second Erlenmeyer flask containing 30 ml of TSB and incubated for 18 h at 37 °C in order to obtain a mid-exponential growth culture. Cells were harvested by centrifugation (15 min, 4000 rpm) and washed twice with ultrapure water. Finally, the cells were ressuspended in phosphate buffer saline (PBS, 8g l^{-1} NaCl 0.2 g l^{-1} KCl 0.2 g l^{-1} KH_2PO_4 1.15 g l^{-1} Na_2HPO_4 pH 7.4) and the concentration adjusted to $6x10^{10}$CFU/ml.

Detachment experiments

Prior to detachment studies, bacterial adhesion was induced by perfusing a bacterial suspension throughout the system during 120 minutes. CLs were fitted to the bottom of the flow chamber

and all tubes filled with PBS, which circulated for 15 minutes. After this period, PBS was switched for the bacterial suspension previously prepared as described in the microorganisms and growth conditions section. The suspension flowed throughout the system for 120 minutes with a flow rate of 2E-4 ml/s, at room temperature. Fresh PBS was then recirculated throughout the system in order to remove the non-adhered or loosely adhered cells. Each CL was observed under phase contrast microscopy and twenty pictures (CCD video camera, Carl Zeiss, Germany) were taken with special care to cover both central and peripheric areas of the lens. Magnification of 1622× and a resolution of 1300×1030 pixels were used. The pixel area of each image was 262144 pixels^2 and corresponds to 1.96E-4 cm^2. Detachment experiments were initiated after capturing images of initially adhered cells.

In order to keep the same hydrodynamic conditions used in the adhesion step, detachment studies were also carried out in the parallel plate flow chamber. The authors are aware that dynamic systems do not reproduce the soaking process normally performed in CL disinfection; however, the goal of this study was mainly to assess cell removal.

Bacterial detachment was stimulated by perfusing the system with the MPS (ReNu MultiPlus®, Bausch & Lomb, Inc.) for 120 minutes. Again, twenty pictures (CCD video camera, Carl Zeiss, Germany) were taken with special care to cover both central and peripheric areas of the lens. Magnification of 1622× and a resolution of 1300×1030 pixels were used. The pixel area of each image was 262144 pixels^2 and corresponds to 1.96E-4 cm^2.

The percentage of bacterial removal was calculated as follows:

$$\%\,\text{Removal} = \left(\frac{\#\,\text{adhered cells} - \#\,\text{adhered cells after MPS action}}{\#\,\text{adhered cells}} \right) \times 100$$

Bacterial viability

Viability of adhered bacteria to worn lenses was investigated through propidium iodide (PI) (Sigma-Aldrich, Germany), staining (5 μg.ml.$^{-1}$), followed by epifluorescence microscopy observation and cell enumeration.

This fluorochrome is capable of staining nucleic acids when the cellular membrane is disrupted and therefore was used to assess cell viability(19). It was expected that PHMB as well as lytic proteins (present in the tear film) would bind to the outermost surface of bacterial cells disrupting them. Membrane disruption followed by leakage of the inner cellular compounds should open a pathway for PI linkage to the nucleic acids.

After bacterial detachment, each CL was covered with a few drops of the dye. They were incubated in the dark for 1 minute and then observed through an epifluorescence microscope (Carl Zeiss, Germany) coupled to a CCD video camera (Carl Zeiss, Germany). The excitation and emission wavelengths were 530 and 615 nm, respectively. Twenty pictures of the adhered bacteria were taken with special care to cover both central and peripheric areas of the lens. The magnification and resolution used were the same as for contrast phase microscopy. Afterwards, the non-viable cells were enumerated.

The percentage of non-viable cells remaining adhered to the lens surface was calculated as follows:

$$\%\,\text{Non viable cells} = \left(\frac{\#\,\text{adhered non viable cells}}{\#\,\text{adhered cells after MPS action}} \right) \times 100$$

Non-viable bacteria were also observed by CLSM (FV 1000 Fluoview, Olympus Europa GMBH, Germany) through a 3 dimensional image of adhered bacteria and their distribution along the lens surface.

Statistical analysis

Bacterial removal was analysed through the non-parametric Mann-Whitney U Test. All tests were performed with 95% confidence level using the statistical program SPSS (Statistical Package for the Social Sciences).

7.4 Results

Bacterial detachment

The % of removed bacteria is detailed in Table 7.2. According to the statistical analysis, bacterial removal was significant for galyfilcon A and etafilcon A materials. Both unworn galyfilcon A and etafilcon A showed a p=0.029, while in worn galyfilcon A and etafilcon A exhibited p=0.006 and p<0.001, respectively.

Table 7.2 Removal and viability of Staphylococcus epidermidis cells adhered to unworn and worn contact lenses (%)

	Unworn lenses		Worn lenses	
	% Cell removal	% Non-viable cells	% Cell removal	% Non-viable cells
Galyfilcon A	59.06*	12.28	63.46*	33.92
Balafilcon A	57.78	6.61	44.22	60.55
Lotrafilcon A	38.07	24.63	50.43	47.06
Lotrafilcon B	39.10	27.49	14.13	72.74
Etafilcon A	62.63*	0	69.29*	23.04

*Statistically significant (Mann-Whitney U with 95% confidence level)

Cell viability

Table 7.2 also presents the percentage of non-viable cells that remained adhered after the passage of the MPS. This percentage was higher for worn CL, ranging from 23.0 % (3.50E5 cells/cm^2) to 72.3 % (1.86E6 cells/cm^2) while unworn ones ranged from 12.3 % (1.19E5 cells/cm^2) to 27.5 % (4.33E5 cells/cm^2). It was not possible to visualise stained bacteria adhered to unworn etafilcon A.

Images obtained by CLSM are present in Figure 7.1. These images show an irregular dispersion of bacteria along the CLs surfaces. Lotrafilcon B CL was not subjected to such analysis due to their surface treatment similarity with lotrafilcon A.

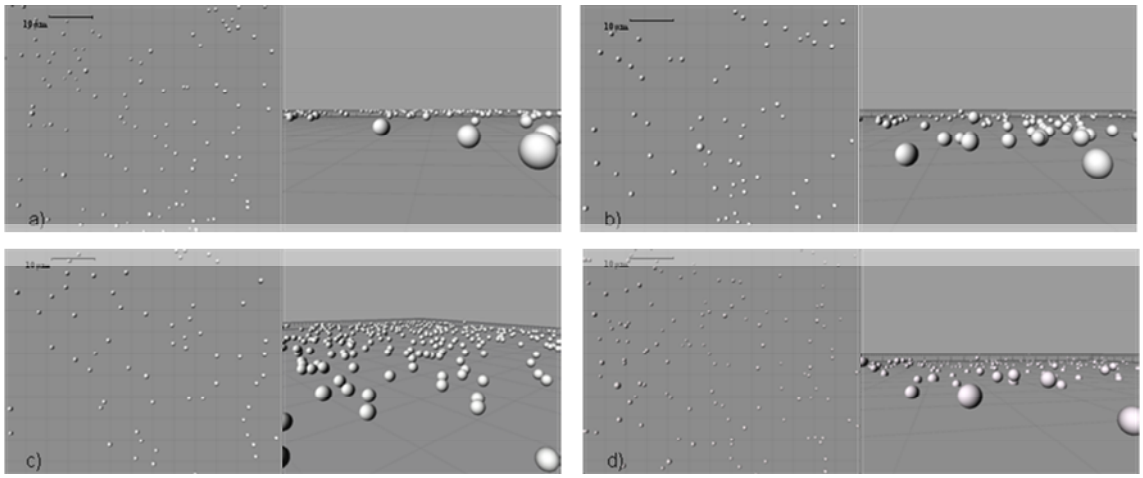

Figure 7.1 Top and perspective images obtained through CLSM of S. epidermidis cells adhered to worn (a) galyfilcon A, (b) balafilcon A, (c) lotrafilcon A and (d)etafilcon A

7.5. Discussion

The present study investigated the influence of lens material and wear on the removal and viability of adhered S. epidermidis to silicone and conventional hydrogel CL. A single PHBM-based solution was used. S. epidermidis is a pathogen normally associated to device-related infections and thus it was considered an appropriate challenging microorganism(20-22). Bacterial disinfection and detachment are two different concepts, but both important from a clinical perspective. While disinfection typically involves cell lysis and death, bacterial detachment promotes cell removal from the lens surface.

Detachment studies revealed that bacterial removal was significant from galyfilcon A and etafilcon A materials (Table 7.2). This result was obtained either for unworn as for worn lenses. Galyfilcon A and etafilcon A are made by the same manufacturer and are the only ones that do not have surface treatment. Although surface treatment was conceive to improve surface wettability, it also adds chemical active groups(23) and seems to enhance roughness(24). These factors might have increased the interaction between the CL and the bacteria making their removal more

difficult. This seems to be true, because in this work surface treated CLs did not show significant cell removal. We also address the hypothesis that PHMB could be operating on the lens surface rather than within the matrix, resulting in a large degree of bacterial removal for galyfilcon A and etafilcon A. PHMB contained in ReNu Multiplus is a large dimensional molecule and its size could limit its adsorption into the lens matrix(25). This seems especially consistent for etafilcon A, which possesses small pores(26). Although few data have been published about this material, it seems plausible that galyfilcon A structure and porosity could also have limited PHMB adsorption. This assumption is based on a study in which the ultra-structure of balafilcon A, lotrafilcon A and galyfilcon A was investigated using Cryo-SEM(27). The authors found that balafilcon A and lotrafilcon A materials exhibit what appears to be a loose network, while galyfilcon A shows a solid bulk. Even though unworn balafilcon A and worn lotrafilcon A exhibited levels of bacterial removal near to the observed for galyfilcon A or etafilcon A, they were not significant from a statistical perspective. Still, we believe that these results could be clinically relevant and thus they should not be disregard.

The determination of the amount of non-viable bacteria remaining adhered is of great clinical importance. This study reveals that although these cells remained adhered after the detachment procedure, they were inactive and thus virtually not harmful. Bacterial viability was asses with the fluorescence dye PI. This assessment is usually made by the classic method of colony forming units (CFU). The CFU method involves total bacterial removal from the lens surface followed by agar platting and counting after 48 hours of incubation. The main disadvantage is that the removal process can kill bacterial cells through excessive vortexing or sonication, or not removing all adhered bacteria. PI staining not only allows “in situ” cell enumeration, but is also a faster and reliable method(13). The results (Table 7.2) show that worn lenses contain a greater percentage of non-viable bacteria than unworn ones. It is generally accepted that lens wear induce the adsorption of lysozyme and lactoferrin which are bacteriolytic proteins(28,29).Therefore, their presence on the lens surface may induce bacterial lysis resulting in this higher percentage of non viable cells. Indeed, a similar result was previously reported by Williams et al.(30), who suggested that lactoferrin killed the attached bacteria on worn lenses. To some extent, we agree that daily CL maintenance with ReNu Multiplus might have assisted this result.

CLSM is a valuable imaging method, which allows living and dead cells observation. Several scans above the lens surface permit a detailed tridimensional image of adhered bacteria. It is possible to observe (Figure 7.1) that S. epidermidis is irregularly distributed among all lenses surfaces, which seems related with the presence of deposited tear film as this assay was performed on worn lenses.

Summarizing, the results obtained in this study suggest that the lens material affects removal of S. epidermidis since the MPS was capable of removing the adhered cells either from unworn as worn galyfilcon A and senofilcon A. The presence of bacteriolytic proteins of the tear film appears to contribute to the non-viability of adhered bacteria.

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Chapter 8 Contact lens material influences the efficacy of disinfection of multipurpose solutions

8.1 Abstract

Purpose: The efficacy of contact lens (CL) disinfection might be influenced by several factors namely the lens material, microbial species and multipurpose solutions (MPS) formulation. The goal of this study consists in evaluating the influence of CL material on disinfection efficacy, by exposing adhered bacteria to dissimilar CLs and MPS.

Methods: Microbiological assessment comprised tests with suspended and adhered cells to CLs. The influence of CL material on disinfection efficacy was assessed by exposing adhered bacteria to MPS of different formulations. The materials used in this study were etafilcon A (Acuvue 2), polymacon (Soflens 38), lotrafilcon A (Focus N&D) and balafilcon A (Purevision). The MPS comprised Opti-Free® Express®, ReNu Multiplus® and Complete®. Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa were the testing microorganisms.

Results: Suspension tests indicated that all MPS were capable of inducing a minimum of 4-log reduction on bacterial concentration ($p<0.05$). Assays performed with adhered bacteria to CLs, demonstrated that polymacon was the CL more susceptible to disinfection ($p<0.05$). Scores of disinfection ranged from 4.30 to 6.44 of log-reduction. Opti-Free Express was the MPS showing best disinfection efficacies. Log reduction in lenses treated with this solution ranged between 4.23 and 5.03.

Conclusions: Disinfection scores were dissimilar among several CL materials. This result should be related with different interactions generated in between the CL material and the multipurpose solutions.

8.2 Introduction

It is acknowledged that microorganisms in planktonic state are more susceptible to antimicrobial activity than adhered ones(1-4). Nevertheless most studies regarding CL disinfection are carried out with suspended microorganisms excluding the lens material from the assessment(5-8). Such tendency arises from the standard ISO 14279:2001, which established that CL disinfection solutions should be primarily tested against suspended microorganisms (stand-alone test). The accomplishment of this criterion does not obligate the MPS' manufacturers undergoing the secondary criterion, in which the disinfection efficacy is evaluated with bacteria deposited on the CLs (regimen test). Hence, suspension or stand-alone tests prevail over regimen tests and therefore studies addressing the influence of CL materials on disinfection are practically inexistent. In addition manufacturers have been reducing the rubbing and rising steps, a procedure that greatly contributes for the elimination of the remaining microorganisms(9). In view of that, it is possible that MPS presents marginal activity during CL disinfection and thus in the prevention of associated infections.

The aim of this work is to assess the influence of CL compositions in the disinfection efficacy of MPS. This evaluation will be carried out on two sets of materials, silicone hydrogel and conventional hydrogel CLs. Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa will be the challenging microorganisms.

8.3 Methods and materials

8.3 Methods and materials 1

Multipurpose solutions

The MPS used in this study and their main components are detailed in Table 8.1. At least two lots from each MPS were tested in this work. Three MPS with different formulations were tested. Opti-Free Express contains two biocides, polyquartenarium-1 (PQ-1) and myristamidopropyl dimethylamine (MAPD) whilst ReNu Multiplus and Complete incorporate polyhexamethylene biguanide (PHMB).

Table 8.1 Multipurpose solutions and main components

Product	Manufacturer	Biocide (%)	Surfactant cleaner	EDTA (%)
Opti-Free® Express®	Alcon	PQ-1 0.001 MAPD 0.0005	Poloxamer	0.5
ReNu Multiplus®	Bausch & Lomb	PHMB 0.0001	Poloxamine	0.1
Complete Comfort Plus	AMO	PHMB 0.0001	Poloxamer	0.02

PQ-1 (polyquartenarium-1); PHMB (polyhexamethylene biguanide); MAPD (myristamidopropyl dimethylamine); EDTA (ethylenediamine tetraacetic acid)

Contact lenses

The CLs used in this study are detail in Table 8.2. Two-silicone hydrogel CL and two conventional hydrogel CL were assayed.

Table 8.2 Contact lens properties

CL material	Brand	Manufacturer	FDA Group	Water content (%)	Main monomers
Etafilcon A	Acuvue®	Johnson&Johnson	IV	58	HEMA +MA
Lotrafilcon A	Focus® Night & Day™	CIBA Vision	I	24	DMA+TRIS+siloxane monomer
Polymacon	Soflens 38	Bausch & Lomb	I	38	HEMA
Balafilcon A	Purevision™	Bausch & Lomb.	III	36	NVP+TPVC+NCVE+PBVC

HEMA(2-hydroxyethyl methacrylate); mPDMS (monofuncional polydimethylmethacrylate); NVP (N-vinyl pirrolidone); TPVC (tris(trimethylsiloxysilyl) propylvinyl carbamate); NCVE (N-carboxyvinyl ester); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); DMA (N,N-dimethylacrylamide); TRIS (trimethylsiloxane)

Test organisms and growth conditions

Three bacterial species were used in this study. Staphylococcus aureus CECT 239 and Pseudomonas aeruginosa CECT 111 were purchased from the Spanish Type Culture Collection, University of Valencia and are respectively equivalent to S. aureus ATCC 6538 and P. aeruginosa ATCC 9027 from the American Type Collection Culture. Although not consider by the standard 14729:2001 as a reference microorganism, Staphylococcus epidermidis 9142 was used. This bacterium is a clinical isolate and a well known producer of the major surface polysaccharide promoting coagulase negative staphylococci adherence and biofilm formation, referred to as either polysaccharide intercellular adhesin (PIA) or by its chemical composition, poly-N-acetyl glucosamine (PNAG) and its adhesion capability was caractherised in previous studies(10;11). This strain was kindly provided by Dr. Gerald B. Pier, Harvard Medical School, Boston USA.

Growth conditions followed the standard ISO 14729:2001 recomendations. Prior to the disinfection assays, bacteria were grown on agar slopes between 18 and 24 hours at 35 °C. The colonies were harvested with DPBST, the suspension vortexed for 1minute and transferred to a sterile polypropylene tube (Orange Scientific, Belgium). Due to the voluntary recall, Complete was not challenged against P. aeruginosa CECT 111.

Disinfection assays

The main goal of this study was to determine whether CLs materials influence the disinfection efficacy of MPS. Still, it was found important to perform disinfection tests against suspended bacteria; thus, suspension tests were also undertaken.

Disinfection tests against suspended bacteria

Disinfection protocols of suspended bacteria followed the standard ISO 14729:2001(12) recommendations and according to the respective guidelines, each MPS must be capable of reducing the bacterial viability in 3-log (99.9%). Briefly, 100-µl of the bacterial suspension prepared as previously described was poured onto a tube containing 10 ml of MPS. The suspension was homogenised in order to ensure the proper dispersion of bacteria. The final

concentration ranged between $1.0 \diamond 10^5$ and $1.0 \diamond 10^6$ colony forming units per millilitre (CFU/ml). Samples were taken after 1 hour, 2 hours, 3 hours and 4 hours of inoculation, and neutralised with DENB. Surviving bacteria grew in the agar plates and were enumerated after 48 hours of incubation at 35 °C**.** At least 3 replicates and 2 independent assays were performed for each combination (MPS and bacteria).

Disinfection tests against adhered bacteria to contact lenses

Assessment of CL disinfection was carried out by exposing cells deposited on CLs to different MPS. The adopted protocol derived from the guidelines established by the standard ISO 14729:2000 for regimen test, with minor modification. Briefly, the inoculum concentration was adjusted between $1.0 \diamond 10^5$ and $1.0 \diamond 10^6$ colony forming units per millilitre (CFU/ml) in DPBST. CLs were removed from the original blisters and placed in a Petri dish. A 100-µl aliquot of bacterial suspension was inserted in the concave side of the lens and another in the convex side. After 10 minutes of contact with the bacterial suspension, each CL was inserted into an individual well of a 24-well culture plate, which was previously filled with 1 ml of MPS. Lenses were soaked for 4 hours at room temperature and then transferred to a filtration apparatus together with the respective amount of soaking solution where neutralisation occurred in 50 ml of DENB, during 15 minutes. After neutralization, three filtrations with 50 ml aliquots of DENB using a nitrocellulose filtration membrane of 0.2 µm pore were made. Afterwards, the CL was placed in an eppendorf containing 1ml of neutralizing solution and vortexed during 1 minute to detach adhered bacteria. The cell suspension obtained was diluted and spread in TSA plates. Following incubation (48 h at 35 °C), the number of CFU was enumerated. At least 3 replicates and 2 independent assays were performed for each combination (CL, MPS and bacterial strain).

Data analysis

Differences in cell number before and after disinfection was compared through the non-parametric Mann-Whitney U test using the statistical program SPSS (Statistical Package for the Social Sciences). A p value of less than 0.05 was considered statistically significant.

8.4 Results

Stand-alone test

Disinfection tests against suspended bacteria were performed according to the stand- alone test guidelines of the standard ISO 14729:2001. The amount of suspended cells that remained viable after several periods of disinfection is displayed in Figures 8.1, 8.2 and 8.3 for S. epidermidis, S. aureus and P. aeruginosa, respectively. All MPS were capable of passing this test, since a 3-log reduction was achieved. Statistical analysis confirmed that this reduction was significant ($p<0.05$).

Figure 8.1 Log reduction of S. epidermidis cells during 4 hours of disinfection (stand-alone test)

Figure 8.2 Log reduction of S. aureus cells during 4 hours of disinfection (stand-alone test)

Figure 8.3 Log reduction of P. aeruginosa cells during 4 hours of disinfection (stand-alone test)

Disinfection tests against bacteria deposited on contact lenses

Prior to disinfection, bacteria were deposited on different CLs. As explained in the Material and Methods section, aliquots of 100-μl were deposited on the upper and bottom side of each CL. Before disinfection, each CL had about $1 \diamond 10^7$ Staphylococci cells and $1 \diamond 10^8$ Pseudomonas cells.

Figure 8.4 present the average log–reductions of the amount of viable cells obtained in disinfection assays. Reduction was significant in all CL materials ($p<0.05$). Nevertheless, the conventional hydrogel polymacon demonstrated greater disinfection scores than the other lenses ranging from 3.64 to 6.44 for S. epidermidis, 3.38 and 4.39 for S. aureus and 5.74 and 6.04 for P. aeruginosa.

Concerning the efficiency of MPS, the PQ-based solution (Opti-Free Express) demonstrated a higher disinfection capacity than the PHMB-based ones, for every lens material.

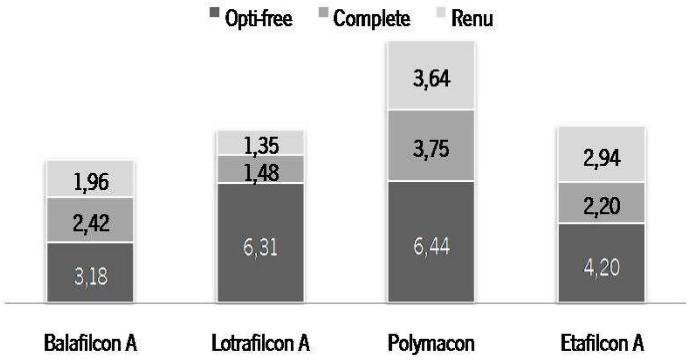

Figure 8.4 Number of adhered S. epidermidis cells (control), and number of survivors after disinfection with Opti-Free, Complete and Renu (CFU/ml)

Opti-Free was effective against S. aureus adhered to all lens types (Figure 8.5). Complete and ReNu were effective against S. aureus adhered to polymacon. Once more, all MPS meet this criterion when challenged against S. aureus adhered to polymacon. From a statistical point of view, disinfection was significant for all lens materials ($p<0.05$), even those where a 4-log to 5-log reduction was not attained.

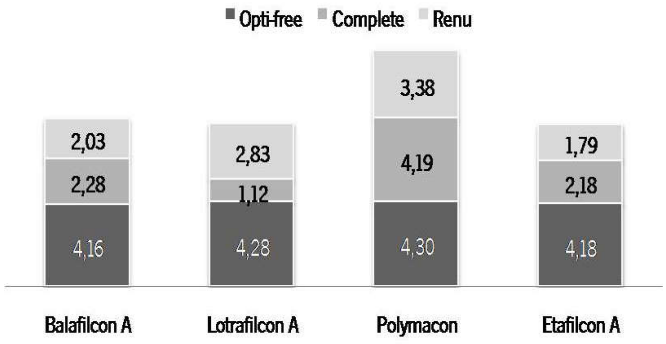

Figure 8.5 Number of adhered cells (CFU/ml) of S. aureus with no disinfection (control), and number of survivors after disinfection with Opti-Free, Complete and Renu

Figure 8.6 refers to P. aeruginosa and demonstrates that Opti-Free was effective against P. aeruginosa adhered to all lenses being lotrafilcon A the exception. ReNu was efficient against P. aeruginosa adhered to polymacon and balafilcon A. Complete was not tested against these bacteria because it was withdrawn from the market. Again, polymacon had satisfactory results

with the tested MPS. From a statistical point of view, disinfection was significant for all lens materials ($p<0.05$), even those where a 4-log to 5-log reduction was not attained.

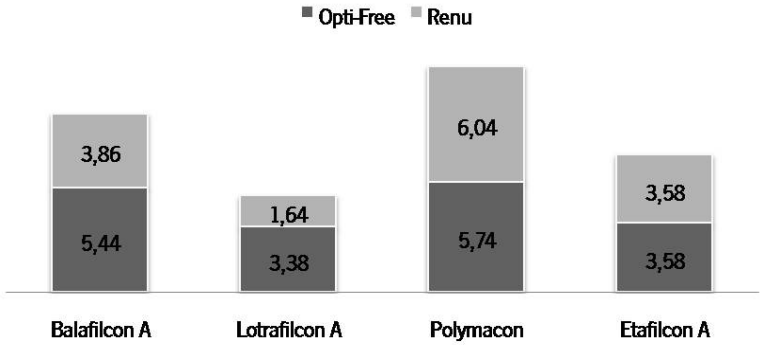

Figure 8.6 Number of adhered P. aeruginosa cells (control), and number of survivors after disinfection with Opti-Free and Renu (CFU/ml)

8.5 Discussion

The outermost surface of bacterial cells are often stabilised by the presence of divalent cations such as Ca^{2+} and Mg^{2+}. Cationic biocides such as PQ and PHMB are capable of displacing these cations, which destabilizes the bacterial membrane and cell wall. Eventually this will lead to the disruption of the lipid bilayer and cell lyses(13). PHBM exhibits one additional feature on this interaction. It tends to become concentrated around any point of maximum charge density within the membrane matching the spots where integral proteins are located. As a result, it changes the boundary phospholipidic(14) environment and causes loss of the protein function(13). It was previously demonstrated that when bacteria adhere to a surface, the antimicrobial activity of biocides immediately decreases(15). The acquisition of a different phenotype and the expression of certain genes seem to be the underlying reason. Other studies sustained that microbes are not the sole entities affecting disinfection efficacy but also the material by itself(16;17). On those studies, the adsorption and release pattern of biocide molecules onto CLs was estimated. The main conclusion was that the retention of MPS within the lens matrix reduces the efficacy of disinfection. Aiming to bring more insight about this issue, the present work investigated the influence of several CL materials including silicone hydrogel materials on the disinfection efficacy of several MPS. Two-silicone hydrogel CL and two conventional hydrogel were assayed.

The results obtained in this work revealed that polymacon was the CL more susceptible to disinfection being this true in almost every combinations bacteria/MPS (Figure 8.4 to 8.6). The biocides PHMB and PQ are both cationic and bacterial cells are negatively charged. Since polymacon is a non-ionic material, the biocide molecules should bond to the bacterial cells rather than to a non-ionic surface. Such attraction towards bacteria will lead to highest scores of disinfection resulting in the overall increase of the MPS antimicrobial activity.

Lotrafilcon A such as polymacon, is a non-ionic material however, their susceptibility to disinfection was not as satisfactory. This might be related with lotrafilcon A roughness since silicone hydrogel CLs tend to be rougher than conventional ones(18;19). In a recent study it was demonstrated that smoother surfaces display highest scores of bacterial detachment than rougher ones supporting the fact that on smoother surfaces the interaction between the biocide and the microbial cells is better(20). Smoother surfaces seems more beneficial, not only by preventing microorganisms from entering into pores or grooves, but also by allowing biocides to reach promptly to the bacterial cell wall or cell membrane.

In the present investigation, Opti-Free was more efficient in terms of disinfection than the PHMB–based ones. Some authors also reported this finding, corroborating the present results. As they explained, the better performance of Opti-Free is given to smaller levels of adsorption on CLs, thus enhancing their antimicrobial activity against microbial cells(16;17). Still, it is also important to stress that Opti-free have greater biocide concentrations than the other MPS, and in addition contains another biocide (MAPD). The biocide concentration is a key factor in disinfection(21). Opti-Free contains 0.001 % of PQ-1, while Renu and Complete have 0.0001 % of PHMB. Opti-Free has 0.5% of EDTA while Renu and Complete have 0.1 and 0.02 %, respectively. It is also though that the presence of two biocides in Opti-Free (PQ-1 and MAPD) instead of one might have assisted their disinfection efficacies by providing a wider antibacterial spectrum(22).

In conclusion, the lens material affects the antimicrobial activity of MPS. CLs having simultaneously a non-ionic character and a smooth surface seem more susceptible to disinfection.

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Chapter 9 General conclusions and suggestions for future work

In this Chapter, the major conclusions of the present Thesis are addressed. Suggestions for future work are also proposed.

9.1 General conclusions

The results presented in this Thesis confirmed that silicone hydrogel lenses are hydrophobic, while conventional hydrogel lenses are hydrophilic. However, the adsorption of proteins and lipids resulting from lens wear, as well as surfactants seems to decrease such hydrophobicity. As result, some materials either coated with tear film or with surfactants presented less adhered bacteria comparing with the respective controls. Deposition of tear film on CL also affected their roughness and topography.

In the first study (described in chapter 3) a group of volunteers wore a silicone hydrogel CL in one eye and a conventional hydrogel CL as contralateral pair. After 30-days of daily wear, the lenses were collected and the number of colonizing microbes as well as the quantity and type of adsorbed proteins were estimated. As for protein adsorption is concerned, different lens materials exhibited different profiles thus suggesting that the lens chemical composition influences the adsorption of tear film molecules. In terms of quantity, silicone hydrogel CLs exhibited similar degrees of adsorbed proteins and considerably smaller than those found on the conventional hydrogel lens. The surface treated CL balafilcon A exhibited a greater number of colonizing microbes, which seems related with their higher hydrophobicity and electron-accepting capacity parameter. This study suggests that the presence of surface treatment in silicone hydrogel materials does not contribute to the decrease of protein adsorption or microbial colonization.

In the second study (described in chapter 4), a group of volunteers wore another set of lenses. Hydrophobicity, roughness, topography and bacterial adhesion were assessed before and after wear. The adhesion of S. epidermidis cells was lower on worn silicone hydrogels CLs when compared to the worn conventional hydrogel material, which seems to result from the decrease of hydrophobicity on silicone hydrogel CLs and increase on conventional hydrogel materials. Bacterial adhesion to worn balafilcon A was significantly lesser when compared with the control (unworn balafilcon A).

It is generally recognised that strains of the same species can act differently in terms of adhesion. One bacterium can exhibit more affinity for one surface than for another, and express its preference by adhering more or less. This is related not only with their virulence

characteristics, but also with the microbial cell surface properties. The "in vivo" microbial colonization study described in chapter 3 showed that balafilcon A had the highest propensity for microbial colonization whereas the subsequent work (chapter 4) revealed that worn balafilcon is equally prone to S. epidermidis adhesion as the other worn silicone-based materials studied. In fact, the strains and methods involved in these two studies were quite different, which explains the different results. In the first study (chapter 3) all colonizing microbes were enumerated after lens wear, and that may have included all sorts of Gram-positive and Gram-negative bacteria and at some extent yeast, whilst in the second one, adhesion of a single strain was performed. Therefore, it is reasonable to assume that a pool of microorganisms and S. epidermidis 9142 adhesion to the same worn material can result in distinct results. Other factor to consider is the method used to quantify microorganisms. In the first work (chapter 3) the number of colonizing bacteria was quantified through colony forming units (CFU), whilst in the second study (chapter 4) adhesion was assessed through a parallel plate flow chamber and the number of adhered cells determined through direct enumeration with the assistance of a phase contrast microscopy and a proper software.

Chapter 5 addresses the propensity of CLs to emcopass oxidized lipids. According to the results, balafilcon A and vifilcon A exhibits significant amounts of oxidized lipids since the fluorescence intensity of conjugated Schiff-bases was greater on these materials. The fluorescence intensity dramatically increased after 14 days of contact with an artificial tear solution suggesting that the length of wear should be shorter in order to avoid excessive deposition of these molecules and compromise the wearers' ocular health.

The work described in Chapter 6 shows that the adsorption of surfactants and the multipurpose solution (MPS) ReNu Multiplus are capable of decreasing lens hydrophobicity. Nevertheless, only octylglucoside successfully inhibited adhesion of S. epidermidis and P. aeruginosa both to silicone hydrogel and to conventional hydrogel CLs ($p<0.05$).

The study described in Chapter 7 reveals that bacterial detachment seems to be influenced by the lens material. Both unworn and worn galyfilcon A and etafilcon A materials exhibited significant levels of removal of S. epidermidis cells. It was also hypothesised that the presence of adsorbed proteins on worn CLs might have played a bacteriolytic and/or bacterostatic role leading to the decrease of the viability of adhered bacteria.

In Chapter 8, CL disinfection was investigated. Three MPS were challenged against 3 bacterial strains, S. epidermidis, S. aureus and P. aeruginosa. All MPS passed stand-alone test. When these solutions were challenged against adhered bacteria, their disinfection efficacy varied upon the lens material. CLs having simultaneously a non-ionic character and a smooth surface seem more susceptible to disinfection.

9.2. Suggestions for future work

Bacterial adhesion to CLs is a process relatively well understood, however bacterial detachment and disinfection are not. In fact, the lack of studies regarding detachment and disinfection especially to silicone hydrogel CLs is remarkable. Accordingly, the investigation of cell detachment and disinfection on silicone hydrogel CLs it is strongly recommended.

One of the issues currently debated concerns the resistance of microorganisms to common biocides. It has been suggested that microorganisms having resistance against biocides might also acquire resistance against antibiotics. This fact is extremely worrying, as during an episode of MK the patient may not respond to the antibiotic therapy as expected. The development of alternative disinfection methods involving the use of bacteriophages and enzymes it is proposed.

Although lipid deposition contributes to the lack of comfort and/or visual acuity, the role of lipid oxidation in the pathogenesis of MK or in other ocular events was not established yet. In view of that, studies adressing the impact of oxidized lipids adsorbed to CLs on the wearers' ocular health should be undertake.