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Faculty of Technology

Polymer Centre

Doctoral Dissertation

Surface Treatment of Collagen-based Biomaterials in Medical Applications

Povrchové úpravy biomateriálů na bázi kolagenu pro medicinální aplikace

Jorge Andrés López García

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Supervisor:	Prof. Ing. Petr Sáha, CSc.
Consultant:	Doc. Ing. Marián Lehocký, PhD.
	Doc. Ing. Petr Humpolíček, PhD.

CONTENT

ABSTRACT

Biomaterials science is an expanding area, which encompasses a wide range of medical knowledge including *i.e.* arthroplasty, cochlear implants, heart valves designing, lenses, dental fixation and tissue engineering. Within this context, tissue engineering is an interdisciplinary field prompted to satisfy requirements, such as skin/nerve regeneration and organ/tissue replacement.

In vitro cell culture on polymer scaffolds is one of the adopted strategies for tissue creation. It consists in a specific cell line which is seeded onto a particular substrate. This scaffold should provide excellent biocompatibility, controllable biodegradability, appropriate mechanical strength, flexibility as well as the ability to absorb body fluids for nutrients delivery. Collagen certainly fulfils these demands, thereby it is often chosen as a starting material. Moreover, this protein is abundant in the animal kingdom and plays a vital role in biological functions, such as tissue formation, cell attachment and proliferation.

An important feature of any potential substrate is its cell-material interaction. This property is surface-selective and intrinsically connected to surface attributes. In this regard, plasma treatment is an effective and economical surface modification technique, which possesses the advantage of materials thin film adjustment without affecting bulk properties. In addition, plasma-based technologies are environmentally friendly. Nonetheless, a serious difficulty in tissue replacement is biofilm formation, which is responsible for infections over the treated areas. It is indeed one of the most serious concerns for several areas, particularly medical devices, healthcare products, dentistry, food packaging and storage, household sanitation and veterinary. In the medical field, nosocomial infections are the foremost worldwide cause of death and disability, which constitutes a threat to patients' lives and high costs.

An efficient way to thwart this problem is by means of materials able to inhibit the growth of pathogenic microorganisms. It may be attained by incorporating antimicrobial agents with high capacity of bacterial abatement that in turn must have a low toxicity against cells that are held on the substrate.

In order to raise awareness of the importance and the high economic impacts that these topics have on science and daily life, this doctoral work embodies a broad review of biomaterials, surface science and their advances in the development of materials suitable for tissue engineering applications. Furthermore, it also delves into a fundamental issue in current medicine, the control of harmful microorganisms in medical implants. The findings of this research seek to enlighten topics related to tissue substitution, plasma treatment, antimicrobial biopolymers and human cell growth.

This doctoral dissertation has a schematic and concise background followed by a synopsis of the obtained results and conclusions. Finally for any further information, the full-version of the framing papers I-III is included.

Keywords: Tissue engineering; Collagen; Plasma treatment; Antimicrobial material; Cell growth.

ABSTRAKT

Výzkum v oblasti biomateriálů zaujímá čím dál větší důležitost s ohledem na jeho aplikace v medicíně jako např. u kloubních náhrad, kochleárních implantátů, srdeční chlopně, čočky, zubní fixaci a tkáňovém inženýrství. V této souvislosti je tkáňové inženýrství interdisciplinární oblastí a výzvou k uspokojení požadavků, jako např. při léčbě kožních poranění, regeneraci nervových tkání nebo transplantaci orgánů. Příprava buněčných systémů na polymerních scafoldech je jednou z nejpoužívanějších technik ve tkáňovém inženýrství, přesto že existuje několik postupů k dosažení obdobných cílů. Tato technika je založena na interakci substrátu se specifickou skupinou lidských buněk. Tento scafold by měl přirozeně vykazovat vynikající biokompatibilitu, řízenou biodegradabilitu a taktéž by měl být schopen absorbovat tělní tekutiny potřebné pro transport živin. Měl by také vykazovat příslušné mechanické vlastnosti jako odolnost a ohebnost. Z těchto důvodů je jako počáteční materiál obvykle zvolen kolagen vzhledem k jeho poměrně vysokému zastoupení u živočichů, kde tvoří přibližně čtvrtinu množství bílkovin a hraje důležitou roli v mnoha biologických funkcích jako tvorba buněk, buněčná adheze a proliferace.

Důležitou vlastností každého potenciálního substrátu je jeho interakce s buňkami a tato schopnost je selektivní na povrchu. Úprava plazmatem je v tomto ohledu poměrně účinná a levná metoda, která spočívá v úpravě tenké povrchové vrstvy aniž by došlo ke změně celkových vlastností. Nadto, techniky založené na úpravě v plazmatu jsou ekologicky šetrné. Nicméně, vážným problémem při náhradě tkání je tvorba biofilmu způsobujícího infekci ošetřených oblastí. Tento problém se týká mnoha oblastí lékařství, zejména potom lékařských přístrojů, výrobků určených ke zdravotní péči, stomatologie, oblasti balení potravin a jejich skladování, hygienických pomůcek užívaných v domácnostech a veterinárního lékařství. V oblasti zdravotnictví jsou především nosokomiální infekce příčinou úmrtí nebo

invalidity pacientů po celém světě. Infekce je tedy hrozba pro život a také navršuje náklady za léčbu. Účinným způsobem, jak vyřešit tento problém, je vyrobit materiály, které mohou být schopné inhibovat růst patogenních bakterií. To může být dosaženo včleněním antimikrobiální látky do materiálu, která musí mít vysokou schopnost zabíjet bakterie, ale zároveň vykazovat celkově nízkou toxicitu vůči buňkám v organizmu.

S ohledem na důležitost a výrazný ekonomický vliv, který tato témata jak ve výzkumném tak každodenním životě představují, zahrnuje tato doktorská práce širokou rešerši o biomateriálech, povrchových analýzách, modifikacích a jejich pokroku v aplikacích tkáňového inženýrství. Mimoto je také podrobně rozebrána základní problematika současné medicíny v oblasti škodlivosti mikroorganismů potenciálně přítomných na implantátech. Výsledky tohoto výzkumu se snaží odkrýt poučné poznatky související s tkáňovými náhradami, úpravou plazmatem, antimikrobiálními biopolymery a růstem lidský tkáňových buněk.

Tato disertační práce poskytuje schematický a stručný teoretický přehled doprovázený výsledky získanými z experimentální činnosti a závěrem, který veškeré poznatky sumarizuje. Nakonec, pro jakékoliv další informace jsou přiloženy plné verze publikovaných článků I-III.

Klíčová slova: Tkáňové Inženýrství; Kolagen; Úprava plazmatem; Antimikrobiální Materiál; Růst buněk.

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FIGURES AND TABLES

ABBREVIATIONS AND SYMBOLS

DDS	Drug delivery system
HaCaT	Human adult low calcium high temperature keratinocyte cells
Copoly(LL-GA)	Copolymer(lactic acid-glycolic acid)
Copoly(LL-CA)	Copolymer(lactic acid-ε-caprolactone)
HAIs	Hospital-acquired infections
LDH	Lactate dehyrogenase
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ATP	Adenosine triphosphate
SRB	Sulphorhodamine B
WST	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
Gly	Glycine
Pro	Proline
Hyp	Hydroxyproline
N	Nitrogen
O	Oxygen
F	Fluorine
Ar	Argon
eV	electronvolt
NYSE	New York stock exchange
XPS	X-ray photoelectron spectroscopy
ESCA	Electron spectroscopy for chemical analysis
ATR	Attenuated total reflection
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy imaging
θ	Contact angle

PUBLICATION OUTPUT

The following papers published in peer-reviewed journals have results from this doctoral research available in full-text at the end of this dissertation as the framing papers of the present doctoral work:

Publication I:

Cell Proliferation of HaCaT Keratinocytes on Collagen Films Modified by Argon Plasma Treatment

Jorge López García, Ahmad Asadinezhad, Jiří Pacherník, Marián Lehocký, Ita Junkar, Petr Humpolíček, Petr Sáha, Pavel Valášek.

Molecules, 2010, vol. 15, p. 2845-2856. DOI: 10.3390/molecules15042845

Publication II:

Enhanced Keratinocyte Cell Attachment to Atelocollagen Thin Films through Air and Nitrogen Plasma Treatment

Jorge López García, Jiří Pacherník, Marián Lehocký, Ita Junkar, Petr Humpolíček, Petr Sáha.

Progress in Colloid and Polymer Science, 2011, vol. 138, p. 89-94. DOI: 10.1007/978-3-642-19038-4_16

Publication III:

HaCaT keratinocytes response on antimicrobial atelocollagen substrates: Extent of cytotoxicity, cell viability and proliferation

Jorge López García, Marián Lehocký, Petr Humpolíček, Petr Sáha.

Submitted to *Journal of Applied Biomaterials and Biomechanics*

1. THEORETICAL BACKGROUND

1.1 Biomaterials technology for tissue engineering applications

Tissue engineering is a multidisciplinary field that integrates both biological and engineering principles orientated towards generating biological substitutes to replace (diseased/damaged) tissue and restore (tissue/organ) function [1, 2].

There are four key approaches in tissue regeneration. The first one promotes cell proliferation and differentiation *in vivo.* The second supplies nutrients and oxygen for cell proliferation. The third is known as drug delivery system (DDS), and releases growth factors that induce tissue renewal; and the latter deals with cell manipulation to obtain cells and cell promoters for transplantation via *in vitro* culture technologies. Likewise, there are three basic tools for the creation of a new tissue; cells, scaffold and growth factor. Cells synthesise matrices of new tissue, meanwhile the scaffold has to offer a propitious environment for cells. The function of growth factor is to assist and promote cells to regenerate new tissue (figure 1) [3, 4].

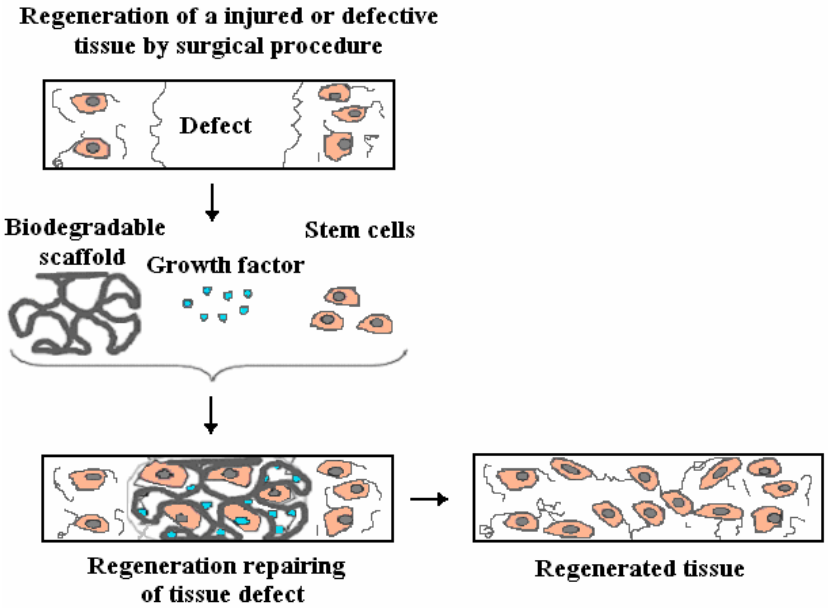

Figure 1. Cell, scaffold and growth factor are the three key materials for tissue engineering [5].

Tissue engineering has three basic targets: ectodermal tissue, which is the outermost tissue that covers the body surface (nervous system, pigment cells, epidermis, and cornea); mesodermal tissue (middle layer associated with connective tissue, muscle and bones) and endodermal tissue, is the innermost of the layers. The following are some of the clinical treatments and investigations that are being carried out at the moment: in ectodermal tissue. Peripheral nerves are capable of renovating after transection injury. Transected nerves may be clinically repaired by end-to-end approximation of the stumps with fine sutures. Synthetic nerve guides (conduits) might help in these cases by protecting the restoring nerve from infiltrating scar tissue or by directing new axons towards their target. Skin comprises essentially three cell types: keratinocytes, melanocytes and fibroblasts. The clinical application of human cells in tissue engineering may be done on skin tissue using fibroblast, keratinocytes, or a scaffold. For example, it is well-established through wound healing, transplantation and cell culture reports that human adult low calcium high temperature (HaCaT) cells are spontaneously transformed to human keratinocytes which have characteristics of basal epidermal keratinocytes and thus, this cell line may be used as an *in vitro* model for highly proliferative epidermis in tissue engineering. Corneal epithelial cells have been pre-seeded on polyvinyl alcohol hydrogels and transplanted into rabbit corneas, having cell attachment and proliferated for 1 to 2 weeks. In mesodermal tissue, synthetic and natural polymers have been explored for arthroplasty and osseous repair. The difficulty lays on material strength and properties optimisation. The ability to generate muscle fibres may be useful in the treatment of muscle injury, cardiac disease, muscular dystrophy and other disorders in smooth muscles and intestines. In endodermal tissue the main effort here has been focused upon liver, pancreas and tubular structures [6-9].

Despite tissue engineering is a nascent industry and many of the tissue-engineered products still being in ongoing stages, it has a potentially huge economic impact and

its revenues may exceed $10 billion by 2013, and just in the USA it is estimated at $80 million annually [10, 11].

Biomaterials play a crucial role in tissue engineering. Throughout history, the biomaterials have been extensively used for medical applications including metals, alloys, ceramics, synthetic/natural polymers or early biomaterials like wooden teeth and glass eyes. Metals and ceramics keep being used, but these are not biodegradable and their processability is limited. On the other hand, numerous polymeric materials have received increasing attention in virtue of their easy biodegradability and processability control (table 1) [12].

Table 1. Basic biodegradable polymers used in tissue engineering

synthetic polymers	natural polymers
poly(glycolic acid)	collagen
poly (lactic acid)	gelatine
poly[lactic-co-(glycolic acid)]	fibrin
polycaprolactone	alginic acid
polyfumarate	chitosan, chitin
copoly(LL-GA)	glycosaminoglycans (hyaluronic acid)
copoly(LL-CA)	
copoly(LLA-ethylene glycol)	

Biodegradation is the phenomenon where the chemical breakdown of materials is caused by a physiological environment. The material is degraded or solubilised by any process in the body to be absorbed from the implanted site. There are two ways of material vanishing. In the first one, the material backbone is progressively cracked by hydrolysis or enzymatic degradation decreasing the molecular weight until it is absorbed. In the second, the material is chemically

crosslinked forming a water-insoluble hydrogel. Thereafter, the crosslinking bond is degraded into small water-soluble fragments, which may be leached from the site implanted. Synthetic polymers are usually degraded by simple hydrolysis, whilst natural polymers are mainly degraded by enzymatic processes [13].

Both synthetic and natural polymers offer good alternatives to biomaterial design: natural polymers (*e.g.*, proteins, polysaccharides and nucleic acids) are easy to get owing to their abundance, and to proffer favourability to cell adhesion. In fact, those have a big potential for cell attachment and transplantation. Nevertheless, their physicochemical manipulation is very limited (even that natural polymers may be chemically altered producing different derivatives). Contrariwise, synthetic polymers may be easily modified by changing their chemical composition and molecular weight. Synthetic polymers are generally more hydrophobic and mechanically resistant than natural ones. As for polymer degradation, this one is slower in synthetic than in natural polymers [14, 15].

1.2 Medical implant contamination

An uncountable amount of undesirable guests, like bacteria and/or fungi may affect substrates' functions. It is manifested by loss of mechanical and physical properties as well as other material damages. Nosocomial contamination, also known as a hospital-acquired infection (HAI) is a widespread cause of implants failure and removal in medicine [16, 17]. Table 2 sums up the main nosocomial infections along with their vehicles of transmission [18, 19].

According to the World Health Organisation, nosocomial contaminations are the major worldwide cause of death and disability. Close to 15% of hospitalised patients are victims of these infections. This problem also signifies an annual cost between $4.5 and $11 billion [20]. In terms of Czech hospitals, there were approximately

260,000 cases in 2010, which represented 12% of the acute care population [21]. Infections are *de facto* one of the most serious challenges in medical implants advancement [22, 23].

Table 2. Overview of the most common hospital-acquired infections

type of infection	contributing factors	primary pathogens
tract infections	urinary tracts	*Escherichia coli*
pneumonia	airborne transmission	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Enterobacteriaceae*
surgical wound infections		*Staphylococci*
bacteremia (blood stream)	intravenous lines	*Staphylococci*

Biomaterials may become resistant to microbial colonisation. For instance, polymers are regularly sterilised via dry/wet heating or irradiation. However, these materials may get contaminated by microorganisms when they are exposed to the atmosphere again. Other method is to endow a biocidal function to the materials. It may be done through three alternatives: the first one is *ab initio* by adding antibacterial agents during the process. The other ones are carried out after processing, either by fixing the biocide on the polymer backbone or by grafting the agents onto polymer surfaces [24-28]. Polymer-containing biocides moieties have also disadvantages, fundamentally problems with cytotoxicity and a limited protection [29-31]. It is worth pointing out that whether a medical implant does not have an optimal cleanliness regime, this antibacterial property will be no longer useful, since HAIs have not been, and probably never will be completely eradicated.

1.3 Viability of antimicrobial materials

An imperative issue once any cell is taken from its natural *in vivo* environment is its viability during an experimental manipulation. Hence, the development of *in vitro* models to assess toxicity of chemicals substances has become crucial. These *in vitro* systems aid in the understanding of drugs-host models, because *in vivo* ones are complicated and difficult to comprehend. Besides, *In vitro* models allow decreasing the number of animals in biological testing, which normally go through painful experiences [32].

Toxicity is the extent of damage that a substance may induce in an organism. It arises either by the effect on a whole organism (*i.e.* animal, bacterium, plant), or the effect on a substructure, as a cell (cytotoxicity) or an organ (organotoxicity) [33]. Definitions of cytotoxicity change according to the nature of the study and whether cells are abated or their metabolism is altered [34].

Antimicrobial agents are substances able to counteract or inhibit the growth of microorganisms [35]. Table 3 lists some natural and synthetic compounds which are frequently set against bacterial strains [36-40]. It should be noted that the yield of these agents hinges on concentration, temperature and stability in the medium.

Various methods have been hitherto designed to examine cell viability and proliferation in cell culture. Colorimetric and luminescence based assays are typically used. Cell death and proliferation are the key issue here; for example, membrane integrity is ascertained by measuring lactate dehyrogenase (LDH) in the extracellular medium. This enzyme is present in the cytosol, and may not be measured extracellularly unless some cell damage has befallen. The LDH assay is based on a coupled enzymatic reaction that results in the conversion of a tetrazolium

salt (INT) to a red colour formazan. LDH activity is determined as NADH oxidation or INT reduction over a pre-establish period of time [41].

Table 3. Antimicrobial agents that are typically employed in medicine and medical devices

synthetic compounds	effective against
benzalkonium chloride	gram positive and negative strains
bronopol	gram negative strains
chlorhexidine	gram positive and negative strains
irgasan	gram positive and negative strains
quinolones	mainly gram negative strains
silver nitrate	gram negative strains
sulphonamides	gram positive strains
tributyl phosphonium salts	gram positive and negative strains
natural and seminatural[†] compounds	
derived from chitin (chitosan and N-alkyl chitosan)	gram negative strains
β-lactams	mainly gram positive strains
aminoglycosides	gram positive and negative strains
terpenoids	gram positive and negative strains

[†] Seminatural: Substances derived from natural sources

Another parameter of colorimetric assays is connected to the metabolic activity of viable cells. The MTT assay consists in the reduction of the tetrazolium salt (MTT) to formazan. It quantifies the number of live cells (figure 2), since this salt is just reduced by mitochondrial succinate dehydrogenase enzyme in the mitochondria of living cells [42]. Adenosine triphosphate (ATP) is ubiquitous in all metabolically

active cells, and it may be estimated by bioluminescent measurement [43]. Other methods, like ^{51}chromium (^{51}Cr) release assay, neutral red, sulphorhodamine B (SRB) and WST are also apt for these type of experiments [44-46].

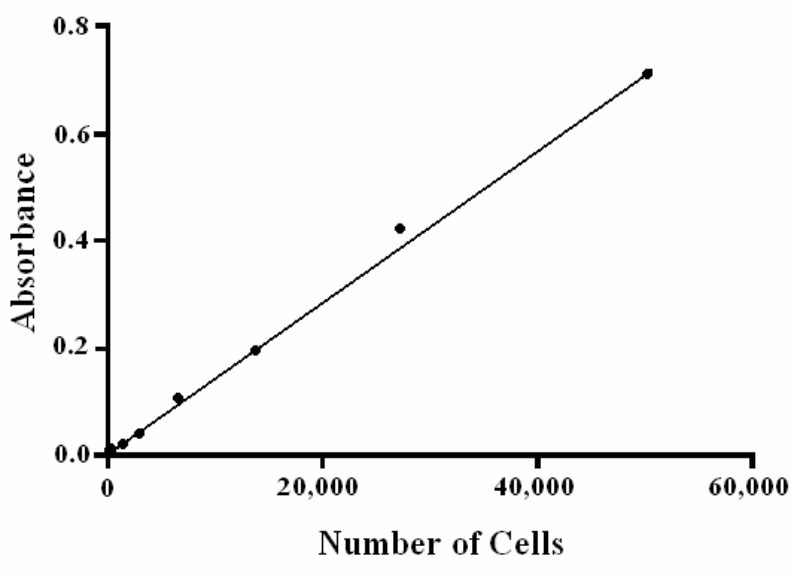

Figure 2. Standard cell quantification curve using MTT cell proliferation assay [42].

1.4 Collagen as a biomaterial

The collagen is a family of fibrous proteins, which are present in nearly all mammalian tissues. These constitute *ca.* 25% of the whole-body protein content. Their abundance is mainly centred on connective tissues, such as tendon, ligaments and cartilage. Skin also contains these proteins, which are involved in prime biological functions, such as tissue formation, cell attachment and proliferation [47]. Around 19 proteins are catalogued as collagen. Moreover, there are several proteins which have collagen domains. In its native form, collagen is a triple helix formed from three polypeptide chains, whose general sequence is $(X\text{-}Y\text{-}Gly)_n$. The presence of glycine (which is the smallest amino acid) as every third amino acid in the repeating sequence of each chain is essential, because other amino acid may not fit in the centre of the triple helix where the three chains come together. Proline (Pro) is recurrently in the X-position of the -X-Y-Gly- sequence, and 4-

hydroxyproline (Hyp) in the Y-position [48]. Indeed, hydroxyproline is almost unique to collagen representing approximately 14% of collagen dry weight. Thus, it is used as an indicator to quantify this protein. Pro and hyp hinder the polypeptide chains rotation and the triple helix is stabilised by hydrogen bonding. Hence, the molecule is relatively rigid [49].

Since the first structure proposed by Rich and Crick in 1955 [50-52], collagen structure has been comprehensively studied, being $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Hyp\text{-}Pro\text{-}Gly)_n$ the most repeated sequences (figure 3). However, table 4 demonstrates that other amino acids may be found in collagen molecular structure [53, 54]. The collagen family may be classified according to the polymeric structures. For instance, collagen that forms small filaments (types I, II, III, V, and XI), collagen that forms network structures (type IV, VIII and X), fibrillar proteins with collagen domains (types VI, VII, IX, XII, XIII, XIV, XVI, XVII and XIX). Collagen types XV and XVIII have been only partially characterised [55].

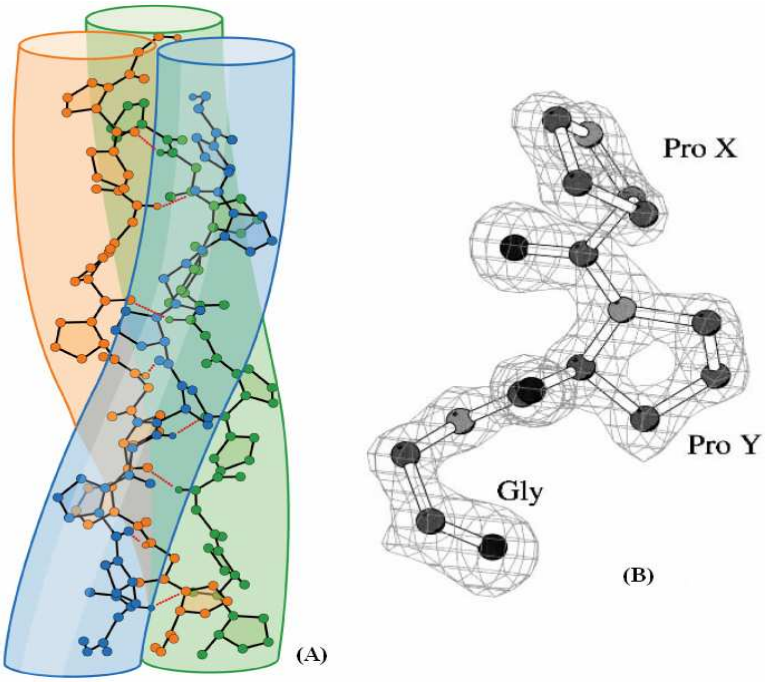

Figure 3. (A) Collagen arranged into a triple helical structure. (B) The $(Pro\text{-}Pro\text{-}Gly)_n$, which is the most widely investigated collagen sequence[51].

Table 4. Amino acid composition of mammalian collagen

Amino acid	Composition (g/100g of protein)
Glycine(gly)	29.7±0.5
*Proline(pro)	13.0±0.3
Glutamic acid(glu)	11.9±0.1
Alanine(ala)	9.3±0.1
Arginine(arg)	8.7±0.5
Aspartic acid(asp)	5.6±0.5
Lysine(lys)	3.6±0.2
Leucine(leu)	3.1±0.1
Serine(ser)	3.0±0.1
Valine(val)	2.5±0.1
Phenylalanine(phe)	2.3±0.1
Threonine(thr)	1.9±0.1
Isoleucine(ile)	1.6±0.1
Glutamine(gln)	1.0±0.3
Asparagine(asn)	0.7±0.1
Histidine(his)	0.6±0.2
Methionine(met)	0.6±0.1
Tyrosine(tyr)	0.5±0.1
Cysteine(cys)	0.2±0.1
Total	99.8±0.1

*The value includes proline(pro) and hydroxyproline(hyp)

1.4.1 Atelocollagen

There are two typical procedures for isolating collagen; enzymatic digestion and salt/acid extraction.

Enzymatic digestion cleaves crosslinks by using proteases (*e.g.*, pepsin, trypsin) which are enzymes that break the crosslinks amongst collagen molecules. It results in soluble triple helices known as atelocollagen, which possesses exactly the same physical properties of the untreated collagen (figure 4). Because of atelocollagen is soluble in acid pH, its liquid form may be moulded in diverse physical shapes, such as atelocollagen films by casting, sponge-like structures by freeze-drying, yarn-like by extrusion, atelocollagen powders, gels, blocks, tubes, pellets-like and other configurations [56-58]. Atelocollagen has been used in distinct branches of collagen research; for example, polymer blends, drug delivery, polymer grafting, tissue engineering, nerve restoration and cosmetics [59-61].

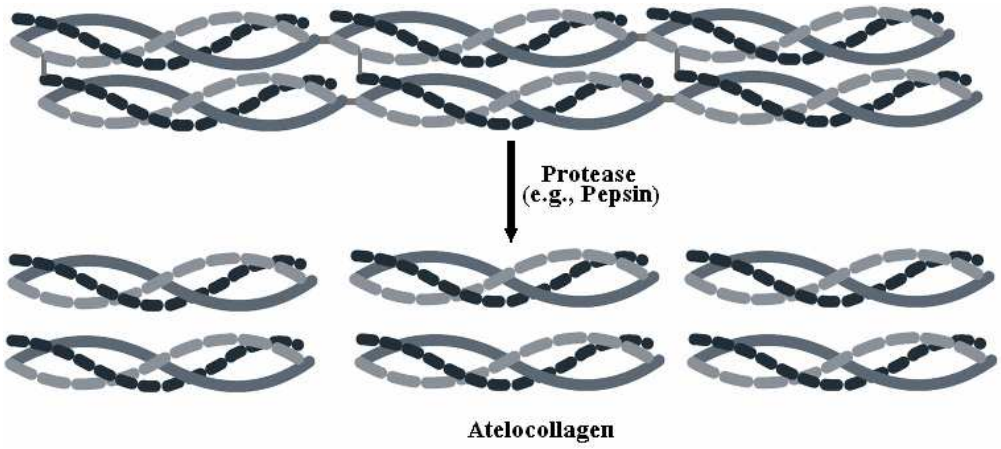

Figure 4. Atelocollagen via enzymatic digestion. The treated collagen keeps its intermolecular bonding; that is why atelocollagen has the same physical properties of untreated collagen.

1.5 Surface modification by plasma treatment

Any biomaterial needs to have an appropriate mechanical strength, malleability and functionality. These parameters are governed by bulk properties, whereas biological responses are controlled by surface composition. As aforementioned, natural polymers are abundant and inherently embedded in biological functions and these are indeed, enormous advantages. Nonetheless, their physicochemical manipulation is restricted by the drastic changes that these may undergo after any kind of treatment. For this reason, plasma surface modification technique is extensively employed in biomaterials science, since surface properties and biocompatibility may be enhanced

confining the treatment to the top layer without affecting the bulk properties [62, 63]. Albeit there is a broad range of values, many authors agree that plasma penetration depth is within the mesoscopic scale (1-1,000 nm) and depends on the substrate and on the operating parameters [64-67]. Figure 5 outlines the main features that were described.

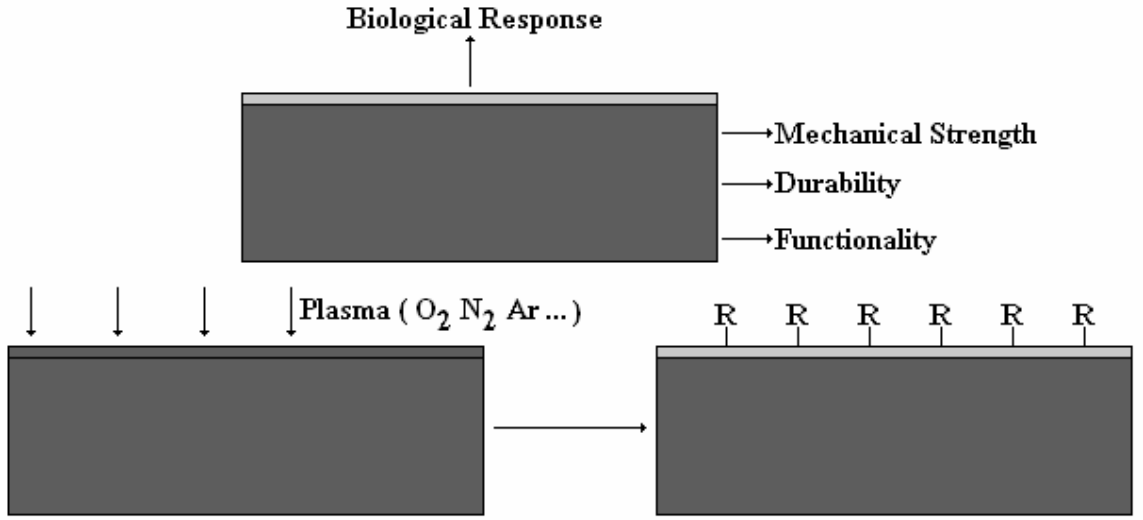

Figure 5. Conceptual approach of plasma treatment in material science.

Plasma treatment is a versatile surface modification technique. Different carrier gases (carbon dioxide, fluorine-containing gases, hydrogen, nitrogen, nitrogen-containing gases, noble gases, oxygen and steam) may produce unique surface properties for various applications. Table 5 displays some of the main advantages and disadvantages of this technique.

Plasma is partially ionised gas, which is composed of neutral atoms or molecules, rays of broad range of energies and positive and negative charged particles with approximately equal charge densities. Plasmas may be generated when an atom or a molecule gains enough energy and is excited into energetic states by radio frequency, microwave, or electrons from a hot filament discharge. As soon as the random molecular kinetic energy exceeds the ionisation energy, collisions then strip some electrons from the atoms, creating a mixture of electrons and ions [68].

Table 5. The main pros and cons of plasma surface modification technique

advantages	disadvantages
▪ modification is confined to the surface layer without affecting bulk properties	▪ It is not a simple process. *i.e.*, it has high-scales difficulties
▪ excited species may modify the surfaces of all polymers, regardless of their nature or chemical reactivity	▪ all the chemical reactions implied are still unknown
▪ versatility: each gas elicits different chemical and physical modifications	▪ It is not a selective reaction. Therefore, it is very difficult to know the amount of organic entities formed on the surface
▪ modification is fairly uniform over the whole surface	
▪ solvents are not required	

Surface reactions are the ones that occur at the interface between a gas and the outermost layer of a material. Plasma treatment conveys reactivity onto the treated surface via plasma species, electron, ions, and UV-radiation [69]. In general, reactions of gas plasmas with polymers may be classified *viz.*:

Plasma polymerisation: it is the creation of a thin film on the polymer surface by organic monomer polymerisation as methane, ethane or tetrafluoroethylene.
Sputtering and etching: in a sputtering process, materials are ablated from the polymer surface by chemical reactions and physical etching to form volatile molecules. Neon and argon are frequently used for eliminating organic contaminants from polymer surfaces. The difference between sputtering and etching is only in the

amount of material that is removed from the surface. When degradation is prominent, etching will take place on the polymer surface [70].

Surface properties hinge on the carrier gas; then the treatment may be performed for cleaning, sputtering, etching, implantation, and deposition. Noble gases are inert and do not react chemically with the treated sample, but these transfer reactivity giving rise to bond breakage and the subsequent origin of free radicals, which may endure for several days and undergo various reactions. In fact, helium, neon and argon are often applied for cleaning and sputtering, whilst argon, krypton, and xenon have found applications in implantation and deposition. Argon is the typical noble gas used in plasma treatment owing to its relatively low cost, availability, and high yield in sputtering processes. Inert gases are also utilised for cleaning before treating the substrate with a reactive gas [71, 72].

Nitrogen is also considered as a low reactive gas on account of its electron configuration ($1s^2\ 2s^2\ 2p_x^1\ 2p_y^1\ 2p_z^1$). However, oxygen functionalities are always incorporated in polymer surfaces after non-oxygen plasma treatments. This phenomenon is a consequence of breaking bonds and free radicals formation that once the samples are withdrawn from the plasma reactor trigger the reaction between atmospheric oxygen and free radicals. Surface wettability, printability, adherence and biocompatibility may be ameliorated by nitrogen-containing plasma treatments [73, 74].

Oxygen and oxygen-containing plasmas are conventionally employed to increase surface energy. Oxygen plasma may react with polymers producing a variety of functional groups, like hydroxyl, carbonyl, carboxyl, ether, and peroxide. Furthermore, oxygen plasma also induces etching on polymer surfaces through reactions of atomic oxygen with the surfaces. Steam plasma is rarely applied, but its effect is akin to the oxygen one [75-77].

Fluorine-containing plasma is also set for surface etching. Nevertheless, it has the opposite effect than oxygen and oxygen-containing plasmas. *Ergo*, this treatment prevents the inclusion of oxygen functionalities decreases surface energy. Although hydrogen plasmas may be employed to raise hydrophobicity, this technique is not effective, since atomic hydrogen reacts with atmospheric oxygen forming oxygen-containing groups on the surface [78]. Figure 6 illustrates the surface topography changes after plasma treatment in individual atmospheres.

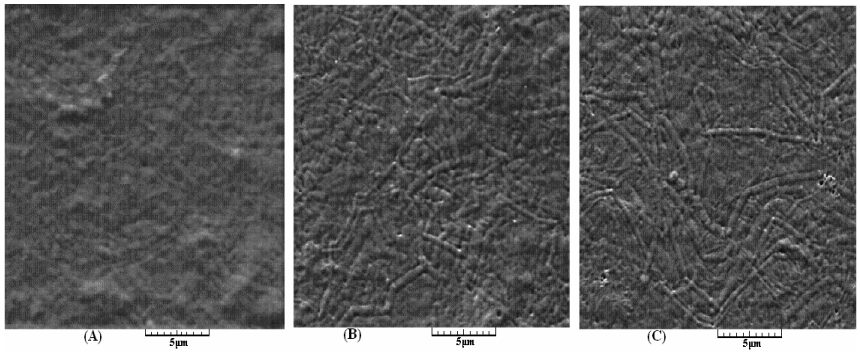

Figure 6. Changes upon atelocollagen surface morphology: (A) untreated surface, (B) Argon, (C) air plasma treated.

1.5.1 Plasma treatment: Applications in industry and medicine

Plasma treatments have been used for numerous applications in the biomedical area: contact lenses, artificial heart valves, vascular grafts, catheters, dialysis membranes, prosthetic devices, and materials for bone joint repair and replacement. Contact lens materials are a classic case, as these materials need high oxygen permeability and surface hydrophilicity. The commercially available lens material is a copolymer of an alkyl acrylate and a siloxane. The siloxane component in the copolymer increases oxygen permeability but reduces the lens surface, resulting in poor patency of the tear film/lens interface. Oxygen-plasma treatment on the lens material increases hydrophilicity, making the lenses more comfortable for wearing [79]. Plasma deposition (including both grafting and polymerisation) may create a new surface setting which promotes cell attachment and cell proliferation on tested surfaces.

Plasma sputtering and etching treatments are applied for cleaning, sterilisation and/or wettability improvement. Table 6 resumes some of the search areas of plasma treatment in biomedicine [80].

Table 6. Applications of plasma treatment in biomaterials engineering

Barriers coatings drug-release, gas-exchange membranes, device protection, protection from corrosion (additives, catalysers, plasticisers)
Biosensors biomolecules immobilised on surfaces
Blood-compatible surfaces vascular grafts, catheters, stents, heart valves, membranes, filter (for blood-cell separation) biomolecules immobilised on surfaces
Non-fouling surfaces contact lenses, wound healing, catheters, biosensors
Tissue engineering and cell culture cell growth, antibody production

Plasma treatment is also used in other realms as diverse as automotive industry (flocking glove boxes, gluing or soft coating dashboards, gluing, painting or metallisation of exterior or interior parts, painting bumpers, bonding filters for automotive engine and connector housing), electronics (deoxidation of contacts, activation of electronic assemblies before encapsulation, pre-treatment of connectors and CD-parts), photography (coating and printability), textile industry (improving dyeability and modifying permeability), mobile phones, aeronautics, sports, superconductors, packaging industry and ceramics [81].

Pursuant to the New York stock exchange (NYSE) plasma technology is a promising industry in the cleantech sector with 125 listed companies from different countries that earned roughly $49 million in 2008 [82].

1.5.2 Plasma technologies linked to tissue engineering

In the previous unit was stated that scaffolds must render a suitable surface chemistry for cell attachment, proliferation and differentiation; and functional groups influence these cellular extents. On account of the reactivity that plasma confers to any material surface, there are two strategies for surface modification. The first one is using plasma treatment for grafting a hydrophilic polymer onto an inert surface in order to increase surface hydrophilicity. The other possibility is by introducing polar entities straight on the surface [83]. As may be noticed, these approaches have one thing in common: the inclusion of functional groups.

Concerning to physical properties, surface morphology also influences cell anchorage. A higher effective surface permits more available sites for cells-substrate interaction [84]. Figure 7 is a schematic illustration of cell-material surface interaction that underlines how surface crystallinity, hydrophilic/hydrophobic character, roughness and chemical compositions all impinge on cell adhesion.

There is an extensive literature which has been devoted to study substrate surface modification to support human cells [85, 86].

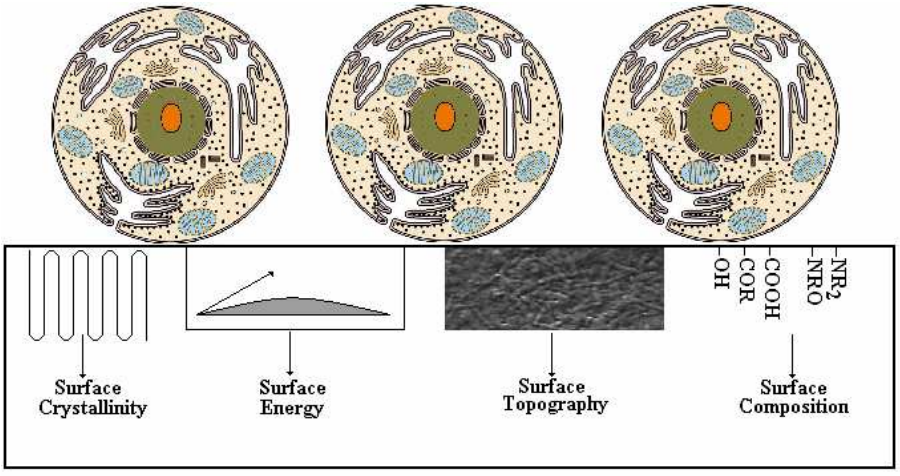

Figure 7. Schematic illustration of cell-material surface interactions.

2. SURFACE CHARACTERISATION TECHNIQUES

To confirm chemical and physical changes in the polymer surface, X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), in combination with attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), scanning electron microscopy imaging (SEM) and measurement of the contact angle with a series of liquids of varying surface tension (Zisman series) are normally employed. There are a lot of surface analysis techniques which may be chosen in conformity with the surface valuation that is required. Therefore, factors as sampling depth, surface diagnosis, universality, cost-effectiveness and sample suitability have to be deemed before selecting the pertinent techniques. It is important to emphasise that each technique supplies distinctive but complementary information.

2.1 X-ray photoelectron spectroscopy (XPS)

The XPS is a quantitative spectroscopic technique that provides the elemental composition, chemical bonding and electronic state of all chemical elements expect hydrogen and helium in a surface. The technique is based on the irradiation of the sample surface by a monochromatic X-ray beam. An electron energy analyser measures the kinetic energy of the emitted photoelectrons from the surface. XPS is surface specific in virtue of it does not extend beyond 7 nm in depth and it has to be performed under ultra-high vacuum conditions. The obtained data from a typical experiment is plotted as electron emission intensity as a function of ejected electron binding energies. There are two types of spectra, the survey spectra are used for elemental analysis, and the photoelectron peaks from each element in the molecule may be identified (figure 8). The area under each peak corresponds to the atomic concentration; thereby surface elemental composition may be calculated by relative peak intensities.

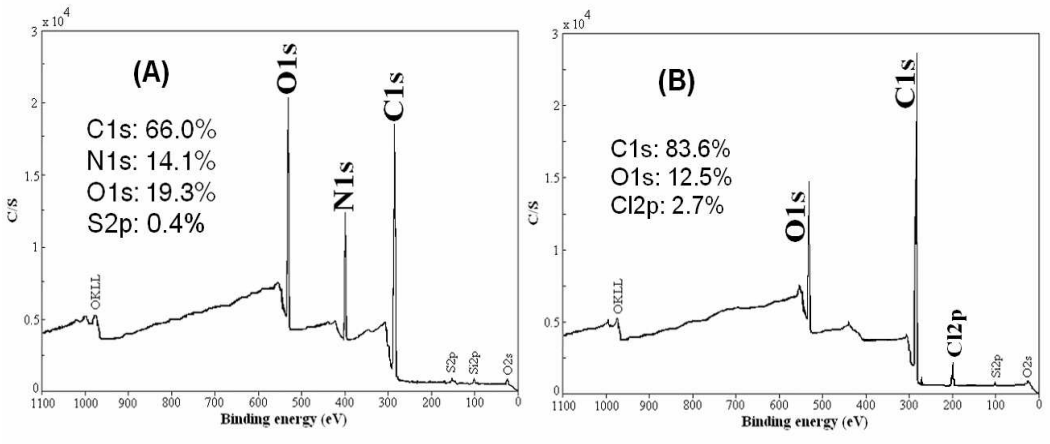

Figure 8. XPS survey-scan spectra of: (A) collagen and (B) PVC.

The high-resolution spectra supply chemical bonding information. The electron binding energy depends not only on photoemission, but also on the chemical state of the element and the chemical surroundings in the molecule. Hence, XPS is also helpful to differentiate organic functionalities through chemical shifts [87]. For example, Poly(vinylidene fluoride), known as PVDF, has two peaks at around 286.3 and 290.8 eV corresponding to CH_2 and CF_2 groups, respectively, which may be easily identified. Nonetheless, the technique is not able to resolve some functional groups, such as C-OH/C-O-C or between carbonyl and carboxyl groups, because these functions have very similar binding energies. Figure 9 shows the range of chemical shifts observed in C1s and O1s core-level binding energies.

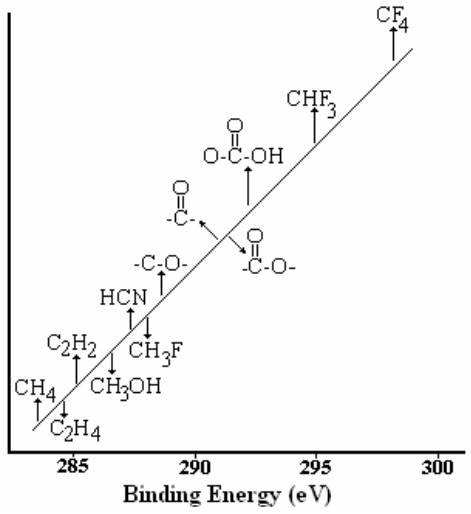

Figure 9. Experimental chemical shifts of some molecules and functional groups [88].

2.2 Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

Infrared (IR) is electromagnetic radiation between 14,290 and 10 cm^{-1}, and it is divided into three regions: near IR (14,290-4,000 cm^{-1}), mid-infrared (4,000- 400 cm^{-1}) and far-IR (400-10 cm^{-1}). The mid-infrared range is used in the study of molecular vibration associated with rotational structure. Radiation in this range is absorbed and converted by an organic molecule into molecular vibration energy, and the absorption frequency or wavelength depends upon relative atomic masses, bond types, bond strength and molecular arrangement. Thereby, this spectroscopic technique may be utilised to elucidate and identify organic functions and compounds, as the example which is given in figure 10.

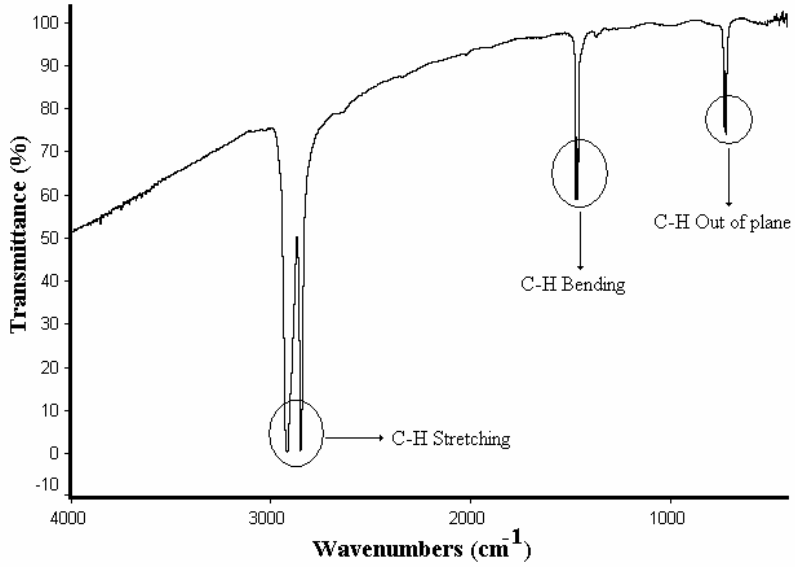

Figure 10. IR spectrum of polyethylene along with its chemical shifts [81].

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) is based on the fact that when a beam of radiation passes from a dense to a less dense medium reflection occurs. This type of surface spectroscopy uses an accessory which measures the changes that take place when an infrared beam is internally

reflected and comes in contact with a sample (figure 11). The depth of penetration is roughly from 0.5 to 5.0 μm making it the least sensitive surface technique. Notwithstanding, it may be employed as a complementary analysis or when surface sensitivity is not necessary [89, 90].

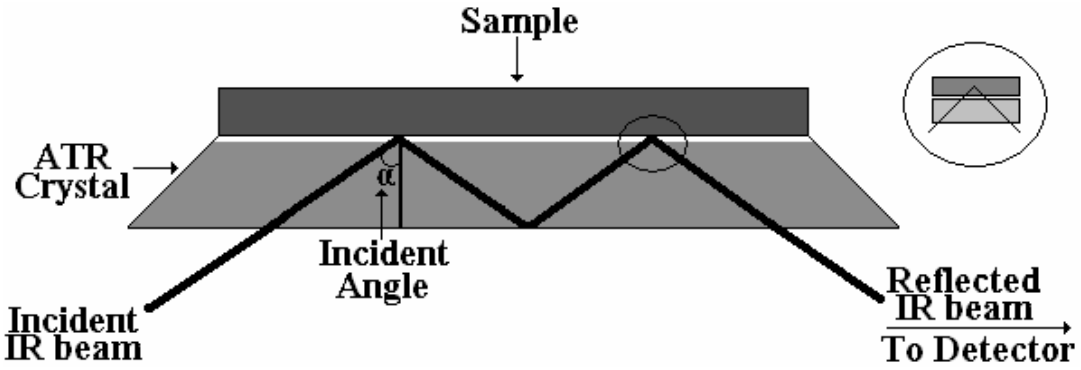

Figure 11. Representation of a multiple reflection ATR system.

2.3 Scanning electron microscopy imaging (SEM)

The scanning electron microscopy (SEM) produces an electronically magnified image of an examined sample. SEM uses a focused high-energy electron beam to generate a variety of signals at the surface of a solid specimen. The signals produced by electron-sample interactions reveal information about sample's surface morphology (figure 12).

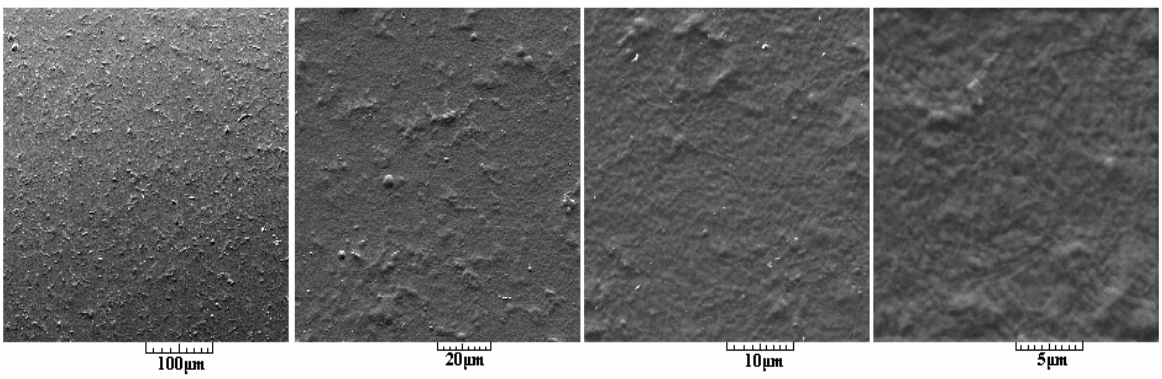

Figure 12. SEM Images of atecollagen films taken at different magnifications.

The data is collected over a selected area giving a two dimensional image. This microscopy has a greater magnification than an optical microscope, as it engages

electrons that have wavelengths about 100,000 times shorter than visible light (photons). Thus, SEM may achieve magnifications 1,000 times greater than light microscopes do (one million times the actual size) [91]. Polymers samples are coated with gold or gold/palladium alloy, on account of this coating brings conductivity to the sample. It is also useful to protect the samples from beam damage which may provoke structural and chemical changes at polymer surfaces.

2.4 Contact angle measurement

Contact angle measurement is a method utilised to determine surface energy of solids. The contact angle θ is the angle formed by the solid-liquid and the liquid-vapour interfaces (figure 13).

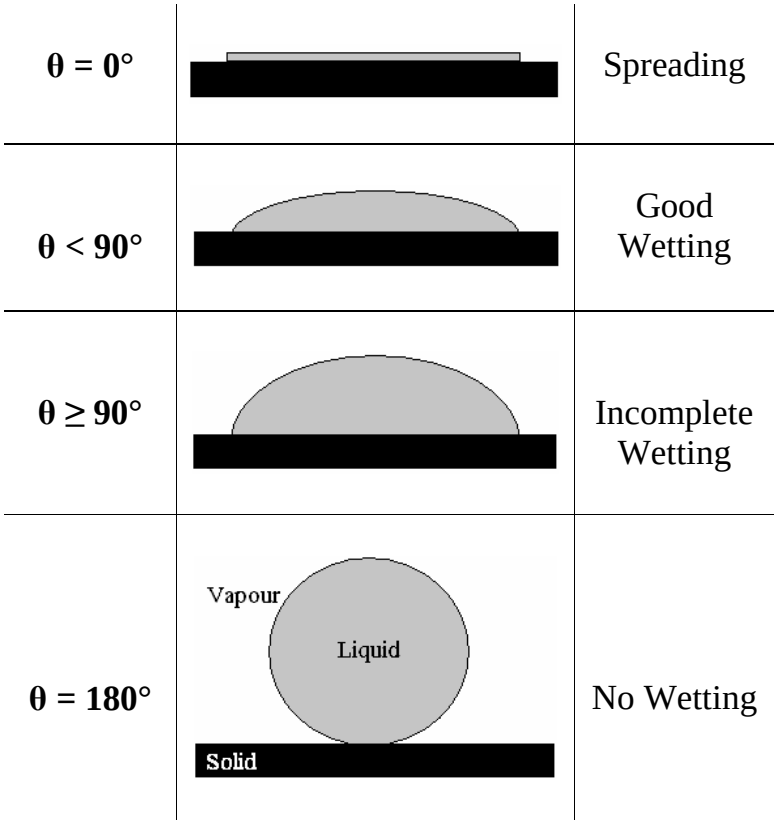

Figure 13. Contact angle measurement: Description of the spreading and wetting states.

It is quantified by measuring the tangent angle of a liquid drop with a solid surface. When the contact angle is 0º, the liquid spreads over the solid surface. In contrast, if θ is 180º, the liquid does not wet the solid surface. A solid surface is considered wettable if the contact angle is less than 90º; whereas the surface is not wettable when θ is above 90º. Solid surfaces may be classified into two basic groups, hydrophilic (wettable with water and high surface energy) and hydrophobic (not wettable with water and low surface energy). The contact angle is measured by goniometer; the measurement may also be made with photographs or video images.

Drop size and volume, liquid density, liquid vapour pressure, surface quality, time of equilibrium, solubility and lab temperature may considerably influence the measurement [92].

3. AIM OF THE WORK

The primary idea of the present research is the development of a novel and cost-effective material for tissue engineering applications. Consequently, the following goals will be pursued within the framework of this doctoral dissertation:

- The preparation of uniform and reproducible collagen thin films by using atecollagen (since further experiments may not be carried out without good quality substrates).
- To activate the aforesaid specimen surfaces via plasma treatment and the subsequent assessment of chemical and physical changes that may occur on by using spectroscopic and microscopic techniques (ATR-FTIR, XPS, SEM imaging and contact angle measurement).
- To explore these surface-modified materials as potential extracellular matrices in terms of cell adhesion and proliferation.
- To introduce the concept of antimicrobial moieties to these samples and evaluate the effect of this property on cell growth.
- To contribute to the scientific discussion surrounding surface science, biomaterials and tissue engineering.

4. FINDINGS SYNOPSIS

The next section intends to highlight the most significant findings of this doctoral attempt. The complete results, discussion and further clarifications are available in the framing publications text.

Paper I dealt with surface modification by using argon plasma treatment on atelocollagen thin films. The effects of the treatment were evaluated on untreated and treated samples by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), Scanning Electron Microscopy (SEM) imaging, and X-ray Photoelectron Spectroscopy (XPS). HaCaT keratinocytes cell line was seeded on these specimens and cell proliferation was measured by MTT assay. The main results were as follows:

Spectroscopy data displayed an increase in the oxygen content as may be substantiated by a higher percentage of oxygen and a raise in O/C ratio (surface oxidation). On the other hand, nitrogen content slightly diminished (etching phenomenon). The ATR spectra showed the typical collagen bands; it means that atelocollagen was retained upon plasma treatment. Nevertheless, the bands decreased in intensity after the treatment, which may be attributed to plasma-induced reactions. In addition, there were notorious changes in the region corresponding to C-O stretching.

As for morphology analysis, treated surfaces were rougher than the untreated ones and presented etched features; this information was in agreement with the spectroscopy data, in which surface etching was observed.

Cell proliferation was *ca.* 1.6 higher on the treated samples as it was reflected by the MTT assay. It evidences that the treated specimens possess higher cell-substrate

compatibility. This biological assay was also supported by light microscope images which corroborated an enhanced cell adhesion.

Paper II had a similar scope than *paper I*, but in this case the atelocollagen films were modified by using nitrogen and air plasma treatment. Both untreated and treated samples were characterised by spectroscopic and microscopic techniques. HaCaT cell growth was also carried out on both group of samples and cell proliferation was determined by MTT assay. The main outcomes were as follows:

The XPS data showed an increment of the oxygen content after air and nitrogen plasma treatment; besides a rise in the O/C ratio confirmed the extent of surface oxidation after each treatment. This extent was higher on the samples treated with air than on the nitrogen-treated ones. Likewise, the nitrogen content increased just in nitrogen plasma treatment, which is most likely related to nitrogen-containing entities.

The ATR-FTIR spectra evinced that the distinctive peptide bands remained, which means that the collagen backbone was not drastically modified. Notwithstanding; the intensity of the bands decreases after the treatments (plasma-induced reactions). For instance, the characteristic amide N-H stretching shifted down denoting an alteration in the surface chemistry. The peaks associated to methyl and methylene stretching deformation shifted and changed in strength and it was particularly noticed on nitrogen treated samples. The nitrogen medium also gave rise to bands which may be assigned to N-O stretching. The bands corresponding to C-O-C linkages underwent evident alterations after both treatments.

In regard to surface morphology, remarkable changes were found in SEM images. *E.g.*, the treated samples depicted rougher surface topography and etched features, whereas the untreated films had a relatively smooth morphology. This was in

agreement with the spectroscopic results where no matter the carrier gas, surface ablation took place.

Higher absorbance values and a more cell aggregates adhered on the treated surfaces sustained that these surface-modified substrates had better conditions for HaCaT keratinocyte cell growth.

Paper III focuses upon the study of HaCaT keratinocytes cell viability and proliferation on potential antimicrobial substrates. Five commercial biocides (benzalkonium chloride, bronopol, chitosan, chlorhexidine and irgasan) were added at different concentrations (2.0, 1.0, 0.5, 0.2 0.1 and 0.02%) in atelocollagen matrices. In order to assess how these antibacterial agents influence the growth of keratinocyte cells on atelocollagen substrates, cytotoxicity and proliferation were determined by MTT assay. This part of this doctoral research provided the following upshot:

Results revealed that the extent of cytotoxicity quantified as percentage of viability was low regardless of the substance or the concentration that was used. The viability range was within 74-99%. The lowest value was found on collagen-benzalkonium chloride 2.0%, whilst the highest one was for the matrix with bronopol 0.02%. The data indicated that these biocides did not drastically inhibit the viability of HaCaT cell line (According to ISO 10993-5, percentages of cell viability above 80% are considered as non-cytotoxicity; within 80-60% weak; 60-40% moderate and below 40% strong cytotoxicity respectively). The percentage of viability with respect to concentration depicted a fall by increasing concentration and the samples with chlorhexidine (2.0 and 1.0%); Irgasan (2.0 and 1.0%) and benzalkonium chloride (2.0, 1.0, 0.5 and 0.2%) were found significant different at a confidence level of 95%.

Concerning to the capacity of these compounds to restrain HaCaT cell growth, it disclosed that chitosan, bronopol and chlorhexidine had lower inhibition in comparison with irgasan and benzalkonium chloride which were the strongest ones in all the cases.

HaCaT cell proliferation on the substrates with and without biocides was also evaluated by using MTT assay. It was found out that cell attachment marginally diminished. The substrates endowed with chitosan and bronopol did not show a statistical significance, whereas the samples with chlorhexidine, irgasan and benzalkonium chloride have statistical significance and three cases were considered as very significant ones (irgasan 2.0%, benzalkonium chloride 2.0 and 1.0%). Cell proliferation under chitosan medium was higher than under the other media followed by bronopol, chlorhexidine, irgasan and benzalkonium respectively. Cell proliferation as a function of concentration exhibited a decreasing trend by increasing concentration of inhibitor, which coincides with the findings obtained in the cytotoxicity study.

5. CONCLUDING REMARKS

5.1 Conclusion

The conclusions and closing remarks pertaining to the present doctoral thesis are summarised as follows:

Despite argon is an inert gas its primary effect on plasma treatments is to supply and deposit energy via plasma species giving rise to crosslinking, bond breaking and diverse intramolecular rearrangements, which in contact with atmospheric air may drastically alter surface properties.

Air contains reactive gases in its composition. Nitrogen is a low reactive gas that only reacts spontaneously with few chemical compounds. In that respect, the exposure on air and nitrogen plasmas gives free rein to many reactions which occur either in the plasma chamber or after the treatment at ambient conditions leading to surface ablation and functionalisation. The O/C ratio under air medium was higher, this points out that the main difference amongst two treatments is that air plasma treatment incorporates more oxygen-containing functionalities and consequently, more hydrophilicity to the samples. It necessary to recall that plasma treatment with any of these gases does not produce a unique functionality on collagen surface.

Keratinocytes cell proliferation is remarkably enhanced after using these carrier gases. It is ascribed to the favourable role of plasma treatment in inducing surface oxidation and increasing surface roughness confirming the strong dependence of cell adhesion and proliferation on surface properties and biocompatibility.

Cytotoxicity is concomitant to concentration and relies on each agent. Bronopol and chitosan emerge as the less hazardous to this cell line; as long as irgasan and benzalkonium chloride exhibit more power of inhibition.

HaCaT cell viability is barely altered by the presence of these substances. Only in eight from thirty samples the cell viability was statistically lower than that found on the substrates without biocides. It means that any of these samples offers an optimal environment for this cell line, which is a key factor in choosing a scaffold for tissue engineering. For this reason, the study of these antimicrobial atelocollagen films is a worthwhile cause in the development of medical implants able to withstand and neutralise harmful microorganisms.

5.2 Contribution

The outcome of this doctoral research strengthens knowledge on the following fields of science and technology:

- The use of atelocollagen as a prospective tissue engineering scaffold due to its tractability.
- The effect of air, argon and nitrogen plasma exposures on chemical, physical and biological properties of biopolymers surfaces.
- Human cell growth on natural polymers and how cell culture may be heightened.
- Strategies and viability studies for rendering biopolymers resistant to microbial colonisation.
- HaCaT keratinocytes cell response on the aforesaid substrates.
- Progress in novel materials as potential candidates for medical implants.

5.3 Future Prospects

Tissue engineering is path towards creativity with eminent progress in the last years. It may be evidenced by the plethora of related publications. There are outstanding breakthroughs which have been attained in clinical trials on tissues and organs. Nevertheless, the final goal of tissue engineering shall be the complete regeneration of organs with complicated physiological and biochemical functions, such as heart, liver, kidneys and pancreas. With regard to human skin, the effort has to be aimed at having a controllable and enhanced cell growth for both epidermal and dermal layer repair in cases of full-thickness burns, non-healing ulcers, pigmentation defects, melanomas, blistering disorders, psoriasis and other chronic wounds. It is well known fact that self-healing is long and with many difficulties.

It is impossible to ignore that nosocomial contamination circumscribes a serious danger for medical implants, since infections may lead to material futility, and also by the fact that any cultured cell material carries the risk of transmitting viral or bacterial sepsis. Hence, the study of antibacterial surfaces by using different kind of antimicrobial agents is something that deserves to be heeded.

The great challenge would be to create novel biomaterials able to simulate the extracellular matrix in inducing attachment that in turn may be capable of counteracting pathogenic microorganisms.

Just few areas of technology demand more interdisciplinary teamwork than tissue engineering or have the impact on quality and length of life; therefore, researchers are facing untold challenges in materials, biology and engineering sciences.

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APPENDICES

This supplementary section includes two appendices where the first one contains the full-text version of the framing publications. The second appendix summarises the *curriculum vitae* of the author.

Appendix A: Framing Papers

This appendix holds the full text version of three framing publications where the reader may find further information of each research along with experimental details, results, discussion and the corresponding references.

Publication I

Cell Proliferation of HaCaT Keratinocytes on Collagen Films Modified by Argon Plasma Treatment

By:

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Article

Cell Proliferation of HaCaT Keratinocytes on Collagen Films Modified by Argon Plasma Treatment

Jorge López García [1], Ahmad Asadinezhad [1], Jiří Pacherník [2], Marián Lehocký [3,*], Ita Junkar [4], Petr Humpolíček [3], Petr Sáha [1] and Pavel Valášek [3]

[1] Polymer Centre, Faculty of Technology, Tomas Bata University in Zlín, T.G.M Sq. 275, 76272, Zlín, Czech Republic; E-Mails: vextropk@gmail.com (J.L.G.); asadinezhad@ft.utb.cz (A.A.); saha@utb.cz (P.S.)

[2] Faculty of Sciences, Institute of Experimental Biology, Masaryk University Brno, Kotlářska 2, 61137, Brno, Czech Republic; E-Mail: jipa@sci.muni.cz (J.P.)

[3] Tomas Bata University in Zlín, T.G.M Sq. 5555, 76001, Zlín, Czech Republic; E-Mails: humpolicek@uni.utb.cz (P.H.); valasek@ft.utb.cz (P.V.)

[4] Plasma Laboratory, Department of Surface Engineering, Jožef Stefan Institute, Jamova cesta 39, SI-1000, Ljubljana, Slovenia; E-Mail: ita.junkar@ijs.si (I.J.)

* Author to whom correspondence should be addressed; E-Mail: lehocky@post.cz; Tel: +420608616048; Fax: +420576031444.

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Abstract: Argon plasma treatment was used to modify the surface of atelocollagen films using a plasmochemical reactor. To evaluate the effects of the treatment, untreated and treated samples were characterized by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), Scanning Electron Microscopy (SEM) imaging, and X-ray Photoelectron Spectroscopy (XPS) techniques. Cell growth was carried out by culturing human immortalized keratinocyte (HaCaT) cells and proliferation was measured via MTT assay. It was observed that argon plasma treatment significantly enhanced the extent of cell proliferation, which was ascribed to the favourable role of plasma treatment in inducing surface oxygen-containing entities together with increasing surface roughness. This can be considered as a potentially promising approach for tissue regeneration purposes.

Keywords: plasma treatment; atelocollagen; surface modification; HaCaT; cell proliferation

1. Introduction

Collagen is a fibrillar protein that exists in nearly all mammalian tissues. It constitutes ca. 25% of whole-body protein content. Its abundance is especially concentrated in connective tissues, such as tendons, ligaments, cartilage as well as skin. Moreover, it is connected with important biological functions, such as tissue formation and cell attachment [1]. On this account, collagen is extensively used in the design of materials with potential applications in the biomedical field.

Skin consists of different types of cells, such as keratinocytes, melanocytes, and fibroblasts [2]. It is well established through wound healing, transplantation, and cell culture studies that human immortalized keratinocyte (HaCaT) cells play a crucial role in epidermal tissue regeneration, since they are spontaneously transformed to human keratinocytes which have the traits of basal epidermal keratinocytes and can be delivered in deep burns. Hence, this cell line can be exploited as an *In vitro* model for highly proliferative epidermis [3-6].

Argon is a member of the noble gases class which is a group of chemical elements of identical properties. They are colourless, odourless, and possess very low reactivity because of a full valence shell [7]. For this reason, plasma treatment by noble gases is of importance since these gases do not react chemically with the treated sample. Nevertheless, it conveys reactivity onto the treated surface via plasma species, electrons, ions, and UV-radiation. In fact, inert gas plasma treatment is used in periods typically from 1s to several minutes, and this exposure is enough to abstract hydrogen and to produce free radicals at or near the surface which then interact to form cross-links and unsaturated groups; notwithstanding, these chemical and physical changes are restricted to the top several hundred angstroms without affecting bulk properties [8, 9].

Depending upon the noble gas and time of the treatment, this type of plasma treatment can then be performed for cleaning, sputtering, etching, implantation, and deposition on the substrates; for example, helium, neon and argon are often used for cleaning and sputtering, while argon, krypton, and xenon have found applications in implantation and deposition. Nonetheless, argon is the most common noble gas used in plasma treatment due to its relatively low cost, availability, and high yield [10].

Noble gases-based plasma treatment has widely been used in a diversity of applications, *e.g.*, surface modification of polymers [11-17], glass [18], carbon fibres [19], superconductors [20], metal and alloys [21,22], and textiles [23-26]. As for medical uses, there are several studies conducted on cleaning surfaces [27-29] and cell attachment [30-35]. In this contribution, focus is directed onto the surface modification via argon plasma treatment and examination of keratinocyte human cell growth on atelocollagen surfaces which has not yet been done. Furthermore, a systematic study of argon plasma treatment effects on HaCaT keratinocyte cell response of atelocollagen films with a view to designing a novel material potentially suitable for tissue engineering applications is undertaken. This is accomplished via surface probe techniques together with pertinent biological assays.

2. Results and Discussion

2.1. Surface Spectroscopic Analysis

X-ray photoelectron Spectroscopy (XPS) spectra have been recorded in order to gain an insight into the chemical modifications of the treated surface. From the analysis of these spectra, carbon (C1s), oxygen (O1s), nitrogen (N1s), and sulphur (S2p) elements were detected on both untreated and argon plasma treated samples surfaces. The respective elemental compositions along with the corresponding atomic ratios are shown in Table 1. The data shows a considerable increase in the oxygen content subsequent to the argon plasma treatment which is also reflected as a raise in O/C ratio. This is ascribed to the occurrence of surface oxidizations which are, stimulated by the argon plasma treatment followed by exposure to the air [36-39]. As suggested by the data, nitrogen content slightly decreases which can be connected with the etching phenomenon, while that of sulphur remains unchanged. The presence of sulphur seems to stem from sulphur-containing amino acids [40, 41].

Table 1. Elemental compositions of the untreated and treated films by XPS measurement.

Sample	C1s%	N1s%	O1s%	S2p%	N1s/C1s	O1s/C1s
Untreated samples	66.0	14.1	19.3	0.4	0.21	0.29
Argon plasma treatment	58.1	11.3	25.0	0.4	0.19	0.43

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectra from untreated and argon plasma treated samples are shown in Figure 1. In the untreated sample spectrum, amide I and II characteristic bands at 3,306, 3,079, 1,630, and 1,549 cm^{-1} can be identified, where the ones at 3,306 and 3,079 cm^{-1} correspond to peptide bond N-H stretching. The C=O stretching interaction with the amide I N-H vibration gives rise to an absorption at 1,630 cm^{-1}, while its interaction with C-N leads to the band at 1,549 cm^{-1}. Characteristic signals due to amide III appear at 1,280, 1,239, and 1,204 cm^{-1} which originate from N-H bending and C-N stretching interactions. In addition, in the spectrum of the untreated sample, one can recognize the other typical amide vibrations assigned to C-N stretching and N-H wagging which appear at 1,400 and 693 cm^{-1}, respectively [42-43]. The absorption peaks within the 3,000-2,800 cm^{-1} spectral range are attributed to aliphatic C-H stretching; likewise, the bands at 1,491, 1,450, and 851 cm^{-1} are associated with C-H vibrations. As for the argon plasma treated sample, the characteristic amide bands are also visible which implies that atelocollagen is retained upon plasma treatment. However, the intensity of the bands decreases after the treatment which can be a consequence of plasma-induced reactions [44-45]. Furthermore, the bands within 1,160-1,000 cm^{-1}, assigned to C-O stretching mode undergo evident alterations after argon plasma treatment. Particularly, a peak at around 1,100 cm^{-1} due to C-O-C linkage gains strength, which means that argon plasma treatment affects the chemical composition of the surface, as earlier discussed in more detail in XPS section. It should also be noted that unsaturated double bonds, such as alkenes (-C=C-) and imino (-C=N-) can possibly arise after the argon plasma treatment [46-47], but cannot be viewed due to overlapping peaks, prospective cross-linking or the insufficient surface-sensitivity of ATR-FTIR.

Figure 1. ATR-FTIR spectra of: (A) untreated and (B) argon plasma-treated films.

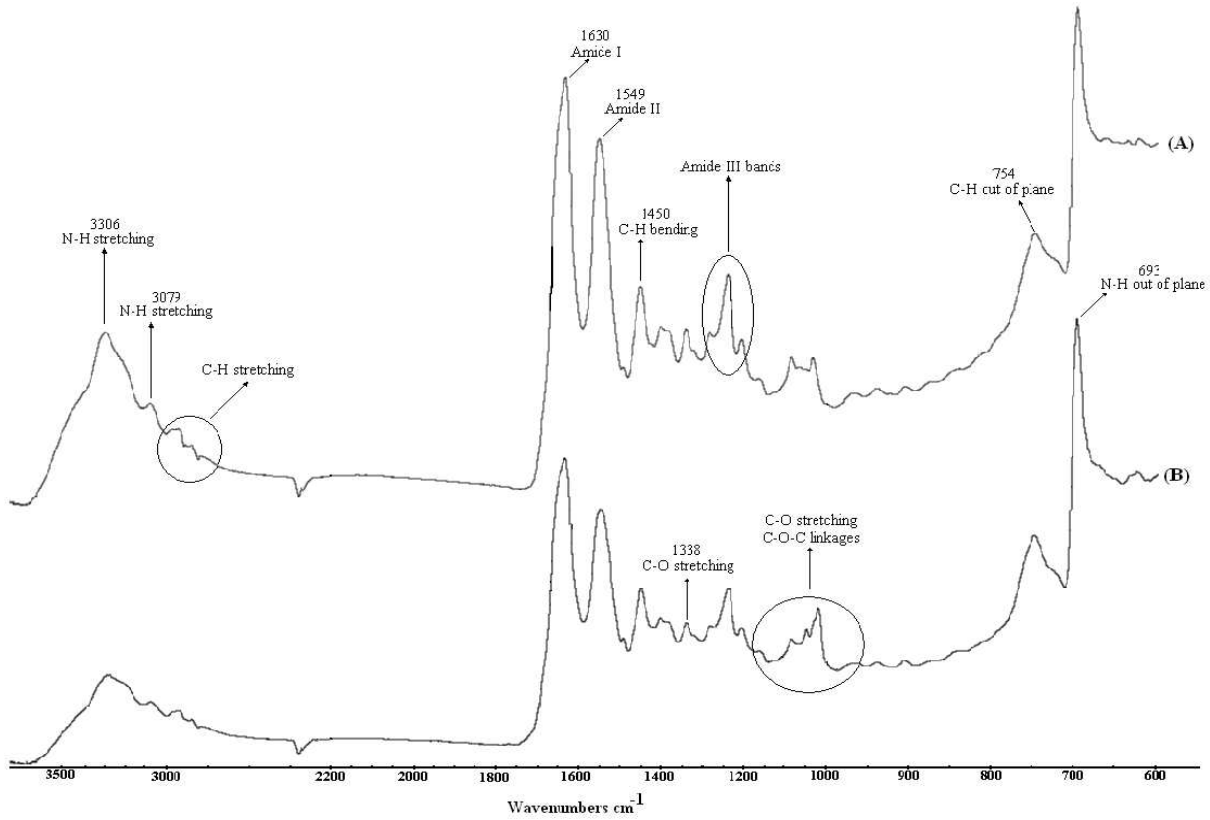
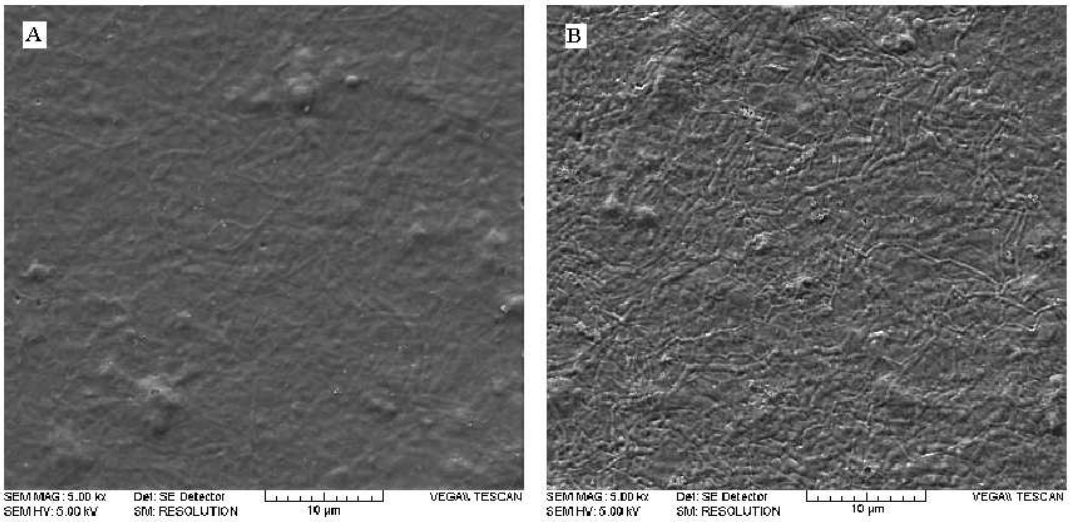


Figure 2. SEM surface topography of (A) untreated and (B) argon plasma-treated samples.



2.2. Effects of argon plasma upon surface topography

Visible changes in surface topography can be observed after argon plasma treatment, as evident from Scanning Electron Microscopy (SEM) images (Figure 2). The untreated film has a relatively smooth morphology, while that of argon plasma treated sample is of higher roughness and shows

etched features. This is in agreement with the results mentioned in previous sections, where surface etching due to the argon plasma treatment was observed. An enhanced roughness is considered as a beneficial factor in adhesion processes, since it is a consequence of surface etching and functionalization [48].

2.3. Effects of argon plasma treatment on cellular behaviour of HaCaT on collagen films

HaCaT cells behaviour on untreated and argon plasma treated samples evaluated using MTT assay is given in Figure 3. It is found out that cell attachment significantly increases, as reflected by the absorbance value which is approximately 1.6 times higher for the argon plasma treated samples than the untreated films. This indicates that argon plasma treated films show higher cell-substrate compatibility and thus, are more appropriate for tissue regeneration applications. This is also supported qualitatively by the light microscope images shown in Figure 4, where the extent of cell adhesion on untreated and argon plasma treated samples can be compared to a control specimen. A higher amount of cell aggregates in form of ripple-like areas adhered on the surface is identified for argon plasma treated sample. Although the mechanisms of HaCaT adhesion and proliferation upon the different substrates are still unclear, it is well known that film roughness and porosity influence cell adhesion and cell proliferation. Besides, surface polar entities content is a crucial factor because HaCaT cells are mainly attached by carbonyl and carboxyl groups, thus their cell growth tends to be favoured in hydrophilic surfaces [49-53]. This attachment is also supported by hydrogen bonding and van der Waals forces, which reinforce the linking between cells and films [54]. An increase in roughness and surface polar functionalities after exposing atelocollagen films to argon plasma promote cell adhesion and proliferation. The results suggest that argon plasma treated atelocollagen films are potentially suitable materials for tissue regeneration.

Figure 3. Comparison of HaCaT cells growth upon untreated and argon plasma-treated films, measured by MTT cell proliferation assay at 570 nm.

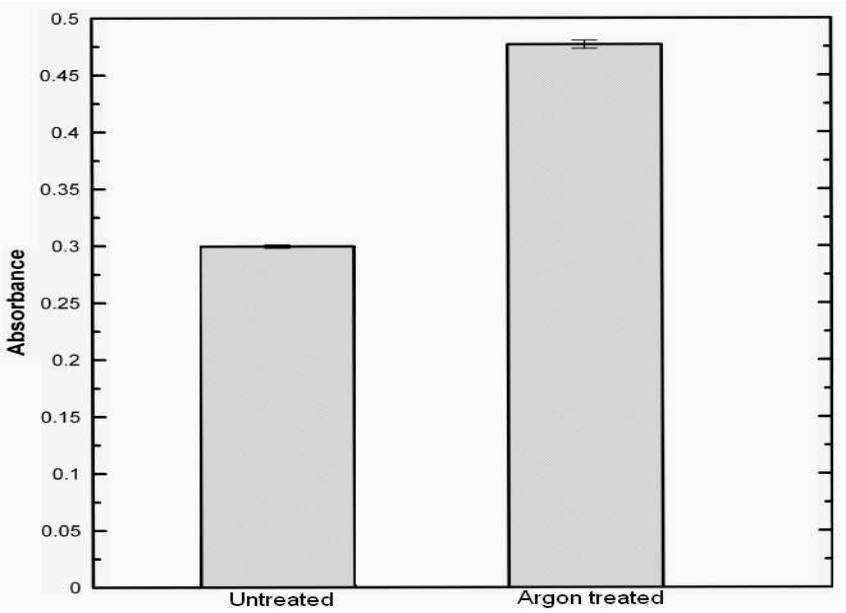
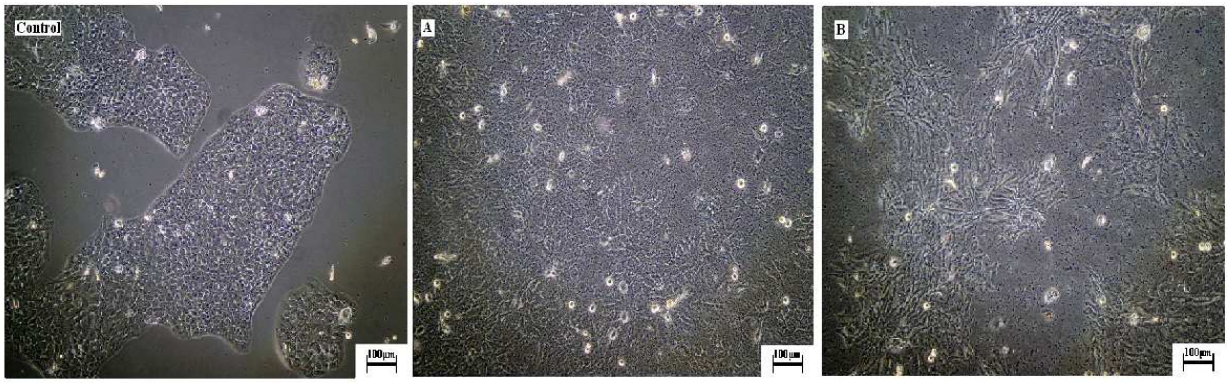


Figure 4. Light micrographs of Human skin HaCaT keratinocytes in culture upon the collagen films compared with control, (A) untreated, (B) argon plasma-treated.



3. Experimental

3.1. Materials

Atelocollagen from bovine Achilles tendon (emulsion which contents 1.43% of atelocollagen, pH 3.5) was supplied by Vipo A.S, Slovakia. Tissue culture dishes of diameter 40 were provided by (TPP, Switzerland). Acetic acid 99% was obtained from Penta, Czech Republic. Vybrant® MTT cell proliferation Assay kit (V-13154) was purchased from Invitrogen Corporation, USA. Human immortalized non-tumorigenic keratinocyte cell line HaCaT [55] (Ethnicity, Caucasian; Age, 62 years; gender, Male and tissue, skin) was supplied by CLS Cell Lines Service, Germany. DMEM (high glucose) supplemented with 2 mM L-glutamine solution and 10% fetal calf serum were provided by Biotech Inc, USA, which was used as the culture medium for HaCaT cell line.

3.2. Preparations of collagen films

The atelocollagen was solubilized in 0.1M acetic acid to prepare a 0.1%w/w solution using an IKA RCT stirring machine (IKA® works, Inc, Germany) for 4 hours at 1,000 rpm. Then, 2 mL of this solution was poured into each of the tissue culture dishes. Thereafter, the solvent was evaporated at ambient conditions for three days.

3.3. Plasma treatment

The plasma treatment of the collagen thin films was carried out by using plasmochemical reactor (Femto, Diener electronic, Germany) with a chamber of 100 mm diameter and 270 mm length, operated at frequency of 40 kHz, pressure of 40 Pa, and power input of 50 W. Argon grade 4.5 was used as carrier gases provided by Linde, A.G., Germany. The feed rate in all experiments was 5 cm^3/min. The duration of the plasma treatment was 5 minutes for each sample.

3.4. Spectroscopic techniques

Surface chemical composition of both untreated and treated collagen films were evaluated by XPS which was performed in a XPS microprobe instrument PHI Versaprobe (Physical Electronics, USA). The base pressure in the XPS analysis chamber was about 6×10^{-8} Pa. The samples were excited with X-rays over a 400 μm spot area with a monochromatic Al $K_{\alpha1,2}$ radiation at 1,486.6 eV. The photoelectrons were detected with a hemispherical analyzer positioned at an angle of 45° with respect to the normal of the sample surface. The energy resolution was about 0.5 eV and survey-scan spectra were made at 187.85 eV. Individual high-resolution C1s and O1s spectra were taken at 23.5 and 0.1 eV energy step for 30 minutes and the concentration of different chemical states of carbon in the C1s peak was determined by fitting the curves with symmetrical Gauss-Lorentz functions. The spectra were fitted using MultiPak v7.3.1 software from Physical Electronics, USA; which was supplied with the spectrometer. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was carried out on a FTIR spectrometer Avatar 320 (Nicolet, USA) equipped with ATR accessory. Each spectrum was obtained by recording 32 scans at a 2 cm^{-1} resolution. The spectral range was within 4,000-650 cm^{-1}.

3.5. Microscopic techniques

The surface morphology of collagen untreated and treated films were analyzed by using SEM on a Vega LMV microscope (Tescan s.r.o, Czech Republic) operated at 5 kV. The specimens were 30° tilted to attain higher resolution and observation. All of samples were coated with a thin layer of Gold/Palladium alloy. The images were taken at magnifications of 5,000 ×.

3.6. Cell culture

HaCaT keratinocyte cells were seeded onto the treated and untreated samples in the culture dishes and incubated at 37 °C for 4 days. The cell culture was performed in 32 tissue culture dishes, 16 for untreated films and 16 for argon plasma treated films.

3.7. Cell proliferation

The HaCaT cell proliferation on treated and untreated films was determined after 4 days of culturing by MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is yellow, to a purple formazan product]. A volume of 10 μL of 12 mM MTT was taken for cell incubation performed at 37 °C for 4 hours in the darkness. The media were then decanted and washed with phosphate-buffered saline solution (PBS). The produced formazan salts were dissolved with dimethylsulphoxide (DMSO, Sigma-Aldrich, USA), and the absorbance was measured at 570 nm to estimate the formazan concentration [56]. The statistical analysis of the recorded data was managed using Student's t-test, where a confidence level of 95% ($p < 0.05$) was considered statistically significant and 99% ($p < 0.01$) was considered very significant.

4. Conclusions

The effects of argon plasma treatment upon atelocollagen surface films and HaCaT cell response have been studied by means of surface probe techniques together with the biological assay. It is assumed that the primary effect of argon plasma treatment is to provide and deposit energy via plasma species, such as electrons, ions and UV-radiation to the substrate surface leading to cross-linking, bond breaking and different kind of intramolecular rearrangements, which in contact with air or other reactive species can drastically alter the surface properties. The spectroscopic techniques show a decrease of the nitrogen content along with the attenuation of the N-H and C-H bands, which can be attributed to the etching phenomenon. It is observed that argon plasma treatment significantly enhances the extent of cell proliferation ascribed to the favourable role of plasma treatment in inducing surface oxygen-containing entities and increasing surface roughness. The keratinocyte HaCaT cell proliferation notably increases confirming the strong dependence of cell adhesion and proliferation on the surface properties and biocompatibility.

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Sample Availability: Contact the authors.

Publication II

Enhanced Keratinocyte Cell Attachment to Atelocollagen Thin Films through Air and Nitrogen Plasma Treatment

By:

García, J.L.; Pacherník, J.; Lehocký, M.; Junkar, I.; Humpolíček, P.; Sáha, P.

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Enhanced Keratinocyte Cell Attachment to Atelocollagen Thin Films through Air and Nitrogen Plasma Treatment

Jorge López García[1], Jiří Pacherník[2], Marián Lehocký[3], Ita Junkar[4], Petr Humpolíček[3], and Petr Sáha[1]

ABSTRACT Collagen films (Atelocollagen from bovine Achilles tendon) were prepared in tissue culture dishes and their surfaces were modified by using air and nitrogen plasma treatment. The treated samples were characterised by surface probe techniques including attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), scanning electron microscopy imaging (SEM) and X-ray photoelectron spectroscopy (XPS). In addition, human immortalised non-tumorigenic keratinocyte cell line (HaCaT) was seeded on the treated and untreated films and cell proliferation was measured by using MTT assay test. The characterisation results confirmed physical and chemical changes on the collagen surface, such as increase in the extent of surface oxidation and surface roughness as well as, the treated samples showed better cell growth than untreated ones, and therefore this approach may be taken into account in the development of promising materials for tissue regeneration applications.

Keywords: *Atelocollagen • Thin films • Plasma treatment • Cell adhesion • Cell growth • Radio frequency discharge*

Introduction

Collagen is a fibrillar protein, which has received remarkable interest by its abundance in the animal kingdom (*ca.* 25% of the whole-body protein content). It is involved in many important biological functions, such as tissue formation, cell adhesion and proliferation. In fact, collagen is present in the skin, tendons and cartilage [1]; thus, it affords extensive possibilities in designing materials for biomedical applications. Several authors have reported on different aspects of collagen research, *e.g.* polymer blends, drug delivery, polymer grafting, tissue engineering, and nerve regeneration amongst others [2, 3].

HaCaT (human adult low calcium high temperature) cells are spontaneously transformed human keratinocytes which have characteristics of basal epidermal keratinocytes; so that cell line may be used as an *in vitro* model for highly proliferative epidermis in tissue engineering [4].Tissue engineering is a multidisciplinary and emerging field focused on providing substitutes that replace tissue and restore functions. It may be reached by combining synthetic/natural polymers with mammalian cells. There are three basic materials for the creation of a new tissue; cells, scaffold and growth factor. Cells synthesise matrices of the new tissue, while the scaffold provides an appropriate environment for cells, and growth factor assists and promotes cells to regenerate new tissue. Hence; the substrate-cells interaction is absolutely relevant and ubiquitous in clinical trials, such as skin transplants for patients with burns, skin ulcers, corneas, cartilage, bone, liver and other tissues [5, 6].

In this regard, plasma treatment is an effective and economical tool in the field of surface modification, which may be used quickly, easily and it does not require relatively expensive devices for its operation. The primary effect of plasma treatment is to convey reactivity to the treated surfaces via electrons, ions and UV-radiation confining the treatment to the top layer without affecting bulk properties. For these reason, plasma surface modification has been done on different materials, such as polymers, carbon fibres, ceramics, and proteins [7-9].

In this work, collagen surface modification was carried out by using air and nitrogen plasma treatment. The treated samples were characterised employing, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, scanning electron microscopy imaging (SEM), and X-ray photoelectron spectroscopy (XPS).

M. Lehocký (□)

[1]Polymer Centre, Faculty of Technology, Tomas Bata University in Zlín, T.G.M Sq. 275, 76272, Zlín, Czech Republic
e-mail:lehocky@post.cz
[2]Institute of Experimental Biology, Faculty of Sciences, Masaryk University Brno, Kotlářska 2, 61137, Brno, Czech Republic
[3]Tomas Bata University in Zlín, T.G.M Sq. 5555, 76001, Zlín, Czech Republic
[4]Department of Surface Engineering, Plasma Laboratory, Jožef Stefan Institute, Jamova cesta 39, SI-1000, Ljubljana, Slovenia

In addition, keratinocyte cells (HaCaT) were seeded onto the treated and untreated collagen samples, and the cell proliferation was measured by MTT assay. The characterisation methods along with the corresponding biological assays confirmed physical and chemical changes as well as better cell proliferation on the plasma-treated samples thus, these materials may prospectively serve for tissue regeneration in medicine.

Materials and Methods

Preparation of Collagen Films

An atelocollagen emulsion from bovine Achilles tendon pH 3.5 which contents 1.43% of collagen (Vipo A.S, Slovakia) was dissolved in 0.1 M water solution of acetic acid to prepare a 0.1% w/w solution using a stirring machine for 4 hours at 1,000 rpm. Then, 2 mL of this solution was poured into each of the tissue culture dishes. Finally, the solvent was evaporated at ambient conditions for three days.

Plasma Surface Treatment

The plasma treatment of such prepared collagen thin films was carried out by using plasmochemical reactor (Femto, Diener electronic, Germany) with a chamber of 100 mm diameter and 270 mm length operated at frequency of 40 kHz, pressure 40 Pa, and power input of 50 W. Air and nitrogen were used as carrier gases. The feed rate in all experiments was $5cm^3$/min. The duration of the plasma treatment was 5 minutes for each sample. Subsequently, the specimens were taken and normally manipulated for the next tests.

X-Ray Photoelectron Spectroscopy (XPS)

Measurements of the collagen samples were performed in a XPS microprobe instrument PHI Versaprobe (Physical Electronics, USA). The base pressure in the XPS analysis chamber was about 6×10^{-8} Pa. The samples were excited with X-rays over a 400 μm spot area with a monochromatic Al $K_{\alpha1,2}$ radiation at 1,486.6 eV. The photoelectrons were detected with a hemispherical analyser positioned at an angle of 45° with respect to the normal of the sample surface. The energy resolution was about 0.5 eV. And, survey-scan spectra were made at 187.85 eV, while for C1s and O1s individual high-resolution spectra were taken at 23.5 and 0.1 eV energy step. The concentration of different chemical states of carbon in the C1s peak was determined by fitting the curves with symmetrical Gauss–Lorentz functions. The spectra were fitted using MultiPak v7.3.1 software which was supplied with the spectrometer.

Attenuated Total Reflection Fourier-transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of both treated and untreated samples were recorded on a FTIR spectrometer Avatar 320 (Nicolet, USA) equipped with a ZnSe crystal at an incident angle of 45º. For each sample 32 scans were recorded within the spectral range of 4,000–500 cm^{-1} in the absorbance mode with a resolution of 2 cm^{-1}.

Scanning electron microscopy (SEM)

SEM images were taken by Vega LMV (Tescan s.r.o, Czech Republic). The operating voltage of the Secondary Electron (SE) detector was 5 kV. All observed samples were coated with a sputtered thin layer of Au/Pd. In order to get higher resolution and observation of the surface topography, the specimens were 30º tilted and the images were taken at 5,000x magnification.

HaCaT Cell Cultivation

Human immortalised non-tumorigenic keratinocyte HaCaT cells (Cell Lines Service, Germany) were seeded onto the treated and untreated samples in the culture dishes and incubated at 37 °C for 4 days [10]. DMEM (high glucose) supplemented with 2 mM L-glutamine solution and 10% fetal calf serum was used as the culture medium.

MTT Proliferation Assay

The cell proliferation was determined after 4 days of culturing employing the MTT cell proliferation assay kit on each sample [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide, which is yellow, to a purple formazan product]. A volume of 10 μL of 12 mM MTT was taken for cell incubation performed at 37 °C for 4 hours in the darkness. The media were then decanted and washed with phosphate-buffered saline solution (PBS). The produced formazan salts were dissolved with dimethylsulphoxide (DMSO, Sigma-Aldrich, USA). The formazan concentration was measured in a spectrophotometer at 570 nm [11].

Results and Discussion

The XPS survey spectra of the untreated and plasma treated films are given in fig. 1. In addition; Table 1 displays the relative peak areas of C1s, N1s and O1s along with O/C and N/C ratios for each experiment. The XPS data shows an increment of the oxygen content after air and nitrogen plasma treatment, besides a rise in the O/C ratio confirm the extent of surface oxidation after each treatment. This increment may be attributed to the oxidation of pendant groups present in collagen backbone. There are two reactions involved, the first ones occur directly in the plasma chamber and these are related to the oxygen and CO_2 content of air (*ca.* 20.94% and 0.031% respectively); on the other hand, the other reactions arise once the films are taken out from the plasma reactor through free-radical reactions. Either of these treatments introduces many radicals which may survive for several days, reacting with oxygen and other reactive species present in the atmosphere leading to surface functionalisation. It is particularly noticed on nitrogen plasma treatment. This gas is considered low reactive due to its molecular stability, and it only reacts spontaneously with few reagents. Nonetheless, oxygen functionalities are always incorporated in nitrogen plasma treatments [12-14]. Other kind of reactions specially centred on N-terminal and C-terminal of the protein might occur giving rise to Nitro R-NO_2, nitroso R-N=O, nitrosamine R_1-N(-R_2)-N=O, amide (RCONH_2) or amines (R-NH_2) entities, which may explain the increase in the N/C ratio after nitrogen plasma treatment. It should be noted that plasma treatment with any of these gases does not produce a unique functionality on collagen surface.

The ATR-FTIR spectrum of the untreated sample divided into two regions along with air and nitrogen plasma treated ones are presented in fig. 2. Peptide bond characteristic bands at ~3,300, 3,080, 1,645, 1,550, 1,240 and 695 cm^{-1} are identifiable and indeed, due to peaks overlapping, plasma treatment effects are rather masked. The amide I is a broad band around 1,640-1,630 cm^{-1} originated from C=O stretching vibrations coupled to N–H bending vibration. According to this absorption range, this band may come from collagen

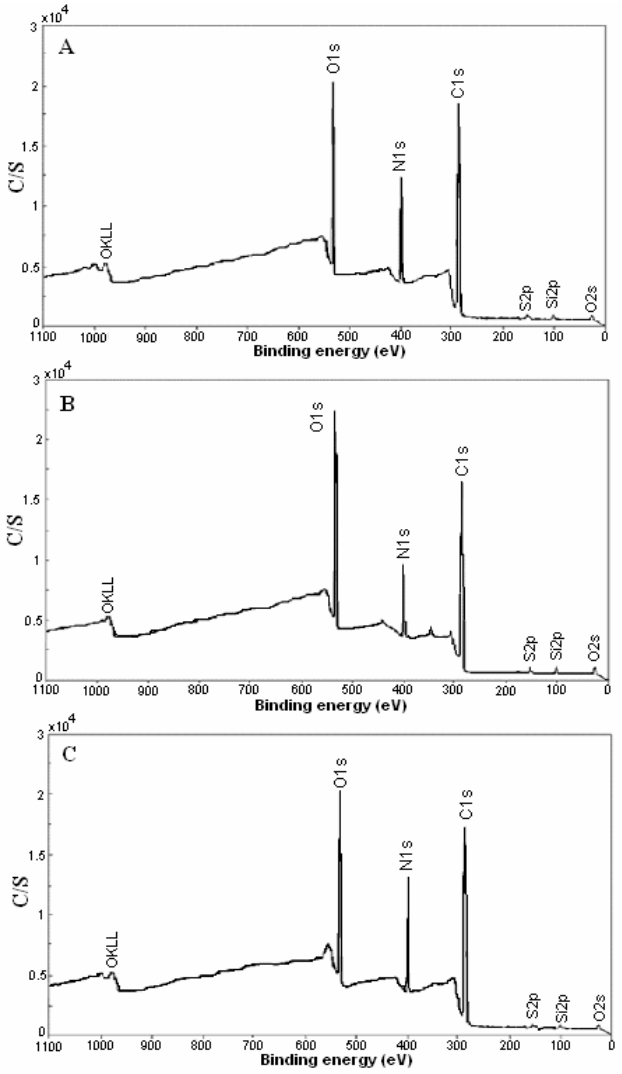

Fig. 1 XPS survey spectra of: (A) untreated; (B) air and (C) nitrogen plasma treated films.

Table 1 Relative Peak Areas of C1s, N1s and O1s before and after the treatment.

Sample	C1s%	N1s%	O1s%	N/C	O/C
Untreated	66.0	14.1	19.3	0.21	0.29
Air treatment	58.1	11.3	25.9	0.20	0.46
N_2 treatment	58.2	18.4	22.7	0.32	0.39

with high proline-proline-glycine sequences [15]. The amide II band ~1,550 cm^{-1} surges of N-H bending vibrations coupled to C-N stretching vibrations, and the amide III characteristics bands appear at ~1,280, 1,240 and 1,200 cm^{-1}

which result from the interaction between N-H bending and C-N stretching. The band at 695 cm^{-1} is a usual amide vibration which arises from out of plane N-H wagging [16]. After each plasma treatment, the typical peptide bands remain visible which means that collagen is retained and its backbone

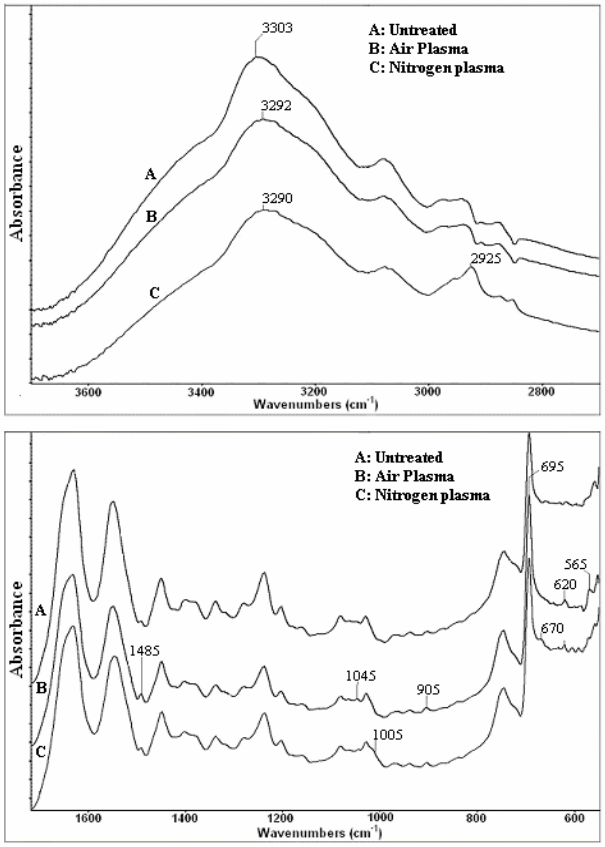

Fig. 2 ATR-FTIR spectra of: (A) untreated; (B) air and (C) nitrogen plasma treated films

is not drastically modified. Nevertheless, the intensity of the bands decreases after the treatments which is probably a consequence of plasma-induced reactions. For instance, the characteristic amide N-H stretching at ~3,300 and 3,080 cm^{-1} shift down indicating of an alteration in the surface chemistry. This is particularly pronounced after treatment in nitrogen medium where the peaks within 2,950-2,850 cm^{-1} corresponding to methyl and methylene stretching deformation shift and change in strength. The intensity of some weak signals at ~1,485, 620 and 565 cm^{-1} increases following plasma treatment which may be assigned to nitro compounds 1,485 N=O stretching, 670 and 562 cm^{-1} NO_2 bending vibrations. Moreover, the bands within the 1,100-850 cm^{-1} spectral range associated with C-O stretching vibrations present clear alterations after both plasma treatments; and it may be observed on the peaks that appear at around 1,045, 1,000 and 905 cm^{-1} respectively. Therefore, it is found out through surface chemistry analysis that plasma treatment, either in air or nitrogen, is capable of affecting the surface chemistry via ablation and functionalisation.

Significant changes on the surface morphology after the plasma treatment may be observed in SEM images (fig. 3). The treated samples show relatively rougher surface topography and etched features, as long as the untreated film has a relatively smooth morphology.

This is in concordance with the spectroscopic results where regardless of the carrier gas, surface ablation was observed.

A rise in roughness and surface polar functionalities after exposing atelocollagen films to air and nitrogen plasma treatment promote cell attachment and proliferation, and it is in agreement with the HaCaT cell proliferation measurement (fig. 4) which was significantly higher for either of these treated samples than for the untreated films.

This previous information is also supported qualitatively by the micrographs in fig. 5, where a higher amount of cell aggregates in form of ripple-like areas adhered on the surfaces is identified. The plasma-treated collagen surfaces offer several favourable sites and polar groups which potentially link human cells.

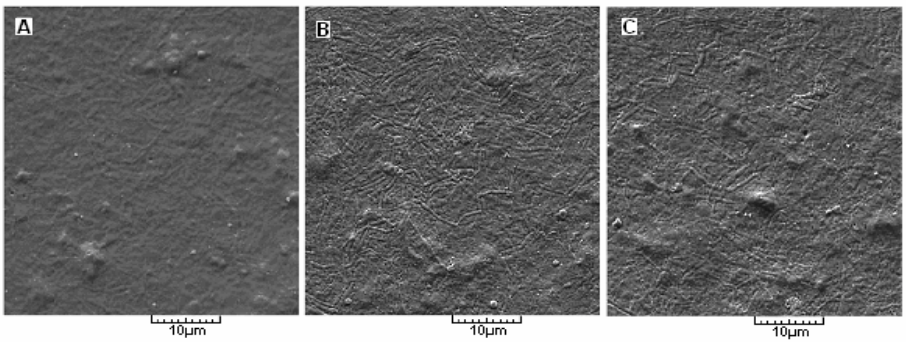

Fig.3 SEM images of (A) untreated, (B) air and (C) nitrogen treated films

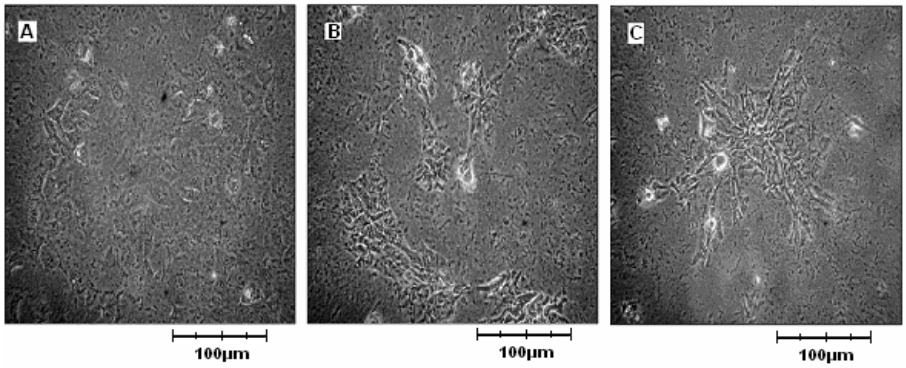

Fig. 5 Micrographs of human skin HaCaT keratinocytes in culture upon the collagen films. (A) untreated, (B) air and (C) nitrogen treated

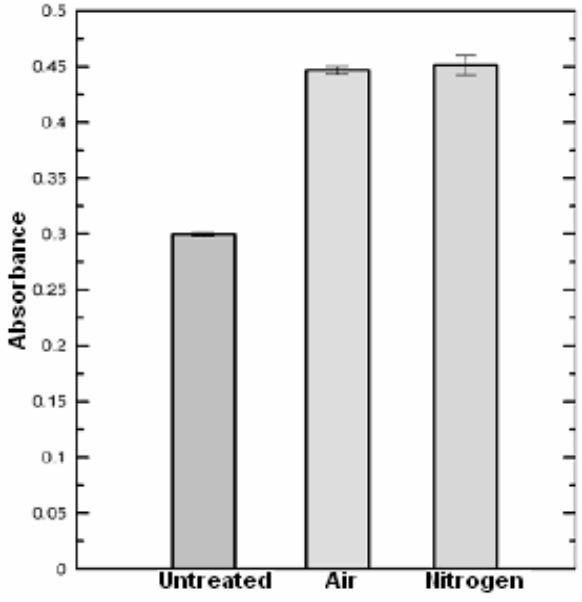

Fig. 4 Absorbance values of Formazan at 570 nm for determining the HaCaT cell proliferation by MTT assay (the error bars depict standard deviations).

It is exactly the case of HaCaT keratinocytes whose cell growth tends to be favoured in hydrophilic surfaces, and their attachment is mainly concern of carbonyl and carboxyl groups along with hydrogen bonding and van der Waals forces, which reinforce the linking between cell and films [17-19].

Surface morphology is a key factor in cell attachment mechanism, since an increase in surface roughness lead to higher effective surface area and thus, more available sites for cells-substrate interaction. This information suggests that those plasma-treated films possess better condition for cell growth and are potentially suitable for tissue engineering applications likewise, the important role of organic entities and surface morphology on cell growth.

Conclusions

XPS and ATR-FTIR results indicated that plasma treatment is able to alter collagen thin films via chemistry surface modification. Other consequence of plasma surface modification was found microscopically. Air and nitrogen plasma treated samples showed an increase in surface roughness.

The keratinocytes HaCaT cell proliferation was remarkably improved after both treatments, pointing up the special connexion between physicochemical surface properties and cell growth. Thus, air and nitrogen plasma treatment are effective tools to increase organic entities (mainly O-containing functional groups) and surface roughness of collagen films providing chemical and physical features which enhance attachment and proliferation of keratinocyte cells.

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Publication III

HaCaT keratinocytes response on antimicrobial atelocollagen substrates: Extent of cytotoxicity, cell viability and proliferation.

By:

García, J.L.; Lehocký, M.; Humpolíček, P.; Sáha, P.

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HaCaT KERATINOCYTES RESPONSE ON ANTIMICROBIAL ATELOCOLLAGEN SUBSTRATES: EXTENT OF CYTOTOXICITY, CELL VIABILITY AND PROLIFERATION

Jorge López-García [1], Marián Lehocký [1,*], Petr Humpolíček [1] and Petr Sáha [1]

[1] Centre of Polymer Systems, Tomas Bata University in Zlín, nám. T.G.Masaryka-5555, 76001 Zlín, Czech Republic.

* Author to whom any correspondence should be addressed; E-Mail: lehocky@post.cz; Tel.: +420608616048; Fax: +420576031444.

ABSTRACT

Purpose: The effective and widely tested biocides: Benzalkonium chloride, bronopol, chitosan, chlorhexidine and irgasan were added in different concentrations to atelocollagen matrices. This contribution seeks to evaluate how these antibacterial agents influence cell viability and proliferation of HaCaT cell line on modified atelocollagen substrates.

Methods: The cell line used in the present study was human immortalised keratinocyte (HaCaT). Cell viability and cell proliferation were assessed by MTT assay.

Results: Acquired data indicated a low toxicity by employing any of these chemical substances. Furthermore, cell viability was comparatively similar to the samples where there were no biocides.

Conclusion: Regardless of the agent, collagen-cell-attachment properties are not drastically affected by the incorporation of those into the substrate. Therefore, these

findings suggest that the samples referred to herein as ‘antimicrobial substrates’ might be suited for tissue engineering applications.

Keywords: atelocollagen; antibacterial surface; cytotoxicity; cell proliferation; MTT assay.

INTRODUCTION

As a biomaterial for industrial application, collagen has been widely used in many fields, such as cell cultures, cosmetics, foods and medicines (1). With regard to medical applications, this protein possesses an excellent biocompatibility, innocuousness and biodegradability. Due to these reasons, this matrix is deemed as a primary resource in biomedical applications and one of the most useful biomaterials that may be prepared in different forms, such as blocks, films, gels (2), pellets, sheets (3), sponges (4) and tubes (5).

Skin comprises essentially three types of cell: keratinocytes, melanocytes and fibroblasts. It is foreseen through wound healing, transplantation and cell culture studies that HaCaT cells may be used as an *in vitro* model for highly proliferative epidermis in tissue engineering (6-8).

A serious difficulty in tissue replacement is biofilm formation, which is responsible for infections over the treated areas. Several implants have to be removed by their poor performances. Indeed, infections are the foremost common cause of biomaterial implant failure in medicine (9-12). Different types of polymers are often sterilised via dry/wet heating or irradiation. Nevertheless, these materials may get contaminated by microbes once they are exposed to atmospheric conditions again. Hence, the preparation of anti-infective polymeric implants is a

powerful way to overwhelm this problem (13-16). One method to develop this kind of materials is by adding organic or inorganic antimicrobial agents in the polymers during processing (17-21).

Antimicrobial agents are substances able to counteract or inhibit microorganisms (22). Benzalkonium chloride (Fig. 1) is a quaternary ammonium compound, which is one of most used and known synthetic biocides in pharmaceutics (23, 24). Bronopol (2-bromo-2-nitropropane-1,3-diol) (Fig. 1) is a chemical compound which has a low toxicity in mammals and a high activity against bacteria, being a popular preservative in many personal care products as shampoos, colognes, deodorants, facial tissues, shaving creams amongst others personal hygiene products (25, 26) Chlorhexidine, (1,1-hexamethylene bis[5-(4-chlorophenyl)biguanide]) (Fig. 1), is recognised as a chemical antiseptic by its effectiveness on both gram-positive and gram-negative bacteria. It is the active ingredient in oral rinses, skin cleansers, topical solution for veterinary use and, in small quantities, it is used as a preservative (27, 28). Another biocide, that holds immediate long term antibacterial efficiency and marginal toxicity in clinical use, is Irgasan (5-chloro-2-(2,4-dichlorophenoxy)phenol) (Fig. 1) (29).

Chitosan is a deacetylated product of chitin, which is produced by chitin alkaline deacetylation (Fig. 2) and this product has properties, such as antimicrobial activity and low toxicity (30). Besides, it is highly synthesised because chitin is the second-most abundant biopolymer in nature. It is found in the cell walls of fungi, the exoskeletons of arthropods, insects, molluscs and cephalopods (31).

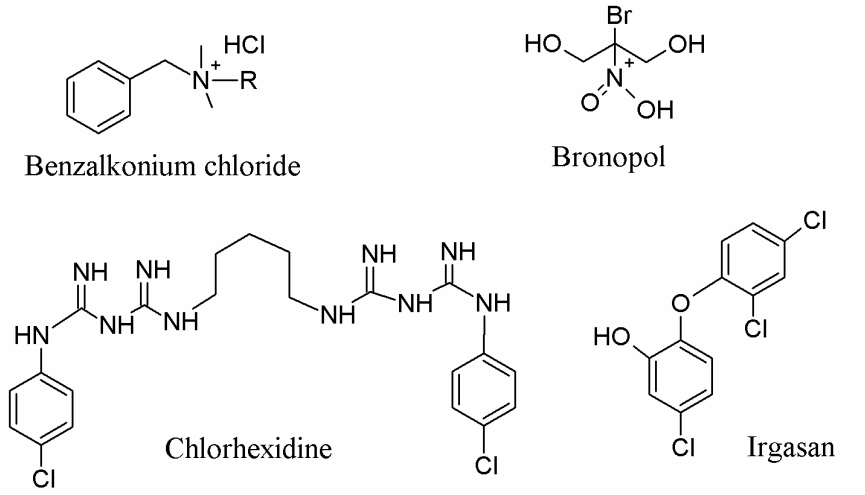

Fig. 1 *Chemical structure of employed antibacterial agents.*

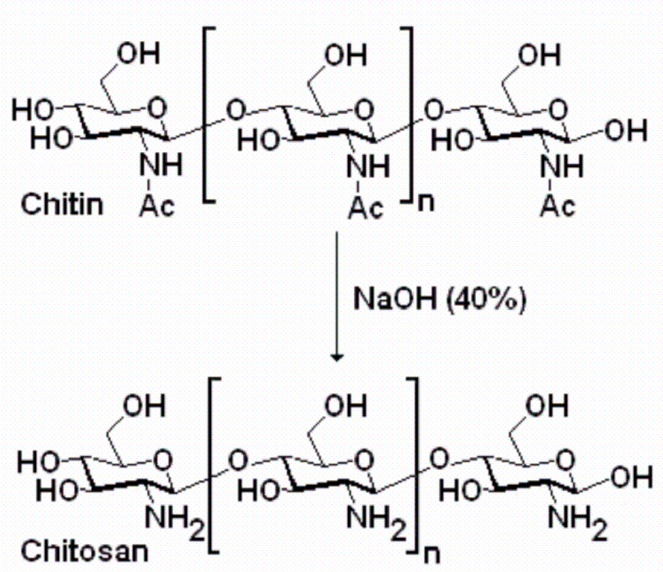

Fig. 2 *Preparation of chitosan from chitin.*

On account of the high impact of nosocomial infections in hospitals, the state of art in antimicrobial polymers is quite extensive and well documented. Nonetheless, there are few publications which have been committed to study the incorporation of the above-mentioned antibacterial agents either into biopolymers bulk or in their surfaces. Therefore, the main focus of this contribution is aimed at the addition of

these chemical substances onto collagen matrices and to evaluate how those may influence keratinocyte cell response on atecollagen films by means of cytotoxicity and cell proliferation studies. The findings of this research may help to strengthen knowledge on fields, such as antimicrobial biopolymers, human cell growth and tissue engineering.

MATERIALS AND METHODS

Materials

Atelocollagen emulsion from bovine Achilles tendon (pH 3.5), which contains 1.4% of atelocollagen was supplied by Vipo A.S, Slovakia. Acetic acid 99% was obtained from Penta, Czech Republic. Bronopol (2-bromo-2-nitropropane-1,3-diol) $C_3H_6BrNO_4$ 98% was purchased from Fluka, USA. Benzalkonium chloride with a predominant formula of $C_{12}H_{25}N(CH_3)_2C_7H_7Cl$ 98%; chitosan 98%; chlorhexidine (1,1-hexamethylene bis[5-(4-chlorophenyl)biguanide]) $C_{22}H_{30}Cl_2N_{10}$ 98%; irgasan 5-chloro-2-(2,4-dichlorophenoxy)phenol $C_{12}H_7Cl_3O_2$ 97% and Dimethyl sulphoxide (DMSO) were provided by Sigma-Aldrich, USA. The reagents in this study were used as received without any further purification. Tissue culture dishes of 40 mm diameter and individual wells of 96-well were acquired commercially from TPP, Switzerland. Vybrant® MTT cell proliferation Assay kit V-13154 was purchased from Invitrogen Corporation, USA.

Preparation of collagen-antibacterial agent substrates

Five mother mixtures of atelocollagen with each antibacterial agent (2.0% weight of agent/weight collagen) were prepared by dissolving these compounds in 0.1 M water solution of acetic acid to obtain a 0.1% weight of collagen/weight of solution, using

an IKA RCT stirring machine (IKA® works, Inc, Germany) for 4 hours at 1,000 rpm. Less concentrated solutions (1.0, 0.5, 0.2, 0.1 and 0.02%) were prepared by simple dilution. Each group of samples was casted on tissue culture dishes and the solvent was evaporated at ambient conditions for three days. Thin films of pristine atelocollagen were prepared and used as experimental blanks.

HaCaT cell incubation

Human immortalised non-tumorigenic keratinocyte cell line HaCaT, (Ethnicity, Caucasian; Age, 62 years; gender, Male and tissue, skin) were supplied by CLS Cell Lines Service, Germany. Dulbecco´s modified eagle medium, contains 4.5 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, 10% foetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml-0.1 mg/ml) was used as a culture medium (Biotech Inc. USA). Cells were incubated at 37℃ for 24 hours wit h 5% CO_2 in humidified air.

Evaluation of cytotoxicity (in-vitro)

Extract preparation. The substrates obtained above were extracted according to ISO 10993-12 in the ratio of 0.1 g of the films per 1.0 ml of culture medium in chemically inert closed containers by using aseptic techniques. Each extract was incubated in DMEM medium at 37 ± 1ºC with stirring for 24 hours (32).

Cell Viability of HaCaT. All cells in the exponential growth phase were seeded in a concentration of 1×10^5 cells/mL onto the substrate extracts (2.0, 1.0, 0.5, 0.2, 0.1 and 0.02%). Cell viability as indicator of cytotoxicity was determined after 4 days of culture by MTT assay. Absorbances were recorded by using a Sunrise microplate

ELISA reader at 570 nm (Tecan group, Switzerland), and all determinations were performed in quadruplicate (33-35).

Cell proliferation test

HaCaT cell proliferation on thin films with the following specifications: collagen-benzalkonium chloride, collagen-bronopol, collagen-chitosan, collagen-chlorhexidine and collagen-irgasan 2.0, 1.0 and 0.5% was determined after 4 days of culture by MTT assay. A volume of 10 µL of 12 mM MTT was taken for cell incubation performed at 37℃ for 4 hours in the dar kness. Thereafter, the media were decanted and washed with phosphate-buffered saline solution (PBS). The produced formazan salts were dissolved with dimethylsulphoxide (DMSO) and its concentration was measured in a spectrophotometer at 570 nm (36).

Statistical analysis

All data were presented as the mean value ± standard deviation (SD) of each sample. Statistical comparisons were performed using Student's t-test with a confidence level of 95% (p <0.05) considered statistically significance and 99% (p< 0.01) considered very significant.

RESULTS

The extent of cytotoxicity from every single concentration of antibacterial agent was quantified as a percentage of cell viability including the absorbance values obtained to each system (Fig. 3). Pursuant to ISO 10993-5, percentages of cell viability above 80% are considered as non-cytotoxicity; within 80-60% weak; 60-40% moderate and below 40% strong cytotoxicity respectively (37). It may be seen in the histograms that these percentages were high and consequently, these

substances were innoxious no matter the concentration that was used. The viability range was within 74-99% and only two samples of benzalkonium chloride 2.0 and 1.0% presented a weak cytotoxicity. The lowest value was found on collagen-benzalkonium chloride 2.0%, whilst the highest one was for the matrix with bronopol 0.02%. Fig. 3.A, B, C depict yields over 80%. For instance, the highest concentration (Fig. 3.A) had a set of values within 74-89%; it may be observed that the substrates endowed with agents at this concentration exhibited the smallest viability rates and the maximum value did not even reach 90%. All the experiments performed with 1.0% of biocide overcame 80% of viability except the sample with benzalkonium chloride (Fig. 3.B 78-93). Fig. 3.C that corresponds to 0.5%, the percentages were between 80 and 94% and the specimens with bronopol, chitosan and chlorhexidine had viabilities over 90%. On the other hand, the lower concentrations (Fig. 3.D, E, F) describe yields above 90% with two exceptions, benzalkonium chloride 0.2 and 0.1%. (Fig. 3.D 85-95%). The histograms of Fig. 3.E disclose that solely the matrix with collagen-benzalkonium chloride 0.1% did not attain 90% and the viability range for this concentration was within 89-96%. As for the samples with the minimum concentration, which is 100 times lower than the most concentrated one, 0.02%, (Fig. 3.F). These showed the highest percentages 90-99%.

The data demonstrates that these biocides do not drastically inhibit the viability of HaCaT keratinocytes cell line. It may be noticed the increase of these values as concentration decreases, which points out the intrinsic connexion between cell growth and amount of cytotoxic drug (38-41).

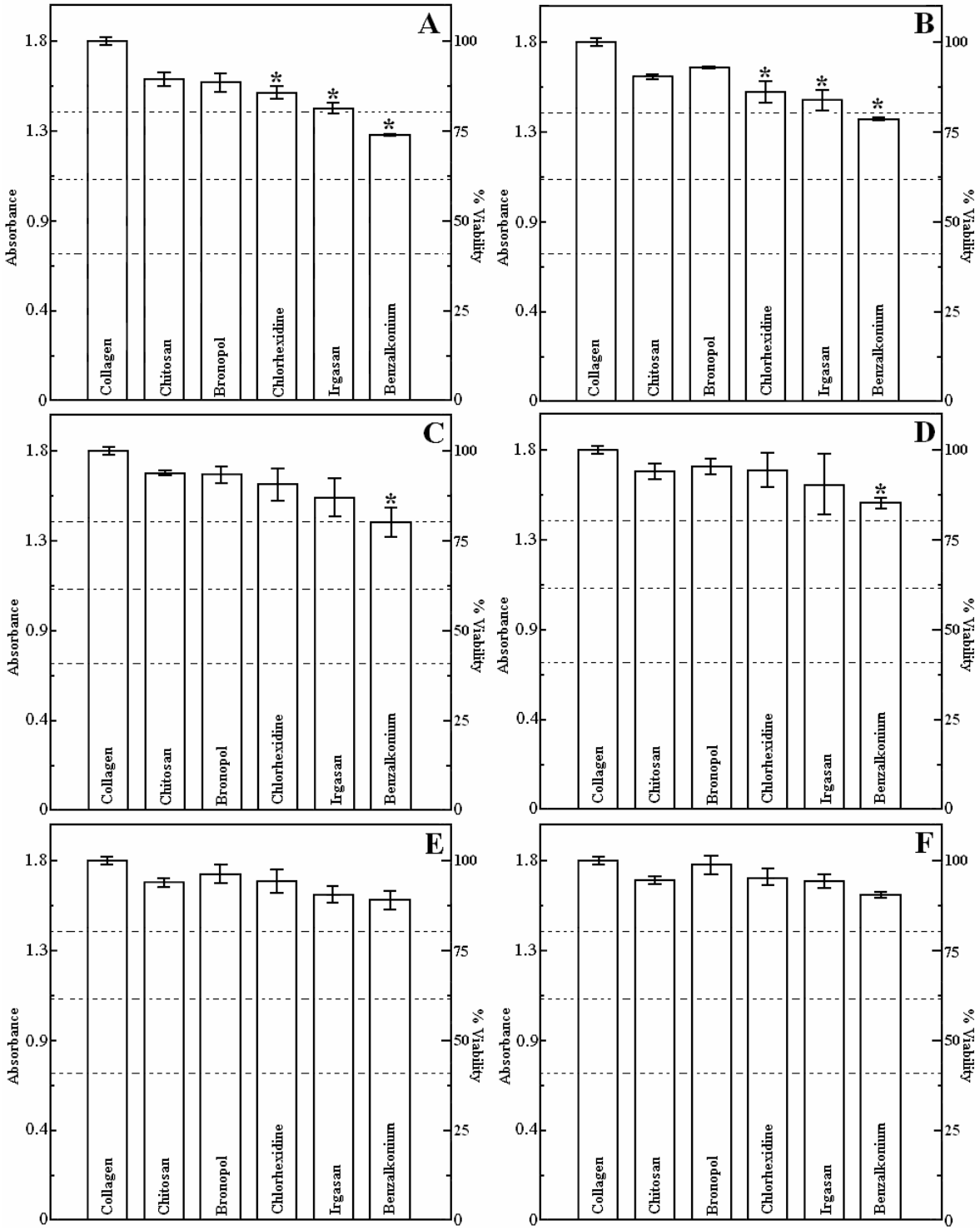

Fig. 3 *Effect of various concentrations of target compounds on HaCaT cell viability. Cell line seeded on atelocollagen matrix with concentration of agent (A) 2.0% w/w (B) 1.0% w/w (C) 0.5% w/w (D) 0.2% w/w (E) 0.1% w/w (F) 0.02% w/w determined by MTT assay (the error bars depict standard deviations and dashed lines define cytotoxicity ranges: non-cytotoxicity >80%; weak >60%; moderate >40%; strong <40%). *p < 0.05, compared with pristine atelocollagen film.*

Statistically speaking, eight sample were found significant different at a significance level of 0.05 (chlorhexidine 2.0 and 1.0%; Irgasan 2.0 and 1.0%; Benzalkonium chloride 2.0, 1.0, 0.5 and 0.2%).

The dependence of cell viability on concentration of agent is an important aspect of this study. Fig. 4 shows the percentage of viability with respect to this variable.

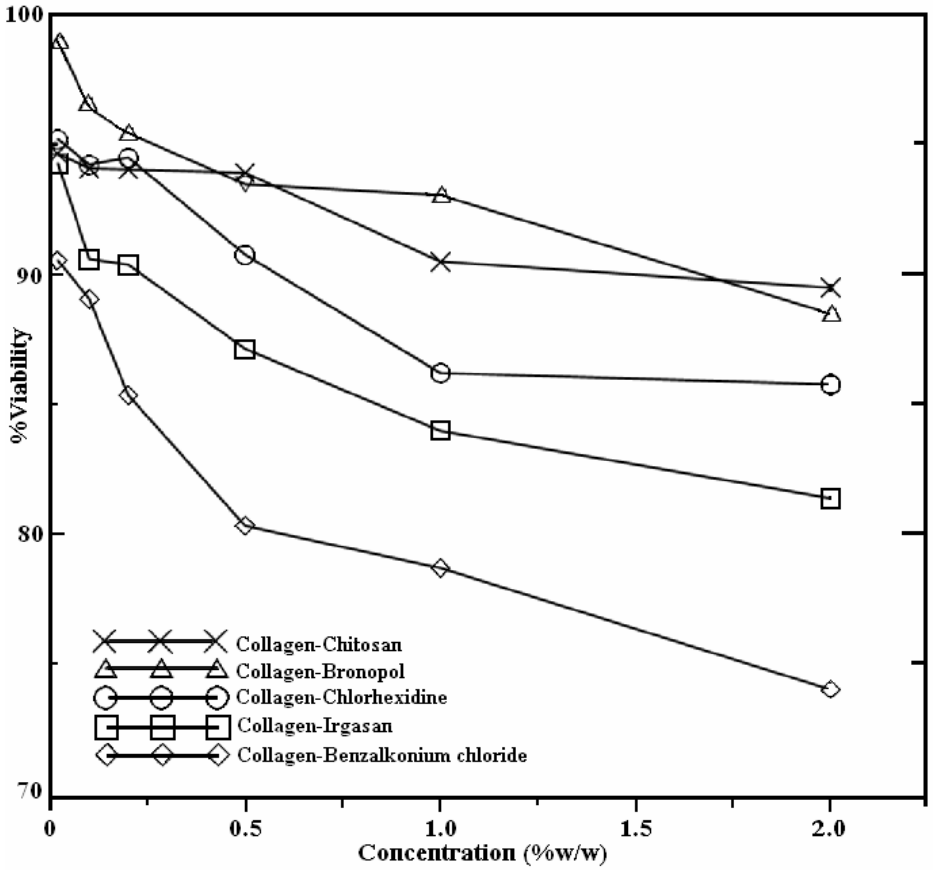

Fig. 4 *Concentration-viability curve of studied agents on HaCaT keratinocyte cell line.*

It reveals a fall in cell viability by increasing concentration. In general all the plots have the same pattern. Chitosan is a particular case, because its curve evinces a plateau followed by a decreasing trend at the highest concentrations (1.0 and 2.0%). The chlorhexidine curve has a local maximum at 0.2%, which is most likely a

consequence of sampling mistakes or an incorrect dilution. Samples free of biocides were taken as the ones with 100% viability.

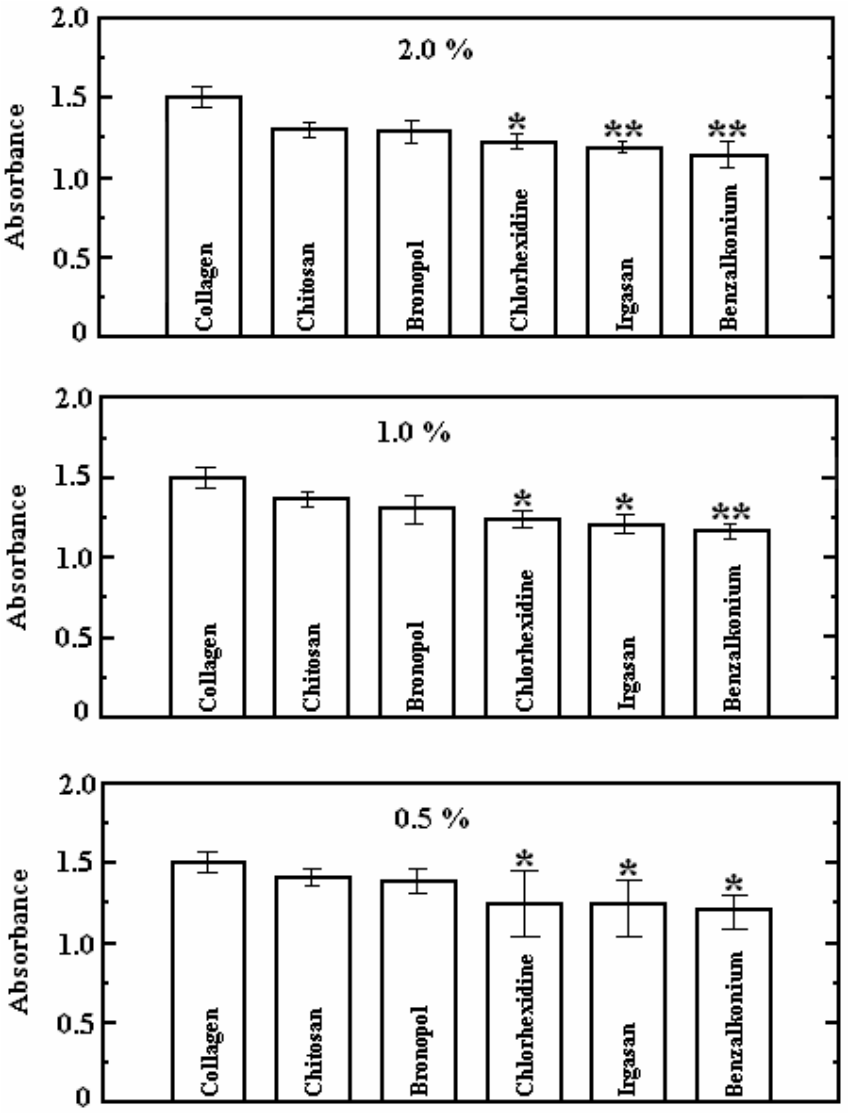

Fig. 5 *Comparison of HaCaT cell growth upon atelocollagen films with and without bactericides determined by measuring absorbance of Formazan product by MTT assay at 570 nm (the error bars signify standard deviation). *p < 0.05 and **p < 0.01, compared with free of biocide film.*

HaCaT cell proliferation on the substrates with and without biocides is given in Fig. 5. It was found out that cell attachment marginally diminished, as reflected by the spectrophotometric data of each test, where all the absorbances corresponding

to the added-agent samples were smaller than the pristine ones. It may be noticed that just the substrates endowed with chitosan and bronopol did not show a statistical significance. On the other hand, all the samples with chlorhexidine, irgasan and benzalkonium chloride have statistical significance and in three cases were considered as very significant ones, irgasan 2.0%; benzalkonium chloride 2.0 and 1.0% respectively.

DISCUSSION

As may be noted across this study, chitosan, bronopol and chlorhexidine have lower inhibition in comparison with irgasan and benzalkonium chloride which are the strongest ones in all the cases. As well as it is important to emphasise the importance of pH and solubility on the yield of each agent, since some of these bactericides do not posses the same effectiveness and stability in acid solution. *E.g.,* chitosan, bronopol and benzalkonium chloride are readily soluble and stable in water and acid solution. In contrast to irgasan, which is slightly soluble in this medium and chlorhexidine is sparingly soluble and unstable in acid pH (42-46). As described in material section, the mixtures were prepared by using a stirring machine for 4 hours at 1,000 rpm. When the biocide is soluble in the employed solvent, a good dispersion (with cohesive character) and distribution of the agent into the mixture is obtained. Contrariwise whether the substance is moderately soluble, the mixture is not uniform having loss of agent during processing, and thus the final concentration is different and lower than the one that was intended (47). It means that irgasan, which has low solubility under the experimental conditions is even able to suppress HaCaT cell growth. Whilst, despite bronopol and chitosan, which are soluble do not represent a serious risk to the viability of this cell line.

It is worth mentioning that for the studied samples preparation, the solvent (acetic acid 0.1 M) had to be completely evaporated, since HaCaT as well as most cells require pHs around 7.0 and the permanent control of pH is essential for optimal culture conditions (48, 49).

Concerning the chosen method to estimate the extent of cytotoxicity, MTT is a rather cost-efficient colorimetric technique, where the measurement strictly depends upon live cells, since the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced to formazan product exclusively by mitochondrial succinate dehydrogenase enzyme in the mitochondria of viable cells. Hence, this assay ensures a good approximation in the study of cell viability and proliferation in cell culture, where dead cells do not participate as interfering species. There are other factors that may induce the reduction of MTT, such as times of incubation, age of culture, media poor in glucose and reagents stability (50).

Cell proliferation under chitosan medium was higher than under the other media followed by bronopol, chlorhexidine, irgasan and benzalkonium chloride respectively. This information coincides with the cytotoxicity assay results, where the studied biocides performed in similar way in both experiments (Chitosan, bronopol and chlorhexidine have lower inhibition ability than irgasan and benzalkonium chloride).

Although the mechanisms of HaCaT cell adhesion and proliferation on different substrates are still unclear, it is well-establish that HaCaT keratinocyte cells proliferate better on rough, porous and hydrophilic scaffolds. The cases of atelocollagen-chlorhexidine and irgasan substrates are a proof of that, since these agents are toxic *per se* to this cell line and also because of their low solubility in the

medium. These substances may alter hydrophilicity, diminishing cell adhesion and proliferation (51, 52).

In culture, keratinocyte cells behave in a similar way they do *in vivo*, where cells migrate towards the air interface to form the epithelial surface. Epidermal substitutes require minimum two weeks to expand keratinocytes population. For these reasons, it is necessary to pay heed to the stability of keratinocytes attachment (53). In that respect, the overall outcome indicates that after four days in culture these substrates hold low marginal toxicity, as well as suitability for cell proliferation.

Surface adherence is a natural tendency which is inherent to bacteria and other microorganisms. It has four basic steps: adhesion, colonisation, formation and the subsequent bacterial biofilm growth, which is independent of the substrate. Biofilms act as defence mechanism against external agents; in consequence, the aim of any antimicrobial materials is at preventing bacterial adhesion and colonisation, which are prerequisites to biofilm formation (54). It is known by literature that benzalkonium chloride, bronopol and chitosan hinder the adhesion of gram-positive strain, but do not behave satisfactorily against gram-negative bacteria (55, 56); chlorhexidine and irgasan are efficacious against both strains (57, 58). According to biothermodynamic studies, bacteria may attach to both hydrophobic and hydrophilic surfaces; notwithstanding, hydrophobic surfaces are colonised faster than hydrophilic ones (59, 60). This feature rises in importance, since the studied substrates are highly hydrophilic, which is favourable to HaCaT cell adhesion but not to bacterial colonisation.

The described phenomenon is largely surface specific and affects material functionality leading to loss of physical and mechanical properties (61, 62).

Consequently, these atelocollagen substrates enhanced by the addition of one or more of these agents may render effectiveness against bacterial stains and biofilm formation, being a promising view in the design of novel antimicrobial biomaterials potentially suitable for tissue engineering applications.

CONCLUSIONS

This contribution delved into the incorporation of bactericides to atelocollagen matrices. The mixtures of atelocollagen with benzalkonium chloride, bronopol and chitosan are uniform and stable. Cell viability of HaCaT is barely altered by the presence of these substances (74-99%). Only in eight from thirty samples the cell viability was statistically lower than that found on the substrates without biocides. It means that any of these substrates provides an appropriate environment for this cell line. Thus, the studied samples are perfectly apt for keratinocyte cell growth. Cytotoxicity is concomitant to concentration and depends upon each agent. Bronopol and chitosan arise as the less hazardous to this cell line having percentages of viability beyond 85% with a negligible cytotoxicity at lower concentrations; whereas irgasan and benzalkonium chloride manifest more power of inhibition with the highest rates of cytotoxicity throughout the study. This inhibition pattern might be observed in both cytotoxicity and proliferation experiments and confirmed by statistic.

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Appendix B: Author's *Curriculum Vitae*

CURRICULUM VITAE

Personal Information:

First Name(s)/ Surname(s)	**Jorge Andrés López García**
Present Address	Nám. TGM 3050. Zlín, 76 001
Telephone	+420776499840
E-mail	vextropk@gmail.com
Nationality	Colombian

Work experience

Dates	Apr 2007 - Nov 2007
Occupation or position held	Quality control laboratory manager
Name of employer	Eterna Inc; which is a plastic production company
Dates	Jan 2006 - Apr 2007
Occupation or position held	Quality control laboratory manager
Name of employer	Carulla Vivero Inc; which is grocery and general merchandising retail chain
Dates	Jan 2005 - Dec 2005
Occupation or position held	Laboratory analyst
Name of employer	INVIMA *Spanish acronym for* (national institute of foods and drugs vigilance), which is an agency of the Colombian ministry of health

Dates	Sep 2003 - Dec 2004
Occupation or position held	Research assistant
Name of employer	FIDIC *Spanish acronym for* (Immunology institute of Colombia)

Education and training:

Dates	since Dec 2007
Title of qualification awarded	PhD Study
Principal branch	Technology of Macromolecular Substances
Name and type of organisation	Tomas Bata University in Zlín, Faculty of Technology
Providing education and training	Polymer Centre. Zlín, Czech Republic

Dates	2006-2007
Title of qualification awarded	MSc Study
Principal branch	Phytochemistry
Name and type of organisation	Javeriana University, Faculty of Science,
Providing education and training	Chemistry Department. Bogotá, Colombia

Dates	2004
Title of qualification awarded	Molecular Biology and Cellular Immunology Course
Name and type of organisation	El Bosque University. Bogotá, Colombia

Providing education and training

Dates	2004
Title of qualification awarded	Chemist
Name and type of organisation	National University of Colombia, Faculty of Science,
Providing education and training	Chemistry Department, Bogotá, Colombia

Research Experience and Publications

HUMPOLÍČEK, P., LEHOCKÝ, M., JUNKAR, I., SÁHA, P., LÓPEZ-GARCÍA, J. Enhanced cell proliferation on collagen films modified by plasma treatment. *International Conference on Advanced Plasma Technologies (ICAPT)*, Strunjan, Slovenia, 2011. ISBN 978-961-92989-3-0

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LÓPEZ-GARCÍA, J. Volatile compounds of Amazonian pineapple (*Ananas comosus [L.]Merr*. Var India) fruit before and after free-concentration process. *Bachelor thesis*. National University of Colombia, Bogotá, 2004.

Communication Skills:

Language	CEFR*
Spanish	C2†
English	C1‡
Portuguese	B2
Czech	A2

*Common European Framework of Reference for Languages
†Native Language
‡Certificated by Oxford English Testing.