

# Developing new interface materials for wound care applications

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# Declaration of original authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

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# Abstract

The aim of my PhD project was the development of novel hydrogels for wound care applications using autoclave-mediated cross-linking in the polymer mixtures. Transparent, bubble-free and flat hydrogels were produced. The two polymers that gave the best results and therefore were used to continue the work, were poly(vinyl alcohol) and poly(methyl ether-alt-maleic anhydride), which is also known as Gantrez<sup>®</sup> AN. These dressings were then fully characterised. We took advantage of the fact that the autoclaving step allowed the gelation in a controlled way, to synthesise another class of hydrogel materials known as 'superporous' hydrogels (SPH), whose production usually involves the use of complicated set up and the addition of initiators and cross-linkers. We developed a straightforward method composed of few simple steps to synthesise SPHs of different shapes and thicknesses from aqueous mixtures of PVA and Gantrez<sup>®</sup> AN. These materials and their physicochemical properties were then investigated. Using the same combination of polymers again it was also possible to produce physically cross-linked hydrogels applying a slightly modified version of the well-known freeze-thawing technique. The cryogels produced were studied and their properties were compared with those of the autoclaved (chemically cross-linked) samples. We developed three new wound dressing prototypes and two novel methods for the synthesis of hydrogels and SPHs that could make a difference, not just in the wound management sector. New products, but above all an innovative and environmentally-friendly approach to biomaterials manufacturing can derive from our work at Reading School of Pharmacy.

From the method of synthesis of the materials we present in this thesis, to their extensive characterisation, our work aimed to give a valid and useful contribution to the wound management field.

## List of publications

E. Caló, and V. V. Khutoryanskiy, Biomedical applications of hydrogels: A review of patents and commercial products, *Eur. Polym. J.* 2015, *65*, 252-267.

E. Caló , When a drug becomes orphan, *UKICRS Newsletter* 2015, 18-19.

E. Caló, J. M. S. De Barros, M. Fernandez-Gutierrez, J. San Roman, L. Ballamy, V.V. Khutoryanskiy, Antimicrobial hydrogels based on autoclaved poly(vinyl alcohol) and poly(methyl vinyl ether-alt-maleic anhydride) mixtures for wound care applications, *RSC Adv.* 2016, *6*, 55211–55219.

E. Caló, L. Ballamy, V. V. Khutoryanskiy, Hydrogels in wound management to be published in *Hydrogels: Design, Synthesis & Application in Drug Delivery & Regenerative Medicine*, CRC Press.

E. Caló, J. M. S. De Barros, L. Ballamy, V. V. Khutoryanskiy, Novel Poly(vinyl alcohol)- Gantrez® AN cryogels for wound care applications, *RSC Adv.*, 2016, *6*, 105487-105494 .

# List of conferences

Malmö University Biointerfaces Research Centre meeting, University of Malmo (Sweden) 19<sup>th</sup> April 2016 – Talk

2<sup>nd</sup> London Polymer Group Symposium, Queen Mary University London 13<sup>th</sup> April 2016- Poster

11<sup>th</sup> International Saint-Petersburg Conference of Young Scientists ‘Modern problem in polymer science’, Saint Petersburg (Russia) 9<sup>th</sup>-12<sup>th</sup> November 2015–Talk

42<sup>nd</sup> Annual Meeting & Exposition of the Controlled Release Society Edinburgh, International Conference Centre 26-29<sup>th</sup> July 2015 – Poster

M4 Colloids Symposium 2015, University of Bristol 2<sup>nd</sup> July 2015 –Talk

Doctoral Research Conference, Reading University 18<sup>th</sup> June 2015 –Three minutes thesis competition (Finalist)

United Kingdom & Ireland Controlled Release Society Symposium, Nottingham University 16-17<sup>th</sup> April 2015 – Poster

Pharmacy PhD Showcase 2015, University of Reading 30<sup>th</sup> March 2015 –Talk

Spanish National Research Council (CSIC) Biomaterials Group meeting, Madrid (Spain) 7<sup>th</sup> November 2014- Talk

United Kingdom & Ireland Controlled Release Society Symposium, Cork University (Ireland) 10-11<sup>th</sup> April 2014 – Poster

Royal Society of Chemistry Biomaterials Conference, Manchester University 7<sup>th</sup>-8<sup>th</sup> January 2014 – Talk

Materials Research Exchange – Exhibition, Ricoh Arena Coventry 25<sup>th</sup> February 2014 – Poster

Young Researchers Pharmaceutics and Biopharmaceutics Meeting, Reading University 25<sup>th</sup> August 2013 –Poster

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# Abbreviations

AA	acetic acid
Ag <sup>+</sup>	ionic silver
AIBN	2,2-azo-isobutyronitrile
AM	acrylamide
AMPS	2-acrylamido-2-methylpropane sulfonic acid
APS	ammonium persulfate
AUL	absorbency Under Load
BHT	butylated hydroxytoluene
CaCl <sub>2</sub>	calcium chloride
CMTKP	carboxymethyl tamarind kernel polysaccharide
CPMAS	cross-polarization magic angle spinning
CPP	central precocious puberty
D	diffusion coefficient
Dk	oxygen permeability
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DVS	divinyl sulfone
E	elastic modulus
EWC	equilibrium water content
FBS	fetal bovine serum
FDA	food and drug administration
FTIR	Fourier transform infrared spectroscopy
GnRH	gonadotropin-releasing hormone

HCl	hydrochloric acid
HEC	hydroxyethyl cellulose
HEMA	hydroxyethylmethacrylate
HFIM	poly(hexa-fluoroisopropyl methacrylate)
IPN	interpenetrating network
J	molar flux of the drug
MDA	N,N'-methylenebisacrylamide
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
MVI	misoprostol vaginal insert
Mw	molecular weight
NaCl	sodium chloride
NaCMC	sodium carboxymethylcellulose
NaHCO <sub>3</sub>	sodium bicarbonate
NaOH	sodium hydroxide
NCTC	national collection of type cultures
NMR	nuclear magnetic resonance (NMR)
NPDC	national patent development corporation
NSAIDs	non-steroidal anti-inflammatory drugs
NVP	N-vinyl-2-pyrrolidone
PAA	polyacrylic acid
PAAM	polyacrylamide
PBS	phosphate buffer
PEO	polyethyleneoxide
PGA	polyglycolide
PGE2	dinoprostone or prostaglandin E2
PHEMA	poly-2-hydroxyethylmethacrylate

PHMB	polyhexamethylene biguanide
PLA	polylactide
PMMA	poly(methyl methacrylate)
PMVEMA	poly(methyl ether-alt-maleic anhydride)
PPO	polypropyleneoxide
PSAs	pressure sensitive adhesives
PVA	poly(vinyl alcohol)
PVP	poly(vinyl pyrrolidone)
RM	level of residual monomers
SAPs	superabsorbent polymers
SEM	scanning electron microscopy
SiHy	silicone hydrogel
SPAK	3-sulfopropyl acrylate potassium salt
SPH	superporous hydrogel
SR	swelling ratio
SSD	silver sulfadiazine
SWF	simulated wound fluid
TEMED	tetramethylethylenediamine
TGA	thermogravimetric analysis
UFLMP	unfrozen liquid micro-phase
UV	ultraviolet
$\alpha$	contact angle
$\beta$ -CD	$\beta$ -cyclodextrin
$\Delta c$	concentration gradient

# 1 Introduction

## Biomedical applications of hydrogels: a review of patents and commercial products

Enrica Caló, Vitaliy V. Khutoryanskiy

Hydrogels have become very popular due to their unique properties such as high water content, softness, flexibility and biocompatibility. Natural and synthetic hydrophilic polymers can be physically or chemically cross-linked in order to produce hydrogels. Their resemblance to living tissue opens up many opportunities for applications in biomedical areas. Currently, hydrogels are used for manufacturing contact lenses, hygiene products, tissue engineering scaffolds, drug delivery systems and wound dressings. This review provides an analysis of their main characteristics and biomedical applications. From Wichterle's pioneering work to the most recent hydrogel-based inventions and products on the market, it provides the reader with a detailed introduction to the topic and perspective on further potential developments.

This chapter was published as:

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## 1.1 Introduction

Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of absorbing large amounts of water or biological fluids. Due to their high water content, porosity and soft consistency, they closely simulate natural living tissue, more so than any other class of synthetic biomaterials. Hydrogels may be chemically stable or they may degrade and eventually disintegrate and dissolve.<sup>[1]</sup>

Hydrogels are called 'reversible' or 'physical' gels if molecular entanglements and/or secondary forces such as ionic, H-bonding or hydrophobic forces play the main role in forming the network. Physical gels are often reversible and it is possible to dissolve them by changing environmental conditions, such as pH, the ionic strength of solution or temperature, etc. In 'permanent' or 'chemical' gels, the network of covalent bonds joining different macromolecular chains can be achieved by cross-linking polymers in the dry state or in solution.<sup>[2]</sup> These gels may be charged or non-charged depending on the nature of functional groups present in their structure. The charged hydrogels usually exhibit changes in swelling upon variations in pH, and it is known that they can undergo changes in shape when exposed to an electric field.<sup>[3]</sup>

Chemical hydrogels are commonly prepared in two different ways: 'three-dimensional polymerization' (Figure 1.1), in which a hydrophilic monomer is polymerized in the presence of a polyfunctional cross-linking agent, or by direct cross-linking of water-soluble polymers (Figure 1.2). Polymerization is usually initiated by free-radical generating compounds such as benzoyl peroxide, 2,2-azo-isobutyronitrile (AIBN), and ammonium peroxodisulphate or by using UV-, gamma- or electron beam-radiation. However, three-dimensional polymerization often results in materials containing significant levels of residual monomers and therefore purification of these materials has to be performed thoroughly because the unreacted monomers are often toxic and could leach out from the hydrogels continuously. The purification of hydrogels containing residual monomers is typically performed by extraction into excess water, and can take up to several weeks to be

completed.<sup>[4, 5, 6, 7]</sup>

There are numerous approaches that could be used to improve or avoid the purification process. One possibility is the use of additional processes that lead to the highest possible degrees of monomer conversion, which could be achieved by conducting three-dimensional polymerization followed by subsequent post-polymerization curing (e.g. by thermal treatment or irradiation of the resulting products).<sup>[8,9]</sup> Alternatively, the selection of non-toxic monomers used for the three-dimensional polymerization, such as oligomers or macromonomers (e.g. polyethylene glycol dimethacrylate) could be a solution.<sup>[10]</sup>

It is also possible to avoid the need for purification of hydrogels after their synthesis by cross-linking ready-made water-soluble polymers. Water-soluble polymers such as poly(acrylic acid), poly(vinyl alcohol), poly(vinylpyrrolidone), poly(ethylene glycol), polyacrylamide and some polysaccharides are the most common systems used to form hydrogels. These water-soluble polymers are non-toxic and widely used in various pharmaceutical and biomedical applications and therefore do not require removal from the system, eliminating the need for a purification step. Radiation induced cross-linking, such as of an aqueous solution of hydrophilic polymers with gamma rays, allows simultaneous polymerization and sterilization. Rosiak et al.<sup>[11,12]</sup> used cross-linking of natural polymers (such as gelatine or agar) and synthetic polymers (such as poly(vinyl pyrrolidone) (PVP) or poly(vinyl alcohol) (PVA) which were cross-linked by gamma radiation for the production of sterile hydrogels used in wound care. Currently their hydrogels are manufactured and marketed as 'Kikgel' and 'Aqua-gel' wound dressings.<sup>[11,12]</sup>

Recently, Khutoryanskiy et al.<sup>[4,13]</sup> reported an alternative method to synthesise hydrogels from ready-made water-soluble polymers in aqueous solutions using thermal treatment or microwave irradiation. In this method the aqueous solutions of specific water-soluble polymers such as poly(methyl vinyl ether-*alt*-maleic anhydride) and poly(vinyl alcohol) are mixed together at room



temperature and the cross-linking process is achieved by thermal treatment under high pressure via autoclaving or microwave radiation. Both radiation and thermal cross-linking methods are inexpensive, safe, do not require a purification step and result in sterile hydrogels if a suitable combination of hydrophilic polymers is used.

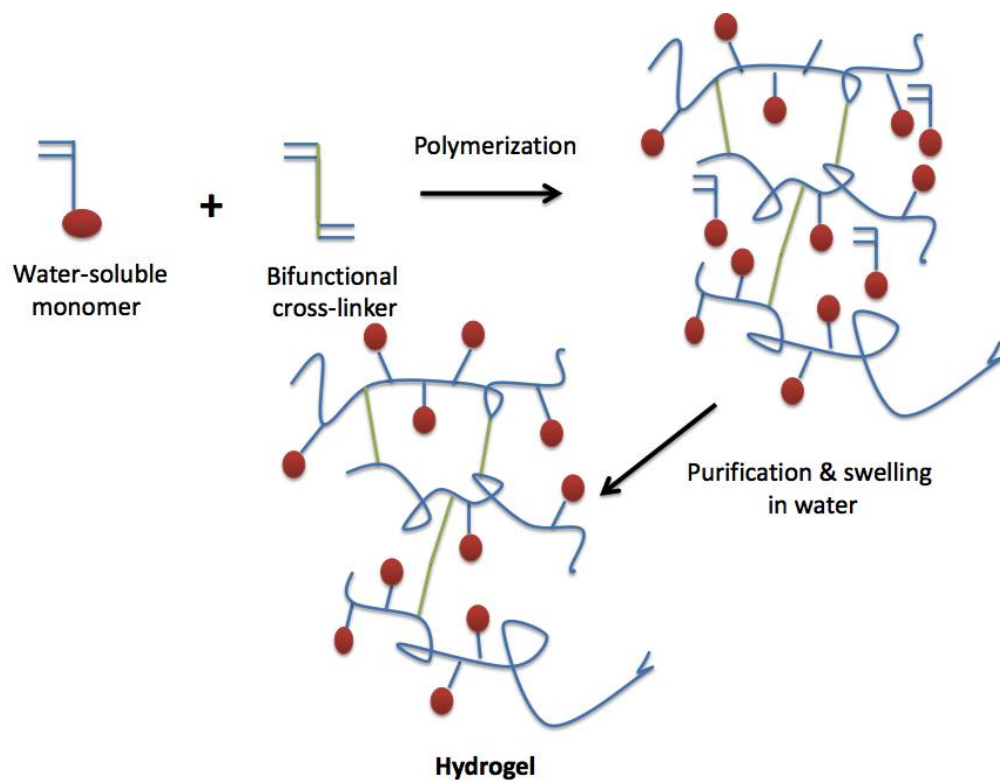


Figure 1.1 Synthesis of hydrogels by three-dimensional polymerization.

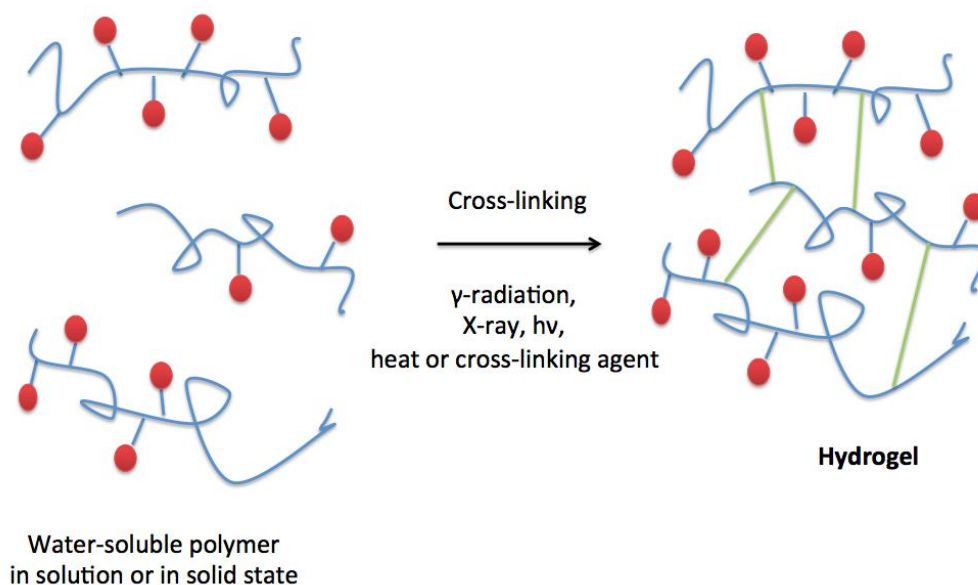


Figure 1.2 Synthesis of hydrogels by cross-linking of ready-made water-soluble polymers.

There are numerous original papers, academic reviews and monographs focused on the synthesis, properties and applications of hydrogels.<sup>[1, 3, 14-22]</sup> This review will consider mostly patent literature on ‘chemical’ hydrogels and their potential commercial applications in biomedical areas. As shown by the considerable number of patents and commercial products, the main areas of hydrogel applications are contact lenses, wound dressings, drug delivery systems, tissue engineering, hygiene products and will be covered in this review.

## 1.2 Contact lenses

In their pioneering 1960 paper, Wichterle and Lim were the first to describe a hydrogel based on poly-2-hydroxyethylmethacrylate (PHEMA) as a synthetic biocompatible material useful for contact lens applications.<sup>[23]</sup> PHEMA lenses were distributed firstly in western Europe in 1962, but with

limited success. In 1965 the National Patent Development Corporation (NPDC) bought the licence to this technology. This was then sold to Bausch & Lomb, which optimised Wichterle's spin-casting process and finally acquired the approval from the Food and Drug Administration (FDA) for their PHEMA lenses in 1971.<sup>[24]</sup>

Contact lenses are mainly classified as 'hard' or 'soft' according to their elasticity. Even though hard lenses are longer lasting, they tend to be poorly accepted by the wearers and can require a lengthier adaptation period. Hard contact lenses are primarily based on hydrophobic materials such as poly(methyl methacrylate) (PMMA) or poly(hexa-fluoroisopropyl methacrylate) (HFIM), whereas soft lenses are based on hydrogels.<sup>[25]</sup>

Soft contact lenses can be produced with different techniques, such as spin-casting, mould-casting and lathe-cutting. In spin- and mould-casting a small amount of liquid monomer mixture is placed into special 'concave' optical moulds in order to shape the lens. During spin-casting the concave mould rotates to form the lens, causing the liquid monomer to flow out uniformly, coating the whole surface. At the same time, polymerization of the monomer is carried out at elevated temperatures, and the residual monomer is carefully removed at the end of the process. The mould-casting technique employs a convex mould which is inserted into the liquid monomer which already contains a mated concave mould, to make the back surface of the lens. The polymerization takes place in the same way as for the spin-casting. This process produces hard lens interposed between the optical surfaces of the two different moulds and once the lens is dry it remains concave.<sup>[26]</sup> Innovative molds, useful for cast molding silicone hydrogel contact lenses, have been described in the US Patent 6,861,123 B2 assigned to Johnson & Johnson Vision Care Inc. Turner et al.<sup>[27]</sup> patented their polyolefin inserts for producing the moulds and the method in which these are used to make lenses. The preferred method for producing the aforementioned lenses was by direct molding of the silicone hydrogels, placing the reaction mixture in a mould having the shape of the

final desired product, and then proceeding with the polymerization.

An alternative method used in the contact lens industry is lathe-cutting (Figure 1.3), in which the lenses are formed from solid 'buttons' of dehydrated material. The liquid monomer mixtures are usually bulk-polymerized in water tanks for some period of time. This type of polymerization is typically started using free-radical initiators which are then decomposed by an increase in temperature. This process results in the formation of longer polymer chains (with higher molecular weights) and potentially more chain entanglements. Oxygen-mediated degradation could occur at the surfaces but a button will have a moderately high volume to surface ratio, so it is possible to remove the surfaces during the lathing process. The lenses are finally collected from the centre of a button.<sup>[24]</sup>

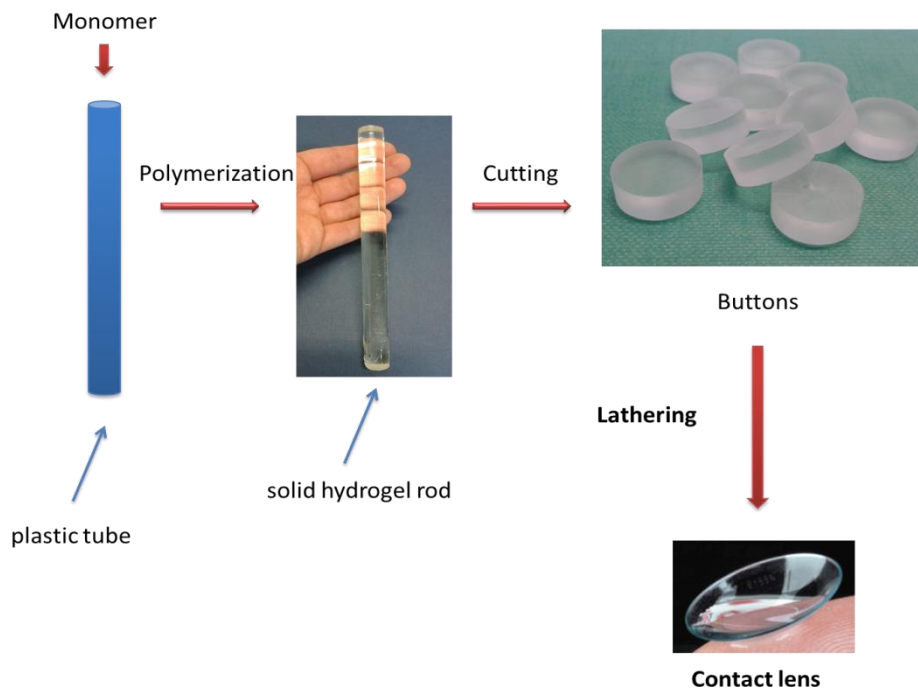


Figure 1.3 Scheme of lathe-cutting technique.

A polymeric hydrogel should have some important physical properties to be used as a contact lens material.<sup>[24]</sup> The ideal characteristics are listed in Table 1.1

Table 1.1 Requirements to the hydrogels used for contact lens applications.

Characteristics	Requirements	References
Luminous transmittance	The minimum luminous transmittance value for contact lenses is 95%. This value significantly affects the transparency of the lens. A slit lamp microscope is typically used to observe any deposit (proteins, lipids, bacteria, minerals) that may cause a lack of the usual transparency of the lens.	[28, 29]
Refractive index	The refractive index of the human cornea surface may vary. Ideal hydrogels should have a refractive index value matching the range 1.372-1.381.	[30, 31]
Sufficient oxygen-permeability	The oxygen permeability of the lens is directly proportional to water content and inversely to thickness. In the contact lens industry the oxygen permeability is expressed as Dk. In order to prevent anoxia throughout the cornea the oxygen transmissibility of the lens (Dk/t, where 't' is the thickness of the lens) required to be 35 for the open eye and 125 for the closed eye.	[32, 33, 34]
Wettability and permeability to water	The water-permeability of the lens is strictly related to thickness. A constant water diffusion rate is normally reached within the first hour of the experimental analysis. It directly depends on the wettability of the lens, which is evaluated by advancing contact angle ( $\Theta_w/a$ ) measurements. The initial value for hydrogel contact lens is usually around 25°.	[35, 36]
Stability	The stability of the material used affects the shelf-life and the manufacturing process of the lens.	[37]
Excellent mechanical properties	The mechanical properties of the lens, such as the elastic modulus (E), have a great impact on their adhesion to the corneal epithelium and on the comfort for the wearer.	[38]

Biocompatibility

The biocompatibility of the material is essential for the [39] ocular health of wearers who tend to use the lenses for an extended period of time.

The equilibrium water content (EWC) of a hydrogel is defined as:

$$\text{EWC (\%)} = m / m_{\text{tot}} \times 100 \quad (1)$$

where  $m$  is the weight of water in polymer and  $m_{\text{tot}}$  is the total weight of hydrated polymer.

EWC could change with temperature, pH and osmolality. For example, PHEMA contact lenses contain approximately 38–40% of water in the fully hydrated state. They typically show low variability with changes in external factors.<sup>[24]</sup>

In US Patent 3,679,504,<sup>[40]</sup> Wichterle disclosed a method of forming coloured soft contact lenses and ophthalmic prostheses. The coloured ingredient was incorporated between two transparent hydrogel layers bound together by polymerizing the hydrophilic monomers mixture. The covering hydrogel layer could also be made from a solution of a hydrophilic macromonomer such as polyethylene glycol mono-methacrylate, which could be manufactured as described in US Patent 3,575,946.<sup>[41]</sup> The use of macromonomers for preparation of hydrogels can potentially eliminate the need for their purification as these materials are often non-toxic.<sup>[40]</sup>

In US Patent 4,472,327<sup>[42]</sup> Neefe proposed a method of making cosmetic hydrogel contact lenses which modified the apparent color of the iris by using small light reflecting particles imbedded in a colored transparent matrix. The lenses described in this patent are of a dual purpose: to correct the visual defects and to change the apparent color of the eye. The whole lens area was transparent, providing peripheral vision and allowing the natural iris pattern to be visible through them. Neefe

discovered that when a small amount of high refractive index fine particles was placed in a matrix of transparent lens material of a substantially lower refractive index, the reflected light had the color of the lower refractive index media.<sup>[42]</sup> Selected particulate material had been employed in the polymerization of HEMA with benzoyl peroxide as an initiator. Furthermore, it was possible to add a selected antimicrobial agent, for example 3-(trimethoxysilyl)propyloctadecylmethyl ammonium chloride, to the liquid monomer mixture before polymerization to ensure that the resulting lenses were more resistant to microbial growth<sup>[24]</sup>.

Numerous attempts have been made to develop new contact lenses with better physical and chemical properties. For instance, Jay Kunzer et al.<sup>[43]</sup> disclosed that certain hydrophobic monomers, such as those shown in Figure 1.4, can act as strengthening agents when copolymerized with hydrophilic monomers such as HEMA, or N-vinyl-2-pyrrolidone (NVP).

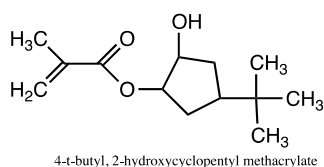
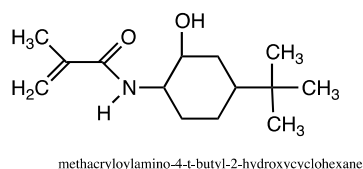
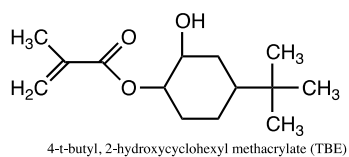


Figure 1.4 Some of the hydrophobic monomers used by Kunzer et al.<sup>[43]</sup>

The soft contact lenses made from these monomers combined with HEMA or NVP are large enough to cover the whole cornea and present good oxygen permeability, ensuring more comfort for wearers.<sup>[43]</sup>

Lai et al.<sup>[44]</sup> proposed the use of a new class of optically clear silicone thermoplastic hydrogel materials in the production of contact lenses. The general formula of the polymers, which includes a silicone-containing segment derived from polysiloxane linked with hydroxyl or amino groups, is shown in Figure 1.5.

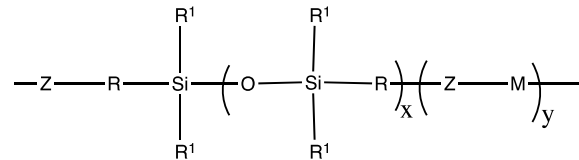


Figure 1.5 General formula of the thermoplastic silicone-containing compositions employed by Lai et al. 'M' is a hydrophilic group; 'R' is an alkyl group with 1 to 10 carbon atoms that can be separated by ether, urethane or ureido linkages; 'R<sup>1</sup>' is hydrogen, monovalent hydrocarbon groups or halogen substituted monovalent hydrocarbon moieties with 1 to 18 carbon atoms; 'Z' may be a divalent urethane or ureido portion; 'x' and 'y' are equal or greater than 1.<sup>[44]</sup>

These materials offered good physical strength and excellent oxygen permeability. A drawback of 'soft' contact lenses in general is their relatively poor gas permeability resulting in oxygen deprivation of the cornea, which receives oxygen only from the atmosphere. In the contact lens industry oxygen permeability is defined as 'Dk', where 'D' is the diffusivity of the lens and 'k' is the oxygen solubility in the lens material.<sup>[24]</sup> Dk essentially depends on EWC in conventional hydrogels because oxygen has the capability to diffuse through water rather than through the gel. The relationship between these two parameters is:

$$Dk=1.67e^{0.0397EWC} \tag{2}$$



where 'e' is the base of the natural logarithm. Oxygen transmissibility of contact lenses may be calculated from the Dk of the material divided by the lens thickness (t). The units of Dk are called Fatt units (named after Professor Irving Fatt) or Barrer:<sup>[24]</sup>

$$Dk \text{ (barrer)} = 10^{-11}(\text{cm}^2 \times \text{mL}_{\text{O}_2}) / \text{sec} \times \text{mL} \times \text{mmHg} \quad (3)$$

$$Dk/t \quad Dk \text{ (barrer/cm)} = 10^{-9} (\text{cm} \times \text{mL}_{\text{O}_2}) / \text{sec} \times \text{mL} \times \text{mmHg} \quad (4)$$

Nowadays, silicone hydrogel (SiHy) lenses have become prevalent on the market (Figure 1.6), due to their higher oxygen permeability and comfortable fit.<sup>[45]</sup>

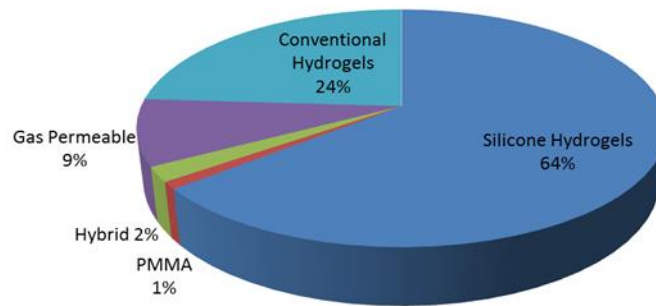


Figure 1.6 Materials used in contact lenses manufacturing in 2012. Data for this figure was taken from.<sup>[45]</sup> Reprinted by permission from Contact Lens Spectrum, published in January 2013. Contact Lens Spectrum is published monthly by PentaVision LLC © 2014 All Rights Reserved. PentaVision is located at 321 Norristown Road, Suite 150, Ambler, PA 19002 (USA). Please visit [www.contactlensspectrum.com](http://www.contactlensspectrum.com) for more information.

One of the drawbacks associated with the use of SiHy lenses is that they often undergo more protein deposition than conventional lenses which leads to problems with lens spoilage. In European

Patent EP 2 365 360 A2, a method for reducing protein deposition on contact lenses has been proposed by adding protein uptake-reducing compounds, such as butylated hydroxytoluene (BHT) or hydroxyamines in the reaction mixture.<sup>[46]</sup> The reaction mixture may include a 'silicone-containing monomer', described in the US Patent 3,808,178 (Figure 1.7).<sup>[47]</sup>

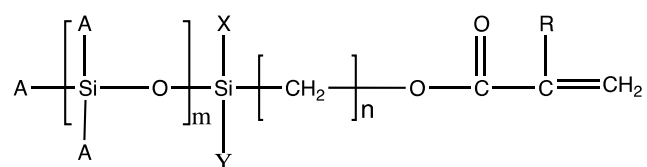


Figure 1.7 General formula of polysiloxanylalkyl ester monomer presented in US Patent 3,808,178. A, X and Y can be C<sub>1</sub>-C<sub>5</sub> alkyl groups or phenyl groups; R is a methyl group or hydrogen; m is an integer from 1 to 5 and n is from 1 to 3.<sup>[47]</sup>

Contact lens surfaces should also have excellent wettability in order to avoid tear-film deposits.<sup>[47]</sup> The SiHy lenses have been made to compensate the hydrophobicity of silicone and to improve its wettability. The silicone hydrogel lenses were moulded and plasma-treated afterwards.<sup>[49]</sup> The clinical performance of any contact lens material depends on its ability to produce a stable pre- and post-lens tear film, which is dependent on its wettability. This can be described as the formation of a continuous superficial fluid film over a solid surface. The wettability index is usually determined by measuring the contact angle ( $\alpha$ ) of water on a lens surface. If  $\alpha = 0^\circ$  then water is able to fully wet the lens, if  $\alpha < 90^\circ$  water wets the lens and if  $\alpha > 90^\circ$  the lens is practically not wettable.<sup>[48]</sup>

'Soft' lenses with greater adherence to the eye have been developed in order to enhance the fit, but on the other hand, they have poor gas permeability and often do not allow oxygen to reach the cornea at a sufficient rate. SiHy contact lenses have been designed to overcome this problem, because they are composed of hydrated, crosslinked polymeric material that contains silicon and a

certain amount of water within the polymer matrix. More recently, Bauman et al. <sup>[50]</sup> disclosed a method for making SiHy contact lenses with a nano-textured surface, imitating the surface of human cornea. The nano-textured surface coating technique has been developed through controlled soaking of the lens into a polymeric material, which can comprise monomeric units of one or more carboxyl-containing vinylic monomers. The nano-textures are then fixed by crosslinking a water-soluble hydrophilic polymeric material onto the prime coating.

One of the more recent products of the UK contact lens industry is 'Gentle 59', promoted by Vista Optics Ltd at the European Federation of the Contact Lens and Iols Industries Congress in Budapest in September 2012. Gentle 59 is made of an acrylic acid-co-acrylamide hydrogel, which seems to have very good tensile properties, moisture retention characteristics and a very comfortable fit.<sup>[51]</sup>

In addition to the applications of soft contact lenses in correction of vision, they can potentially be used for drug delivery to the eye. However, conventional hydrogel-based contact lenses exhibit relatively low drug loading capacity and often show a burst release upon ocular administration.<sup>[52]</sup> Many methods have been developed to modify the conventional contact lenses to improve their drug loading and release. These include modifying the polymeric materials with a controlled hydrophilic/hydrophobic copolymer ratio, impregnating drug-containing colloidal structures, incorporating ligand-including hydrogels and developing multilayered hydrogels.<sup>[52]</sup> Venkatesh et al. <sup>[53]</sup> showed the potential of 'biomimetic hydrogels' as carriers to load relevant amounts of H1-antihistamines. They also show potential to release therapeutic dosages of drug in vitro in a controlled manner for a period of 5 days, with a possible extension in the presence of proteins. Xu et al. <sup>[54]</sup> incorporated  $\beta$ -cyclodextrin ( $\beta$ -CD) into hydrogels for contact lenses, observing an increase in the equilibrium swelling ratio and tensile strength. Puerarin was used as a model drug to study loading and release from PHEMA/beta-CD hydrogels. It was established that puerarin

loading and the in vitro release rate depended on the amount of beta-CD in the hydrogel. In rabbit eyes the PHEMA/beta-CD hydrogel contact lenses demonstrated longer residence time of puerarin in the tear fluid compared to conventional PHEMA contact lenses and 1% puerarin eye drops.

Developing safe and cost-effective contact lenses is the focus of the eye care industry. Contact lens materials with optimal characteristics such as oxygen permeability, comfort, compliance, hygiene and disinfection have still not been achieved, which opens exciting opportunities for further developments in this area.

### 1.3 Wound dressings

A wound is a defect or a break in the skin which can result from trauma or medical/physiological conditions. Wounds can be classified, depending on the number of skin layers and on the area of the skin affected, as superficial (if only the epidermis is involved), partial-thickness (if the epidermis and deeper dermal layers are affected) and full-thickness wounds (when subcutaneous fat and deeper tissue has been damaged).<sup>[55]</sup> Wounds are usually sub-divided into 'acute' or 'chronic' wounds. Chronic wounds require dedicated nursing care that represents a significant cost for national health systems. Design of effective dressings relies on an understanding of the healing process, as well as the specific conditions of a patient and the effect that each material used could have on the wound.<sup>[55, 56]</sup> Wound healing can be hindered by various factors such as desiccation, infection or abnormal bacterial presence, maceration, necrosis, pressure, trauma and edema.<sup>[57]</sup>

The 'ideal' wound management product should absorb excess exudate and toxins, keep a good moisture between the wound and the dressing, preserve the wound from external sources of infection, prevent excess heat at the wound, have good permeability to gases, be supplied

completely sterile and be easy to remove without further trauma to the wound.<sup>[58]</sup>

Recently, the wound dressing industry highlighted the importance of providing comfort and conformability of dressings, the need for infrequent changes, cost effectiveness and a long shelf life.<sup>[58]</sup> The choice of the right dressing to suit a particular wound is therefore fundamental for optimum healing and the quality of life of the patient.<sup>[59]</sup> The majority of the currently available products can be classified as low adherent dressings, semipermeable films, hydrocolloids, hydrogels, alginates, foam dressings or antimicrobial dressings.<sup>[57]</sup> Although plain gauze is still one of the most commonly employed products in hospitals, new wound dressing research and development has produced advanced materials with better physical and chemical properties (Table 1.2). Gauze is certainly cheap, readily available and suitable for a lot of wounds. In particular the gauzes impregnated with some active ingredients such as iodine, zinc oxide/zinc ions, or petrolatum show enhanced performance. Iodine provides antimicrobial properties, whereas zinc oxide could promote wound cleansing and re-epithelialization.<sup>[60, 61]</sup> However, the use of gauze often results in problems associated with its removal as it may cause trauma by stripping off newly formed epidermis.<sup>[62]</sup>

Table 1.2 Advanced wound dressings (reprinted from P. S. Murphy, G. R. D. Evans, *Plastic Surgery International* 2012, 2012, 1).<sup>[60]</sup>

<b>Protective dressings</b>	<b>Notes</b>
Gauze	Inexpensive; readily available
Impregnated gauze	Nonadherent; preserves moisture
<b>Antimicrobial dressings</b>	
Antibacterial ointments	Reapply often to maintain moisture
Iodine based	Absorbent; Not for use with thyroid disorders

Silver based	Many forms; Broad spectrum; low resistance
<b>Autolytic debridement</b>	
Films	Occlusive; allows exchange of gasses
Hydrocolloids	Not for exudative or infected wounds
Hydrogels	Rehydrates to soften dry wounds
<b>Chemical debridement</b>	
Papain/urea	Availability issues in US
Collagenase	Selective debridement
<b>Absorbent dressings</b>	
Foam	Absorbs moderate exudate
Hydrogels	Absorbs minimal exudate
Hydrofibers	Absorbs heavy exudate
Alginates	Absorbs heavy exudate

Advanced dressings are designed to maintain a moist environment at the site of application, allowing the fluids to remain close to the wound but not spread to unaffected, healthy skin areas.<sup>[62]</sup> The relevance of the moist wound environment as a factor accelerating the healing process was first observed by Winter in 1962, but only recently has received more serious attention.<sup>[63]</sup> Dressings designed for moist wound healing are represented by hydrogel and hydrocolloid products but only the latter can absorb mild to medium exudate or drainage. Both induce autolytic debridement, which facilitates the elimination of the dead tissue.<sup>[57]</sup> Hydrocolloids are usually composed of sodium carboxymethylcellulose, gelatin, pectin, elastomers and adhesives. Hydrofiber<sup>®</sup> (ConvaTec) dressings allow moisture to be captured because they form a swollen gel structure and conform to the wound site forming a 'seal'. Hydrofiber<sup>®</sup> may be in the form of a hydrophilic, non-woven flat sheet dressing that can be converted to a soft gel sheet by absorbing the wound exudate.<sup>[58]</sup>

Hydrogels are widely used as debriding agents, moist dressings, and components of pastes for

wound care. However, they do not need further wound fluids to become gels and are suitable for dry wounds.<sup>[60]</sup>

The so-called ‘moisture donor’ effect of hydrogels helps autolytic debridement, increasing collagenase production and the moisture content of necrotic wounds.<sup>[62]</sup> They can absorb and retain contaminated exudate within the gel mass through expansion of crosslinked polymer chains resulting in isolation of bacteria, detritus and odour molecules in the liquid. Their high water content allows vapour and oxygen transmission to the wounds such as pressure sores, leg ulcers, surgical and necrotic wounds, lacerations and burns. They seem to play an important role as emergency burns treatment alone or in combination with other products, thanks to their cooling and hydrating effect.<sup>[63]</sup> For example, Burnshield hydrogel burn dressing (Levtrade International) present even in first aid kits is a polyurethane foam containing 96% of water and 1.06% *Melaleuca alternifolia* extract.<sup>[64]</sup>

Hydrogel dressings are also used for granulating cavity wounds.<sup>[65]</sup> Amorphous gels are generally reapplied every day while sheet hydrogels are usually changed 2–3 times a week.<sup>[66]</sup>

Table 1.3 Some examples of hydrogels and hydrogel sheets as wound dressings.

<b>Product</b>	<b>Main constituents</b>	<b>Main characteristics</b>
Granugel® (ConvaTec)	Pectin, carboxymethylcellulose and propylene glycol.	A clear, viscous hydrogel for the management of partial and full-thickness wounds, may be used as a filler for dry cavity wounds to provide a moist healing environment. <sup>[67]</sup>
Intrasite Gel® (Smith & Nephew)	Modified carboxymethylcellulose (2.3%) and propylene glycol (20%)	Amorphous sterile hydrogel dressing for use in shallow and deep open wounds. <sup>[68]</sup>

Purilon Gel® (Coloplast)	Sodium carboxymethylcellulose and more than 90% of water	Indicated in conjunction with a secondary dressing for necrotic and sloughy wounds and first and second degree burns. <sup>[69]</sup>
Aquaflo™ (Covidien)	Polyethylene glycol and propylene glycol	It has a disc shape that maximizes wound coverage and helps to fill shallow cavities. Translucent gel that allows wound visualization. <sup>[70]</sup>
Woundtab® (First Water)	Sulphonated copolymer, carboxymethylcellulose, glycerol and water	The dressing contains a superabsorbent polymeric gel able to absorb bacteria and retain them in its structure. Described as a wound 'kick-starter' patch for chronic wounds, it can also be used as a secondary absorbent. <sup>[71]</sup>

In 1992 Cartmell et al.<sup>[72]</sup> proposed a transparent wound dressing as thin-film, with a non-adhesive central portion containing hydrogel material which included polypropylene glycol or polyethylene glycol, and isophorone diisocyanate. This product is described as being flexible in order to facilitate its removal, and transparent to permit constant observation of the wound healing process. Cartmell describes that the edges of this dressing adhere to the skin due to the adhesive layers that protect the wound site from bacteria and foreign bodies. Two years later in the US Patent 5,423,737 Cartmell et al.<sup>[73]</sup> disclosed an improved version of this transparent wound dressing. In this case there was a release tab inserted between the transparent layer and the release liner. The invention was intended to respond to a need for a cost-effective product which was simple to manufacture and easy to handle and apply. A similar device has been presented by Holm et al.<sup>[74]</sup>, in which a hydrogel pad is included within an adhesive dressing. This demonstrates that many attempts have been made using new technologies but having the same patient goals.

If local or systemic infection is compromising the wound, or could compromise the healing process, one possible therapeutic approach would be to use dressings containing antimicrobial agents, such as iodine or silver. Silver is useful against a large range of microorganisms, including



*Pseudomonas aeruginosa* and *Staphylococcus aureus*.<sup>[75]</sup> These two opportunistic pathogens are frequently present in chronic wounds and their mechanism of action includes a biofilm-based infection in the host.<sup>[76]</sup> A 'critical colonisation' resulting from a multiplication of bacteria is normally accompanied by an increase in pain. Even if the correct treatment is chosen, the healing process could be delayed by a 'critical colonisation' which can result in the formation of a thick slough that is not responsive to standard debridement techniques and a malodour. Bacteria levels should be reduced to a minimum to allow the wound to heal, and the topical application of an antimicrobial dressing is one of the most common ways to achieve this effect.<sup>[75]</sup> US Patent 8,431,151 B2 proposed a method to manufacture a hydrogel antimicrobial non-woven fibrous dressing with controlled release of silver ions. The inventors describe a PEG-based multi-block thermoplastic polyurethane incorporating polyhedral oligomeric silsesquioxane, forming organic-inorganic hybrid hydrogels with unique mechanical properties and adjustable swelling ratios. In this case a nanofiber network, produced with the electro-spinning technique, was used to deliver silver ions. AgNO<sub>3</sub> was directly incorporated into polymer/dimethylformamide solutions to prepare the antimicrobials scaffolds.<sup>[77]</sup>

Hydrogels have been included in the structure of some wound dressings together with other materials, forming composite products suitable for many types of wounds. Shah et al.<sup>[78]</sup> described a material composed of a cotton gauze, or other fibrous substrate, impregnated with a thermoplastic hydrogel forming polymer. The polymers included A-B-A block copolymers, multiblock copolymers, graft copolymers and polymer blends each incorporating a hydrophilic (such as polyethylene oxide or poly(hydroxyalkyl methacrylate)) and a hydrophobic component (such as polystyrene, poly(methyl methacrylate) or polyesters). The hydrogel showed microphase separation of the hydrophobic portion becoming water-insoluble but remaining water-swellaable. By absorbing the wound exudate, the composite dressing could assume a slimy consistency avoiding the adherence to the wound surface that could cause further trauma, and allowing more infrequent changes.

Future developments in wound care products will depend on continued demands from public and healthcare professionals.<sup>[79]</sup> The important challenge for the future is to establish the appropriate wound care strategy for every single patient, and this can be achieved only by offering the optimal products. Innovative dressings need to be developed while their production costs must be kept low.

## 1.4 Drug delivery

Many patents and academic papers about possible applications of hydrogels in drug delivery have been published, however, only a few have resulted in commercial products. Hydrogels have attracted noticeable interest for their use in drug delivery due to their unique physical properties.<sup>[80-82]</sup> The high porosity that characterizes hydrogels can easily be adjusted by controlling the density of cross-links in their matrix and the affinity to water. Their porous structure also allows drugs to be loaded and then released. The advantages offered by hydrogels for drug delivery applications include the possibility for sustained release, which results in maintaining a high local concentration of an active pharmaceutical ingredient over a long period.<sup>[80]</sup> The drug can be loaded into a hydrogel and then its release may proceed through several mechanisms: diffusion controlled, swelling controlled, chemically controlled and environmentally responsive release.

The diffusion controlled release systems can be represented by reservoir or matrix devices. Both allow the drug release by diffusion through the hydrogel mesh or the pores filled with water. A reservoir delivery system (Figure 1.8) includes a drug-containing core coated with a hydrogel membrane, commonly available as capsules, cylinders, spheres or slabs. The concentration of the drug is higher in the centre of the system to allow a constant release rate.<sup>[83]</sup>

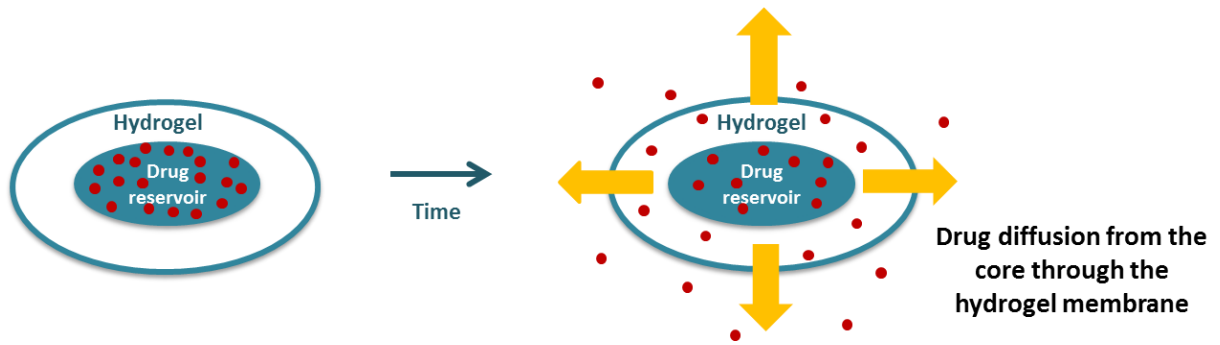


Figure 1.8 Scheme of drug release through a hydrogel membrane in a reservoir system.

In matrix systems the drug is dispersed or dissolved uniformly throughout the three-dimensional structure of the hydrogel (Figure 1.9). Drug release is achieved through the macromolecular mesh or the pores, and the initial release rate in this case is proportional to the square root of time, rather than being constant and time independent as happens in reservoir systems.<sup>[83]</sup>

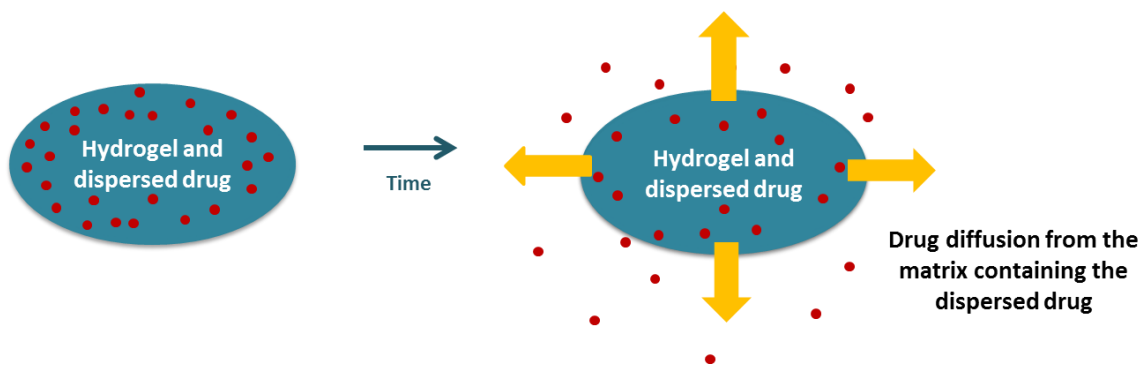


Figure 1.9 Drug release from matrix systems.

In swelling-controlled release devices the drug is dispersed within a glassy polymer as in a matrix device, and when the polymer is in contact with a bio-fluid it starts swelling. The material then expands beyond its boundary allowing the diffusion of drug with the relaxation of polymer chains.<sup>[83]</sup>

This process is also called Case II transport and it shows constant, time-independent kinetics of release. It is known as ‘anomalous transport’, one that combines swelling-controlled release with diffusion.<sup>[84]</sup> The gradient existing between the dispersed drug in the hydrogel and the surrounding environment permits the diffusion of the active ingredient loaded from the high concentration through the hydrogel, to the lower one.<sup>[85]</sup> The molar flux of the drug in this case,  $J$  (mol/cm<sup>2</sup>s), is proportional to the concentration gradient ( $\Delta c$ ) as the driving force for this process:

$$J = -D \cdot \Delta c, \quad (5)$$

where  $D$  is the diffusion coefficient in the polymer (cm<sup>2</sup>/s), and  $c$  is the concentration of the drug within the polymer (mol/cm<sup>3</sup>). The release rate normally depends on the time so the release kinetics is determined from:

$$\partial c / \partial t = -\Delta \cdot J = \Delta \cdot (D \cdot \Delta c) \quad (6)$$

This equation describes the transport of drug out of the hydrogel when the boundary is static (static drug delivery).<sup>[86]</sup>

The hydrogel-based dosage forms can have different designs and shapes depending on the route of drug administration (Table 1.4).

Table 1.4 Main types of hydrogel-based products applied via different routes of drug administration.

Route of administration	Shape	Typical dimensions	References
Peroral	Spherical beads	1 $\mu$ m to 1mm	[87, 88]
	Discs	Diameter of 0.8 cm and thickness of 1mm	[89]
	Nanoparticles	10-1000 nm	[90]
Rectal	Suppositories	Conventional adult suppositories dimensions (length $\approx$ 32 mm)  with a central cavity of 7 mm  and wall thickness of 1.5 mm	[91]
Vaginal	Vaginal tablets	Height of 2.3 cm, width of 1.3 cm and thickness of 0.9 cm	[92]
	Torpedo-shaped pessaries	Length of 30 mm and thickness of 10 mm	[93]
Ocular	Contact lenses	Conventional dimensions  (typical diameter $\approx$ 12 mm )	[94]
	Drops	Hydrogel particles present in the eye drops must be smaller than 10 $\mu$ m	[95]
	Suspensions	N/A	
	Ointments		
	Circular inserts	Diameter of 2 mm	[96]

and total weight of 1 mg [97]

(round shaped)

Transdermal	Dressings	Variable	[1]
Implants	Discs	Diameter of 14 mm and thickness of 0.8 mm	[98]
	Cylinders	Diameter of 3 mm and length of 3.5 cm	[99, 100]

The topical application of hydrogels can effectively be used to deliver drugs that can help to alleviate the symptoms of many pathological conditions. For instance, Nho et al. <sup>[101]</sup> proposed a therapeutic hydrogel made of poly(vinyl alcohol) or poly(vinylpyrrolidone) for the treatment of atopic dermatitis. This product contained an extract from medicinal plants such as *Houttuynia cordata*, elm, celandine and *Canavalia gladiata*, which could be used for the treatment of dermatitis. To prepare this hydrogel poly(vinylpyrrolidone) and poly(vinyl alcohol) were dissolved in the medicinal plant extract. Then, the solution was left to set to produce a gel. It is possible to freeze/thaw the cast and introduce physical cross-links into the gel. Finally the physical gel must be treated with gamma, UV- or electron beam-radiation to initiate chemical cross-linking and to sterilize the final product. The hydrogel was supported by a hydrophilic non-woven fabric sheet and an air-permeable polyethylene film.

Furthermore, hydrogels are suitable for transdermal iontophoretic delivery of drugs, as was demonstrated in the European Patent Application EP 0 524 718 A1, where polyurethane hydrogel matrices were used as monolithic drug reservoirs. These hydrogels were synthesized from mixtures prepared by adding a prepolymer solution containing an isocyanate-capped oxyalkylene-based

prepolymer in anhydrous aprotic organic solvent to water. When the organic solvent has evaporated completely, the hydrogel matrix can be loaded with a drug. Transdermal iontophoresis is defined as the transport of ionic drugs through the skin, driven by a very weak electric current. The applied current helps to transfer the ionized drugs through the stratum corneum into the dermis, in which the active ingredient can diffuse into capillaries and then into the systemic circulation. Alternatively, hydrogel compositions can be employed as passive transdermal reservoirs. The hydrogels used in the aforementioned work showed a high swelling ratio, good flexibility, strength and transparency.<sup>[102]</sup>

Hydrogels could be useful as ocular drug delivery carriers, not only in the form of lenses as previously discussed. The US Patent 8,409,606 B2 presented a system that provided the release of specific drugs through punctal plugs. In this work very soft biodegradable covalently cross-linked hydrogels with high-swelling capability were used, in order to be able to remain in situ (in the punctum or lacrimal canal) with greater comfort for the patient. The system could be designed to be 'temporary' or 'permanent' and the plugs could be accordingly made of collagen or silicone, respectively.<sup>[103]</sup>

Ocular therapeutics™ produces ophtalmic drug delivery systems and medical devices using poly(ethylene glycol) hydrogels. For instance, dexamethasone punctum plug is designed for the controlled release of the corticosteroid in case of post-operative inflammation and pain and it has entered the Phase 3 trials. After a four-week treatment period, during which the plug releases the drug from the canaliculus to the ocular surface, it is naturally removed via the nasolacrimal system.<sup>[104]</sup>

Ideally a drug delivery system should be synchronized with the physiological status of the patient and should provide drug release in response to changes in environment. Moreover, if the drug exhibits some side effects, its release when it is not required can cause additional problems. Hydrogels can show changes in their swelling behaviour, structure, permeability or mechanical

properties in response to various internal and external stimuli.<sup>[87]</sup> Bae et al.<sup>[105]</sup> proposed a delivery device capable of releasing a drug enclosed within a hydrogel, which deswells responding to a chemical or physical stimulation (change in temperature, pH, ionic strength or glucose concentration). It utilises either temperature- or pH- sensitive hydrogels already used in drug delivery as cross-linked homopolymers or copolymers, such as the N-isopropylacrylamide based copolymers or cross-linked weak polyelectrolytes. The system presented by Bae et al.<sup>[105]</sup> was composed of a 'sponge-like' porous gel confined in a walled structure permeable to the loaded drug. Thus, it was possible to obtain a self-regulated drug delivery, which could be pulsatile if needed.

Biodegradable and nontoxic multi-block hydrogel copolymers have been used as drug delivery matrices and described in the US Patent 5,514,380. They were synthesized from a hydrophilic soft block and a hydrophobic, biodegradable hard block. Their degradation could be achieved with the hydrolysis of intramolecular ester and amide bond that easily occurred in the human body. Polyethyleneoxide (PEO) and/or copolymers of PEO/polypropyleneoxide (PPO) with molecular weight of 600-30,000 Da met the required qualities of the hydrophilic, non-biodegradable polymers employed in the mentioned patent. The biodegradable block could instead be represented by polylactide (PLA), polyglycolide (PGA) or a PLA/PGA copolymer.<sup>[106]</sup>

US Patent 8,383,153 B2 describes a poly(amidoamine) based hydrogel for application as drug carriers. This temperature- and pH-sensitive hydrogel had a molecular structure developed to avoid the initial burst drug release and was instead capable of providing a sustained release. The material can be produced by a one-step process by coupling between secondary amine groups (-NH) of a diamine compound (such as piperazine) and vinyl groups (CH<sub>2</sub>=CH-) of an alkylene bisacrylamide compound (e.g. N,N'-methylenebisacrylamide (MDA) or N,N'-ethylenebisacrylamide). This hydrogel can be used as a carrier for different types of physiologically active compounds, using different routes of administration.<sup>[107]</sup>



A drug delivery system comprising a hydrogel and a catheter were also proposed in US Patent 7,066,904 B2. The catheter allows the incorporation and the immobilisation of a relevant amount of drug into the hydrogel, and then its release by a triggering agent or different condition in the desired location. In this case the polymers used, such as (hydroxyethyl)methacrylate-co-methacrylic acid, are pH-sensitive in order to produce hydrogels able to undergo a volume phase transition at a specific pH. A salt solution, such as sodium phosphate or sodium bicarbonate can be used to alter the microenvironment within the device and trigger the release of the active ingredient. In fact, the pH of this solution could be in the range of 7.5 to 8.4 or in the range of 6.4 to 7.3, and could cause alternatively swelling or contraction of the hydrogel.<sup>[108]</sup>

One of the successful examples of hydrogels for drug delivery is the vaginal insert Cervidil<sup>®</sup> for cervical ripening, which has been on the market since 1995. This controlled release formulation has been used to induce or bring on labour in patients who are at or near the time of delivery. Each insert contains 10 mg of dinoprostone (prostaglandin E<sub>2</sub> or PGE<sub>2</sub>) in 271 mg of cross-linked polyethylene oxide/urethane polymer and it releases the drug over a period of 12 hours at approximately 0.3 mg/hr. The drug release is triggered by the hydrogel swelling when placed in a moist vaginal environment.<sup>[109]</sup>

Controlled Therapeutics Scotland Ltd has developed a misoprostol vaginal insert (MVI) that uses the same delivery system as the Cervidil<sup>®</sup>, but contains misoprostol, a cytoprotective agent active on the cervix and uterus to induce labour. The same company is currently developing a modified release hydrogel polymer buccal patch (Pilobuc<sup>TM</sup>) containing pilocarpine, for the treatment of symptoms of Sjögren's syndrome, a systemic autoimmune disease in which exocrine glands that produce tears and saliva are destroyed by the immune cells.<sup>[110]</sup>

A hydrogel subcutaneous insert in the form of reservoir system, called SUPPRELIN LA (by Endo Pharmaceuticals Solutions Inc.), for the release of histrelin acetate is available on the market.

Histrelin acetate is a gonadotropin-releasing hormone (GnRH) agonist indicated for the treatment of children with central precocious puberty (CPP). It produces a decrease in luteinizing hormone (LH) levels and sex steroids serum concentration within the first month of treatment. The implant is made of 2-hydroxyethyl methacrylate, 2-hydroxypropyl methacrylate, trimethylolpropane trimethacrylate, benzoin methyl ether, Perkadox-16, Triton X-100 and contains 50 mg of histrelin, which is delivered over 12 months time (approximately 65 mcg per day). After this period the device needs to be removed as it is nonbiodegradable.<sup>[111]</sup>

Park et al.<sup>[112-115]</sup> had proposed the use of superporous hydrogel compositions as gastric retentive devices for long-term oral drug delivery. These hydrogels were produced starting from (meth)acrylic acid or (meth)acrylamide, a so-called 'disintegrant', represented by a natural or synthetic cross-linked hydrophilic polymer such as cross-linked carboxymethylcellulose or poly(vinyl pyrrolidone) and a cross-linking agent such as N,N'-methylenebisacrylamide. They were synthesized using the gas blowing technique where polymerization and foaming (with sodium carbonate or bicarbonate as foaming agent) take place at the same time. More specifically, in this process the polymerization has to start only a few minutes after the beginning of foaming in order to entrap the gas bubbles in the network. The final device was able to remain in the stomach up to more than 24 hours allowing the slow release of the drug loaded.

Hydrogel devices were suggested for oral delivery of different active ingredients, e.g. non-steroidal anti-inflammatory drugs (NSAIDs).<sup>[116]</sup> They can be used to protect drugs or proteins (e.g. insulin) susceptible to the proteolytic degradation that occurs in the stomach.<sup>[117,118]</sup> In the US Patent application WO1998043615 A1<sup>[119]</sup> a hydrogel matrix made of poly(methacrylic acid-*g*-ethylene glycol) cross-linked with tetraethylene glycol dimethacrylate is presented. This hydrogel could be loaded with insulin simply by immersing it into its solution at pH 7.4. When administered orally, insulin will be protected from the acidic environment of the stomach by the formation of inter-chain

complexes within the hydrogel network. Hydrogen bonding between the carboxyl and the ether groups on the grafted chains stabilized these complexes at acidic pH. These hydrogels exhibited pH-sensitive swelling behaviour: once in the upper small intestine (at higher pH), the complexes dissociate increasing the pore size and allowing the insulin to be released from the matrix. Additionally, the ability of these hydrogels to strongly adhere to the intestinal mucosa significantly improves the release and absorption of the protein. <sup>[117, 119]</sup>

In the future, hydrogel-based products could represent a significant proportion of drug delivery systems, to successfully administer drugs at the desired rate and site in the body. Specific release rates and dissolution profiles could be achieved with the development of new hydrogels with different hydrophobicity/hydrophilicity and structural characteristics. These systems could improve the delivery of more sensitive molecules and be employed in the treatment of pathologic conditions such as diabetes or even cancer. Specifically, more developments are expected in the use of hydrogels for delivery of therapeutic proteins and peptides.

## 1.5 Tissue engineering

There are millions of patients suffering from the loss or failure of an organ or a tissue caused by an accident or a disease every year. Over 8 million surgeries are conducted to treat these patients in the U.S. each year, and the overall cost of these issues to the U.S. economy is estimated to be around \$400 billion per year. Tissue and organ transplantations represent generally accepted therapies, but they are dramatically limited by donor shortages. <sup>[120]</sup>

The term “tissue engineering” was originally defined in 1988 as the “application of the principles and methods of engineering and life sciences toward fundamental understanding of

structure-function relationship in normal and pathological mammalian tissues and the development of biological substitutes for the repair or regeneration of tissue or organ function”.[121] In other words, it involves the improvement or replacement of specific tissues or organs using engineered materials and synthetic strategies.

Tissue engineering is a more recent application of hydrogels, in which they can be applied as space filling agents, as delivery vehicles for bioactive substances or as three-dimensional structures that organize cells and present stimuli to ensure the development of a required tissue (Figure 1.10). Space filling agents are the most commonly used group of scaffolds and they are employed for bulking, to prevent adhesion, and as a biological ‘glue’. Drugs can be delivered from hydrogel scaffolds in numerous applications including promotion of angiogenesis and encapsulation of secretory cells. Additionally, hydrogel scaffolds have also been applied to transplant cells and to engineer many tissues in the body, including cartilage, bone, and smooth muscle. [122]

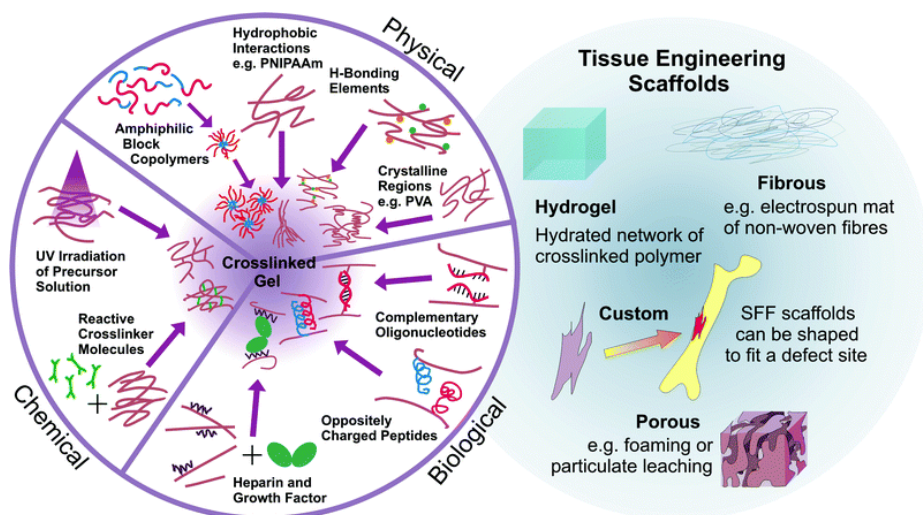


Figure 1.10 Hydrogels in tissue engineering (reprinted from E. S. Place, J. H. George, C. K. Williams, M. M. Stevens, *Chem. Soc. Rev.* 2009, 38, 1139-1151<sup>[123]</sup> with permission from the Royal Society of Chemistry).

An indispensable property is the biocompatibility of hydrogels, which could be defined as the ability of a material to be in contact with the body organs without any damages for the surrounding tissues and without triggering any undesirable response. <sup>[114]</sup> Synthetic materials capable of forming hydrogels suitable for tissue engineering include poly(ethylene oxide), poly(vinyl alcohol), poly(acrylic acid), poly(propylene fumarate-co-ethylene glycol), and polypeptides. Agarose, alginate, chitosan, collagen, fibrin, gelatin, and hyaluronic acid are naturally derived polymers that could also be used for this purpose. <sup>[124, 125]</sup>

In European patent EP 1 664 168 B1, an interesting hydrogel-based composition for manufacturing porous scaffolds has been presented. It was composed of a biodegradable unsaturated self-cross-linkable polymer such as poly(propylene fumarate), biodegradable hydrogel microparticles (diameters of 1 to 1000 micrometers) entrapping water and a free-radical initiator promoting the cross-linking process. The microparticles were made of cross-linked collagen or gelatin and can contain a biologically active substance. The method disclosed produced 'super-absorbent semi-solid' hydrogel microparticles, able to swell in water but not to flow as a liquid, with a defined shape due to the cross-linking. After the polymerization process, the scaffold formed with the mixture could be used directly for the treatment of skeletal defects without leaching out the hydrogel porogen. <sup>[125]</sup>

Harris et al. <sup>[126]</sup> described a tissue engineering scaffold with the benefits of microporous and nanoporous scaffolds, comprising a nanofibrous and nanoporous hydrogel formed from self-assembling peptides, which are non-immunogenic, biodegradable, and capable to interact with cells. They were able to stimulate tissue ingrowth and vascularization, and furthermore, this hydrogel could be used for slow-diffusion drug delivery. The self-assembling peptides used to form hydrogels should have alternating hydrophobic and hydrophilic amino acids (more than 8). For instance, one of them had the following amino acid sequence: Arg-Ala-Asp-Ala-Arg-Ala-Asp-Ala-Arg-Ala-Asp-Ala-Arg-Ala-Asp-Ala. This peptide is commercialized as 'PURAMATRIX' (3-D Matrix, Inc., Cambridge, Mass.) Its

self-assembly could easily take place in tissue culture medium (Dulbecco Modified Eagle's Medium, Gibco BRL, Gaithersburg, Md) containing calf serum. The scaffolds presented might also be applied to open wounds or be surgically implanted. It was established that scaffolds made of only one component or phase may not produce the ideal environment for supporting tissue regeneration. Conversely, the so-called 'hybrid' materials were found to give better results, in terms of cell proliferation, differentiation and migration.

Hydrogels scaffolds are used for cell-sheet and tissue production. Kumar <sup>[127]</sup> has recently disclosed a method to produce biodegradable poly(vinyl alcohol) hydrogels complexed with phenylboronate-containing polymers able to encourage cell and tissue growth. PCCs include a phenylboronate ligand (such as 4-vinylphenylboronic acid), an acrylic monomer (such as N-isopropylacrylamide or acrylic acid) and an alkaline tertiary amine (such as N,N-dimethylaminoethylmethacrylate). The cells which could be represented for example by keratinocytes or fibroblasts, are cultured for 5-20 days on the hydrogel scaffolds. It is then possible to collect the cell layers formed by simply dissolving the hydrogel scaffolds using a saccharide solution (such as fructose or mannitol solution). This saccharide biodegradation is possible due to the presence of phenylboronate ligands that are derivatized forms of phenylboronic acid, which can establish reversible covalent interactions with 1,2 or 1,3-cis-diol-containing compounds such as carbohydrates.

Blanchard et al. <sup>[128]</sup> has reported the use of pure cross-linked keratin-based hydrogels for tissue engineering cell scaffolds. Keratin is biocompatible, and non-immunogenic biopolymer that promotes epithelialization process and can be extracted from patient hair or nails. After purification and partial oxidization of the keratin, the sulfonic acid residues of the protein, which are hydrophilic, form disulfide cross-links between backbones and bind water. Additional hydrogen bonds are then formed in this hydrogel. The material was shown to be suitable as nutrient support and scaffold for

cell growth.

Song et al.<sup>[129]</sup> has proposed beta-glucan-based hydrogel scaffolds for tissue engineering produced by radiation fusion technology. Beta-glucan (beta-1,6-branched-beta-1,3-glucan) can promote cell regeneration and collagen biosynthesis, and it is recognized to be safe and biocompatible. It could be extracted from different fungi such as *Schizophyllum commune* or *Ganoderma lucidum* and dissolved in distilled water. This aqueous solution was then cast in petri dishes and irradiated for the cross-linking step using electron, gamma or UV beam at a dose of 5 to 50 kGy to form a gel. Stem cells could rapidly adhere, grow and differentiate on the scaffold formed.

One of the most important future challenges in tissue engineering is how polymers could be used to stimulate the blood vessel network formation in the desired tissue, essential to supply its needs. Hydrogels could represent a valid option to effectively control the vascularization process, by local delivery of both angiogenic factor and endothelial cells to the intended area.<sup>[120]</sup> Additionally, many types of tissue such as bone, muscle or blood vessels are located in areas requiring excellent mechanical properties that the majority of the currently available hydrogels do not show, so new approaches should be investigated in the future to achieve better results.

## 1.6 Hygiene products

Superabsorbent polymers (SAPs) have been introduced into the agriculture and diaper industry about thirty years ago, and since then their uses have been extended to several other applications due to their excellent water retention.<sup>[130]</sup> SAPs have been firstly commercially produced in Japan in 1978 for use in feminine napkins, and this early material was represented by a cross-linked starch-g-polyacrylate.<sup>[131]</sup>

At the end of the 90s, 'superporous hydrogels' (SPHs) were introduced and presented as a

different type of water-absorbent polymer system. As SAPs, SPHs are formed by covalently cross-linked hydrophilic polymers, but unlike SAPs, they show an exceptional size-independent fast swelling kinetics. The first generation of SPHs was generally made from highly hydrophilic acrylamide, salts of acrylic acid and sulfopropyl acrylate. Later generation of SPHs are represented by 'hybrid SPHs' produced by adding a so-called 'hybrid agent' (natural or synthetic water-soluble or dispersible polymer capable of chemical or physical cross-linking) to the SPH previously made. With this method it is possible to generate an interpenetrating polymeric network. For example, acrylamide-based SPH is synthesized in the presence of sodium alginate and after that, a cross-linking occurs between alginate chains and calcium ions forming a 'hybrid SPH'. These more recent SPHs have shown better and more useful qualities, such as high mechanical strength and elasticity even in swollen state.<sup>[130]</sup> Superabsorbent hydrogels, in particular the acrylate-based materials, are extensively used in hygiene products to absorb fluids. In fact they are able to hold moisture away from the skin, promoting skin health, preventing diaper rash and providing a comfortable use. Parents in all the industrialized countries as well as hospitals around the world employ disposable diapers containing SAPs.<sup>[132]</sup>

A further increase in the use of these materials is observed in training pants and adult incontinence product markets. SAPs can also prevent the colonization of germs, reducing the risk of fecal contaminations and potential spread of gastrointestinal infections. The first use of SAPs in the diaper industry was proposed in 1982 by Unicharm in Japan, with its subsequent use in sanitary napkins. After that, diapers became thinner and also had improved water retention performance. It was possible to develop diapers with leakage values below 2% and the standard weight of a medium size diaper could be reduced by about 50%, with some obvious advantages in terms of environmental issues and reduced manufacturing costs.<sup>[132]</sup>

Regarding the ecological impact of disposable diapers and similar products, it is relevant to consider current diaper consumption. For instance, a child within the 30<sup>th</sup> month uses approximately six diapers a day and each of them has a volume of 500 cm<sup>3</sup>, so only one child produces on average



3,000 cm<sup>3</sup> of litter a day, *i.e.* 1,092 cubic meters every year<sup>[132]</sup>. Making recyclable disposable diapers, napkins, hospital bed sheets, sanitary towels and other similar products is therefore one of the vital targets for the modern industry. An innovative solution to this problem has recently been proposed, which involves the use of cellulose-based hydrogels, which are totally biodegradable. Novel types of hydrogels, containing sodium carboxymethylcellulose (NaCMC) and hydroxyethyl cellulose (HEC) cross-linked with divinyl sulfone (DVS), can swell like SAPs, and exhibit high water retention under centrifugal loads. These improvements were achieved by introducing microporous structures into the hydrogel, which increases water retention and swelling kinetics due to capillarity effects.<sup>[132]</sup>

US Patent 32,649 describes one of the first hydrogel-forming polymer compositions suitable for hygiene products manufacturing. It consisted of a water-insoluble, slightly cross-linked polymeric material, which could be prepared from carboxylic acids and acid anhydrides, or olefinically unsaturated sulfonic acids, using a free-radical polymerization in the presence of a cross-linking agent in an aqueous solution. This material could be dried to result in polymer compositions capable to form hydrogels upon contact with water or bodily fluids.<sup>[133]</sup> Only a few years later, in US Patent 5,009,653, Osborn proposed a product consisting of a thin and flexible sanitary feminine napkin with an absorbent core placed between two air-laid tissue sheets. The core was composed of a hydrogel-forming material, prepared from acidic monomers such as acrylic acid, methacrylic acid or 2-acrylamido-2-methyl propane sulfonic acid. This material was highly absorbent, could withstand medium to high menstrual flows and was very conformable to the body of a user, preventing the risk of leakage and staining.<sup>[134]</sup>

Many attempts have been made to develop new products, which could not only swell, but also retain the fluids absorbed under external pressure or against an applied restraining force. An absorbent material composed of a porous matrix of fibers and superabsorbent hydrogel is described

in the US Patent 5,147,343, which has the capability to initially imbibe fluids and swell, while being exposed to a load. The matrix can be formed from wood pulp or cotton linters as well as synthetic fibers (polyethylene, polypropylene polyesters etc.) and the hydrogel could be produced from polyacrylamides, polyvinyl alcohol, ethylene-maleic anhydride copolymers or polyvinyl ethers. The 'Absorbency Under Load' (AUL) is defined as the volume of 0.9 wt % NaCl solution which the superabsorbent composition could absorb per 1 g in one hour, being subjected to a load of 21,000 dynes/cm<sup>2</sup>. Hence, the work (W) performed by the material could be calculated using the following formula:<sup>[135]</sup>

$$W = (\text{AUL}) \times (\text{Restraining force}) \quad (7)$$

Pampers (owned by Procter & Gamble) and Huggies (from Kimberly-Clark) are the two most widely used disposable diaper brands, with about 35% and 22% global market share, respectively. Both are sold in over 50 countries and they have wide range of products. Manufacturers have been focusing their efforts on enhancing the production and engineering of SAPs with better properties, i.e. higher AUL, lower levels of residual monomers (RM) and soluble fractions.<sup>[136]</sup> Further developments in this area are expected with the formulation of the materials containing enzymes and other additives to prevent infections and unpleasant smells. Additionally, taking the scale of production of these materials into consideration, there is a clear need in environmentally friendly hygiene products that undergo biodegradation.

## 1.7 Conclusions

Hydrogels are widely present in everyday products though their potential has not been fully explored yet. These materials already have a well-established role in contact lenses, hygiene products and wound dressing markets but commercial hydrogel products in tissue engineering and drug delivery are still limited. Many hydrogel-based drug delivery devices and scaffolds have been designed, studied and in some cases even patented, however not many have reached the market. More progress is expected in these two areas. Limited commercial products with hydrogels in drug delivery and tissue engineering are related to some extent to their high production costs.

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## 2 Antimicrobial hydrogels based on autoclaved poly(vinyl alcohol) and poly(methyl vinyl ether-alt-maleic anhydride) mixtures for wound care applications

Enrica Caló, Joao M.S. de Barros, Mar Fernández-Gutiérrez, Julio San Román, Lucy Ballamy, Vitaliy V. Khutoryanskiy

Novel antimicrobial hydrogels with good mechanical and physical properties were synthesized by autoclaving aqueous mixtures of poly(vinyl alcohol) and poly(methyl vinyl ether-alt-maleic anhydride). The structure of these materials was studied by infrared spectroscopy, scanning electron microscopy and solid state nuclear magnetic resonance. The swelling behaviour, mechanical properties and adhesion of the hydrogels to porcine skin were evaluated. It was established that these hydrogels exhibited antimicrobial properties and inhibited bacteria growth against *Staphylococcus aureus*. The biocompatibility of the hydrogels was confirmed using an MTT assay (indirect cytotoxicity) and by monitoring cell proliferation in contact with the gels (direct cytotoxicity).

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## 2.1 Introduction

Hydrogels are three-dimensional networks produced by chemical (chemical hydrogels) or physical (physical hydrogels) cross-linking of water-soluble polymers, which can be achieved using different synthetic approaches.<sup>[1]</sup> The starting materials for making hydrogels can be represented by natural or synthetic monomers or polymers that can form hydrophilic networks alone, or with the addition of cross-linking agents or/and initiators.<sup>[2]</sup> Hydrogels are widely used as biomaterials for drug delivery, tissue engineering and wound dressings, due to their unique mechanical and physical properties.<sup>[3-5]</sup>

Hydrogels have drawn interest from the wound care industry because of their resemblance to living tissue as well as their ability to absorb and retain exudate which improves the balance of hydration of the wound bed.<sup>[6,7]</sup> A major advantage of hydrogel products is their transparency which allows the user to visually monitor the wound without removing the dressing. Unlike traditional wound care products, such as common plain gauze, hydrogel dressings do not require daily changes.<sup>[8,9]</sup> Their high water content facilitates vapour and oxygen diffusion to the wound which may accelerate the healing process.<sup>[9]</sup> Hydrogels are also well known to promote autolytic debridement, which is a process in which the body breaks down and removes dead tissue, carried out by specific enzymes called matrix metalloproteinases which migrate to the wound site after injury.<sup>[10]</sup>

Hydrogel-based dressings can be designed as amorphous gels or as flat sheets ready to be applied. In both cases they conform and slightly adhere to the surface of the wound, therefore avoiding the formation of the so-called 'dead space' where bacteria can proliferate causing infections.<sup>[11]</sup>

Hydrogels are typically synthesized via three-dimensional polymerization of hydrophilic monomers. This method requires extensive purification of the hydrogel product from any unreacted monomers, which are often toxic. Purification is achieved by extraction using excess water and may take up to

two weeks. An alternative method for the preparation of hydrogels is the cross-linking of water-soluble polymers. This is achieved by using ionizing radiation such as gamma rays or accelerated electrons.<sup>[12]</sup> The advantage of this method is that the hydrogel formation and its sterilization are achieved in a single step. However, the application of radiation requires the use of expensive facilities.

Recently we pioneered the synthesis of hydrogels using microwave-mediated thermal cross-linking in aqueous mixtures of functional water-soluble polymers. This method offers the advantage of hydrogel formation with simultaneous sterilization without the need for their purification because no toxic monomers or cross-linkers are used.<sup>[13]</sup> In microwave processing, energy is supplied by an electromagnetic field and results in rapid heating of aqueous solutions. The use of this method commercially is often limited because of the need for access to industrial microwave facilities. Autoclaving is a viable alternative to microwave processing and is a more common method of heating aqueous solutions at industrial scale. Autoclaving is widely used for sterilisation of medical devices.

The present work is the first report on the synthesis of 'ready-to-use' hydrogels by autoclaving aqueous mixtures of poly(vinyl alcohol) (PVA) and poly(methyl vinyl ether-alt-maleic anhydride) (Gantrez® AN or Gant). The hydrogels developed in this work have a unique combination of properties such as excellent swelling ability, good mechanical strength, adhesiveness to skin, as well as intrinsic antimicrobial activity.

## 2.2 Experimental section

### 2.2.1 Materials

PVA of 98-99% hydrolysis and of medium (Mw 57-66 kDa) and high (Mw 88-97 kDa) molecular weights were purchased from Alfa Aesar (UK). The hydrogel compositions will be then referred to as PVAm-Gant and PVAh-Gant (with their molar ratios) in figures and tables. Actual degree of PVA deacetylation was verified by  $^1\text{H}$  NMR and found to be 98.7%, and the actual Mw of the PVA used were found to be 51.3 kDa (for medium Mw PVA) and 66.3 kDa (for high Mw PVA) by gel permeation chromatography. Nutrient agar used in the antimicrobial test was purchased from Oxoid Ltd UK. Hoechst 33342 nucleic acid stain was purchased from Life Technologies (Molecular Probes<sup>TM</sup>). Gantrez<sup>®</sup> AN 139 (Mw ~ 216 kDa) and all the other chemicals used were purchased from Sigma-Aldrich (UK). Aquacel<sup>®</sup> Ag dressings (used as a control dressing during antimicrobial properties and biocompatibility tests) were kindly provided by ConvaTec Ltd.

### 2.2.2 Synthesis of autoclaved hydrogels

The aqueous mixtures of polymers were prepared using different PVA-Gantrez<sup>®</sup> AN molar ratios: 1:1, 1:2, 2:1 (base moles of PVA per base moles of Gantrez<sup>®</sup> AN). For hydrogel synthesis 17.4 % w/v aqueous solution of Gantrez<sup>®</sup> AN was added to 4.4 % w/v solutions of PVA (with a final volume of 15 mL) and the mixtures were stirred for 205 minutes at room temperature. Then they were cast in 8 cm diameter glass petri dishes which were autoclaved at 125°C, 1.4 bar for 90 minutes (CertoClav Multicontrol 12 L).

### 2.2.3 Synthesis of freeze-thaw hydrogels

Mixtures of 10% w/v aqueous solution of PVA and 17.4 % w/v aqueous solution of Gantrez<sup>®</sup> AN with a 1:2 molar ratio of the two polymers, containing 0.25% w/v of NaOH (with a final volume of 18 mL)

were frozen at  $-18^{\circ}\text{C}$  for 9 hours and then thawed at room temperature resulting in physically cross-linked hydrogels.

#### 2.2.4 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectra of PVA powder, Gantrez<sup>®</sup> AN powder and a freeze-dried gel sample were recorded using a Nicolet IS5 ID5 ATR infrared spectrometer and data analysed using OMNIC software with 16 scans and a resolution of  $4\text{ cm}^{-1}$ .

#### 2.2.5 Solid state nuclear magnetic resonance (NMR)

Hydrogel samples, and individual PVA and Gantrez<sup>®</sup> AN solutions were freeze-dried and used for recording solid state  $^{13}\text{C}$  NMR spectra.  $^{13}\text{C}$  solid state cross-polarization magic angle spinning (CPMAS) NMR spectra were recorded on Bruker Avance III spectrometer operating at Larmor frequency of 125.78 MHz (11.75T). The standard bore 4 mm MAS probe was spun at 15 kHz rate. The CP contact time was 1.4 ms, and the  $90^{\circ}$  pulse width was  $3.7\mu\text{s}$ . A total of 2048 signal transients were accumulated with 6 s relaxation delays at ambient temperature. All spectra were referenced to external signal as a secondary reference (frequency peak at 38.5 ppm with respect to TMS).

#### 2.2.6 Boiling experiment

Disc shape ( $\sim 2\text{ cm}$  in diameter) autoclaved hydrogels and freeze-thaw hydrogels were immersed in boiling water for over 2 hours; observations were registered using a digital camera.

#### 2.2.7 Swelling

Swelling of the autoclaved hydrogels was measured in deionized water, in an ion-containing solution (8.298g of sodium chloride and 0.368g of calcium chloride dissolved in 1 L of deionized water) to simulate wound fluid, and in phosphate buffer (PBS) at  $37^{\circ}\text{C}$ . The hydrogels were kept in excess



media for 11 days and weighed regularly to calculate the swelling ratio (SR in %) using the following formula:

$$SR (\%) = \frac{W_s - W_i}{W_i} \times 100 \quad (1)$$

where  $W_s$  is the weight of the swollen gel and  $W_i$  is its initial weight.

#### 2.2.8 Mechanical properties

Mechanical properties of the hydrogels were studied with the autoclaved samples. Tensile tests were conducted using a Texture Analyser XT Plus (Stable Micro Systems Ltd, UK) in sample stretching mode. Rectangular hydrogel samples of 20 mm in length and 3 mm in width were used with a 'grip-to-grip' separation of 30 mm. Each sample was subjected to an extension at 100 mm/min to its breaking point.

#### 2.2.9 Scanning electron microscopy (SEM)

The gels were synthesized, freeze-dried and cut before gold sputter coating. Scanning electron microscopy (1000x and 10000x magnification was used) was carried out using an FEI Quanta FEG 600 Environmental Scanning Electron Microscope (ESEM).

#### 2.2.10 Adhesion test

Adhesion tests were carried out using a Texture Analyser XT Plus (Stable Micro Systems Ltd, UK). The hydrogel samples (20 mm diameters) were attached to the probe and a piece of shaved porcine skin (supplied by Vicars & Son butchers, Reading) was placed on the platform below. Porcine skin from the back of the animal was used for these tests. The tests were performed with the following settings: pre-speed test 1.0 mm/s; test-speed 0.5 mm/s; post-test speed 1.0 mm/s; applied force 0.5 N; contact time 60.0 s; trigger type auto; trigger force 0.1 N; and return distance of 10.0 mm. Force versus distance curves were recorded using the Texture Analyser software (T.A. Exponent). The

samples were put in contact with the porcine skin for 1 min and then detached in order to collect the following data: maximum force required for the detachment (adhesive strength), total work of adhesion (represented by the area under the force versus distance curve) and the cohesiveness (maximum distance travelled by the gel to be detached from the porcine skin) as defined in Boateng et al. .<sup>[14]</sup>

#### 2.2.11 Antimicrobial activity

The disk diffusion method<sup>[15]</sup> was employed to evaluate the antimicrobial properties of all PVA-Gantrez<sup>®</sup> AN hydrogel samples. *Staphylococcus aureus* NCTC 8532 strain from the National Collection of Type Cultures was used in these experiments. Nutrient agar and nutrient broth were prepared following manufacturer instructions and were sterilized by autoclave (121°C for 15 minutes). *Staphylococcus aureus* was grown overnight (37 °C with rotary shaking at 200 rpm) in nutrient broth. Serial dilutions were performed to determine initial cell numbers and testing concentrations. Nutrient agar plates were inoculated with a *Staphylococcus aureus* concentration dose of either 10<sup>8</sup> CFU/mL or 10<sup>4</sup> CFU/mL spread onto agar surface. Disks of hydrogel sample (~1 cm in diameter) were cut and placed on the agar surface and incubated at 37°C for 24 hours. The diameter of the growth inhibition zone was measured using a ruler. Six samples and three repeats each were tested in parallel for each experiment. All experiments were performed aseptically.

#### 2.2.12 Cytotoxicity analysis

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed to indirectly test the cytotoxicity of the hydrogel samples.<sup>[16-19]</sup> The hydrogel samples (~5 mg each) were immersed in 8 mL of Dulbecco's modified Eagle's medium (DMEM from Sigma) containing 2 % v/v L-glutamine and 50 µg/mL gentamicin, and incubated at 37°C. On the 1<sup>st</sup>, 2<sup>nd</sup> and 7<sup>th</sup> day of the experiment, media was removed and collected (these represent the gel extracts) in sterile conditions

and replaced with fresh media. A commercial wound dressing (Aquacel® Ag by ConvaTec Ltd) was used as a control (~5 mg).

Human dermal fibroblasts passage 3 were seeded at a density of  $8 \times 10^4$  cells/mL in complete medium (10% Fetal bovine serum (FBS), 2% L-Glutamine and 50 µg/mL of gentamicin were added) in a sterile 96-wells culture plate and cultured until confluence was reached. The medium was then replaced with the hydrogel (and control) extracts and incubated at 37°C and 5% CO<sub>2</sub> for 24 h. The MTT solution was prepared in warm PBS (0.5 mg/mL) and added to the plate (100 µL per well), which was incubated at 37°C for 3 h. The MTT and medium was removed and 100 µL of DMSO were used to solubilize the formazan retained by the cells. The absorbance was measured with a Biotek Synergy HT detector at 570/630 nm.

Human fibroblasts were also seeded at the same density ( $8 \times 10^4$  cells/mL) in complete medium in the presence of ~5 mg hydrogel samples for direct biocompatibility testing. A hydrogel sample prepared by autoclaving PVA-Gantrez® AN aqueous mixtures (1:1) containing 0.05%v/v Triton® was used as a positive control; this sample is expected to cause significant cytotoxicity. A commercial Aquacel® Ag (ConvaTec Ltd) dressing was used as a negative control. The cells were cultured and the media changed every day. Culture growth was monitored and the cells were stained for nuclei fluorescence using Hoescht 33342 and the Zeiss A1 Inverted Epifluorescent Microscope with Nikon NIS Elements photo-capturing system was used to take images of the cultures.

#### 2.2.13 Statistical analysis

One-way ANOVA with Bonferroni post-hoc test on Prism (Graphpad, USA) was used to carry out all the statistical analysis to compare different groups of data.

## 2.3 Results and discussion

### 2.3.1 Synthesis of hydrogels and mechanism of cross-linking in polymer mixtures

Transparent, bubble-free, flat hydrogel samples were successfully produced from aqueous mixtures of PVA and Gantrez® AN using autoclaving. In order to establish the nature of cross-linking in the mixtures of Gantrez® AN and PVA, the autoclaved hydrogel samples were freeze-dried and subjected to FTIR (Figure 2.1) and solid state  $^{13}\text{C}$  CP-MAS NMR (Figure 2.2) for spectroscopic characterisation. The results were compared with the spectra of the individual polymers.

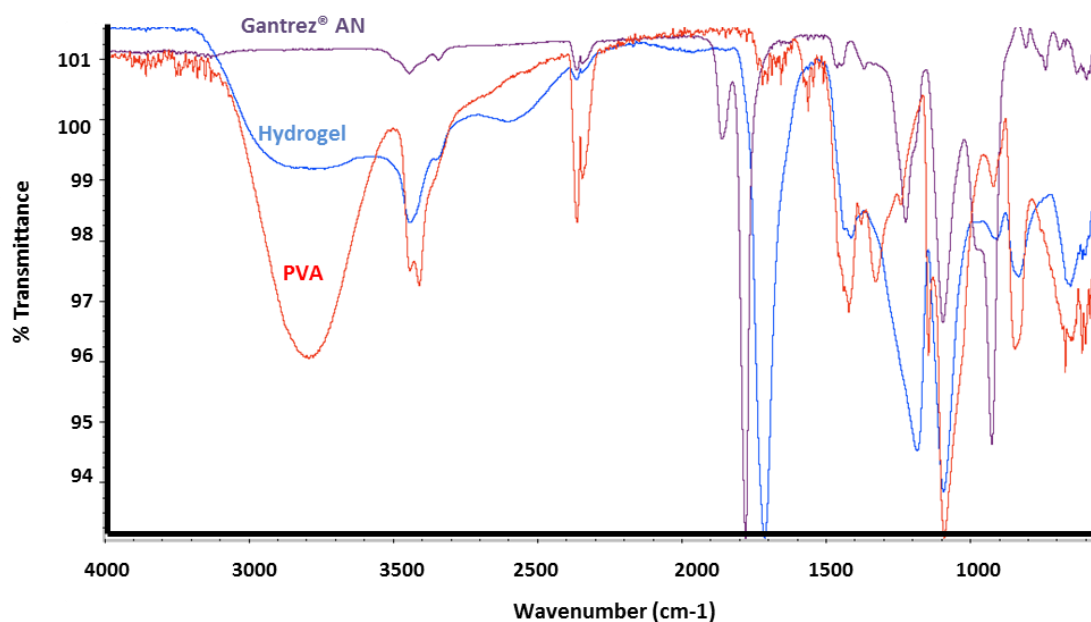


Figure 2.1. FTIR spectra of Gant powder (purple), PVAm powder (red) and freeze-dried PVAm-Gant (1:1) hydrogel sample (blue).

In the FTIR spectrum of Gantrez® AN the absorption peak of the anhydride carbonyl group is clearly visible in the region  $1760\text{-}1820\text{ cm}^{-1}$ ; OH stretch peak ( $3300\text{-}2500\text{ cm}^{-1}$ ) and CO stretch peak typical for carboxylic acids ( $1320\text{-}1000\text{ cm}^{-1}$ ) are visible as well as the OH bend peak characteristic for carboxylic groups at  $950\text{-}910\text{ cm}^{-1}$ ; C-O-C stretch peak of the methyl vinyl ether unit is visible at  $1092$

$\text{cm}^{-1}$ .<sup>[20]</sup> In the PVA spectrum it is possible to observe the characteristic peaks of the hydroxyl groups (3200-3400  $\text{cm}^{-1}$ ), CH stretch (3000-2850  $\text{cm}^{-1}$ ), CH bend (1470-1450  $\text{cm}^{-1}$ ) and CO stretch peak of alcohol groups (1320-1000  $\text{cm}^{-1}$ ). The peaks present in the freeze-dried hydrogel spectrum result from the spectral features of both polymers. For instance, in the hydrogel's spectrum the peak attributable to the carbonyl of the maleic anhydride is shifted to lower wavenumbers, typical for the carbonyl of the carboxylic acid (1710  $\text{cm}^{-1}$ ) or the ester (1735  $\text{cm}^{-1}$ ). The peak typical for the hydroxyl groups of the PVA is present in the same region 3200-3400  $\text{cm}^{-1}$  in the hydrogel's spectrum as well, even if less pronounced. The peak at 1172-1177  $\text{cm}^{-1}$  could support the esterification reaction between the COOH formed after the ring opening and the OH groups of PVA.<sup>[20]</sup> A peak at 2400-2300  $\text{cm}^{-1}$  is visible in all the three spectra and it is attributable to carbon dioxide present in the atmosphere.<sup>[13]</sup> Figure 2.2 shows the solid state  $^{13}\text{C}$  CP-MAS NMR spectra of Gantrez<sup>®</sup> AN, PVA and freeze-dried hydrogel sample with related assignments.

In the spectrum of Gantrez<sup>®</sup> AN: C1 and C2 of the two carbonyl groups of the anhydride cycle give a single peak at 190-170 ppm; C3 and C4 of the maleic anhydride cycle give a double peak at 35-55 ppm; C5 and C7 of the lateral chain give signals at 85-70 and 25-35 ppm, respectively; C6 of the methyl group gives a peak at 60-50 ppm. In the PVA spectrum: C8 gives a double peak at 70-60 ppm and C9 a single one at 50-40 ppm.

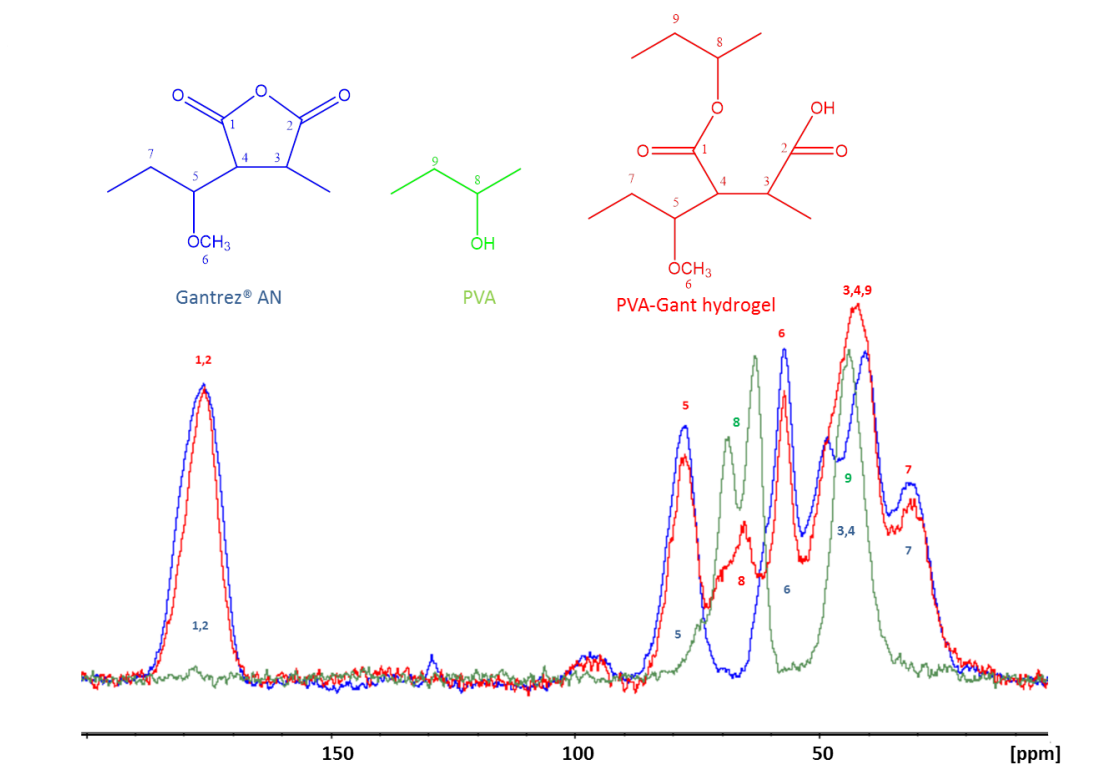


Figure 2.2 Solid state  $^{13}\text{C}$  CP-MAS NMR spectra of Gant, PVAm and PVAm - Gant (1:1) hydrogel sample.

Moving on to the hydrogel's spectrum: C1 and C2 of the now open maleic cycle give a single peak in the same region (190-170 ppm) as in the spectrum of Gantrez<sup>®</sup> AN, as well as C3 and C4 that in this case give a higher peak at 55-35 ppm (for the contribution of C9), C5 (85-70 ppm), C6 (60-50 ppm) and C7 (25-35 ppm). The peak given by C8 in the hydrogel's spectrum is still found at 70-60 ppm (as in PVA) but is now single.

Since the spectroscopy did not provide a conclusive evidence on the formation of covalent linkages between Gantrez<sup>®</sup> AN and PVA, additional experiments were conducted with PVA- Gantrez<sup>®</sup> AN hydrogels cross-linked via non-covalent interactions through the formation of hydrogen bonds and crystalline domains. These physically cross-linked samples were prepared using freeze-thawing procedure, well-known for the preparation of hydrogels from PVA.<sup>[21]</sup> For this purpose solution

mixtures of Gantrez® AN and PVA, containing 0.25% w/v of NaOH, were frozen for a period of 9 hours and then thawed at room temperature leading to opaque hydrogels. Immersion of hydrogels produced via the freeze-thaw technique into boiling water results in completely different phenomena to the hydrogels produced via the autoclaving technique. The physically cross-linked freeze-thaw hydrogels undergo quick dissolution within 20 seconds due to disruption of intermolecular hydrogen bonds at high temperatures (Figure 2.3). On the other hand, the autoclaved hydrogels are not dissolved upon immersion into boiling water and only minor additional swelling occurred within two hours. The results of the boiling experiments clearly demonstrate that autoclaving leads to the formation of stable bonds between Gantrez® AN and PVA, whose nature is likely to be covalent. We propose that autoclaving leads to the formation of ester bonds between the carboxylic groups of Gantrez® AN and hydroxyl groups of PVA. These bonds are not detected by spectroscopic techniques because their concentration in the hydrogels is very low. This is also consistent with our previous report on the synthesis of hydrogels using microwave processing.<sup>[13]</sup> Typically 1-2 ester bonds per macromolecule will be sufficient to make a network and the concentration of these new bonds will be very low and impossible to detect by spectroscopic techniques. The presence of these bonds is further confirmed by degradation and complete dissolution of autoclaved hydrogels in boiling 10% NaOH solution within approximately 45 minutes.

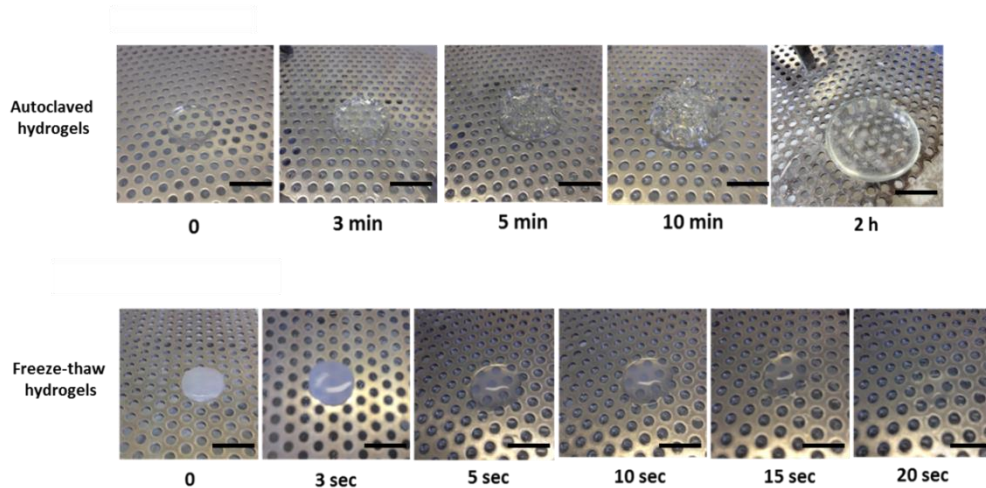


Figure 2.3. Comparison between the behaviors of chemically and physically cross-linked PVAm-Gant (1:1) hydrogels after being immersed in boiling water. Scale bars are 2 cm.

It should also be noted that autoclaving solutions of individual PVA and Gantrez® AN does not result in formation of hydrogels. Autoclaving-mediated cross-linking is only possible when two polymers are present in an aqueous mixture, which further confirms the formation of ester bonds between carboxylic groups of Gantrez® AN and hydroxyl groups of PVA.

Depending on the nature of cross-linking, the same mixture of polymers can be used to produce hydrogels with completely different properties (Figure 2.4). For instance, physically cross-linked hydrogels produced by freeze-thaw technique are not fully transparent, have reversible properties and form at low temperatures, making them promising for tissue engineering and drug delivery due to their excellent mechanical strength.<sup>[22]</sup> Chemically cross-linked autoclaved hydrogels, on the contrary, are less suitable for tissue engineering applications because of high temperatures used for their preparation; but they are highly promising for wound care. The properties of these hydrogels and their applicability for wound care shall be discussed in detail.



### 2.3.2 Physicochemical properties of autoclaved hydrogels

The autoclaved hydrogels immersed in deionized water undergo significant swelling, taking over 100 g of water per 1 g of initial sample within 11 days (Figure 2.5). Even after this relatively long period of time the hydrogels have not yet reached their maximum swelling capacity and continue to absorb water. The swelling ratio (SR) of the autoclaved hydrogels was significantly higher in deionized water compared to the ion-containing solution which was used in order to more closely resemble the ionic strength of wound fluid (Figure 2.6 and 2.7). Only a modest 5-times swelling was observed in the ion-containing solution. This significant reduction in SR with the ion-containing solution compared to deionized water is typical for polyelectrolyte hydrogels and can be explained by the charge screening effects and the lower osmotic pressure due to the difference in mobile ions present between the hydrogel matrix and the external medium.<sup>[23]</sup>

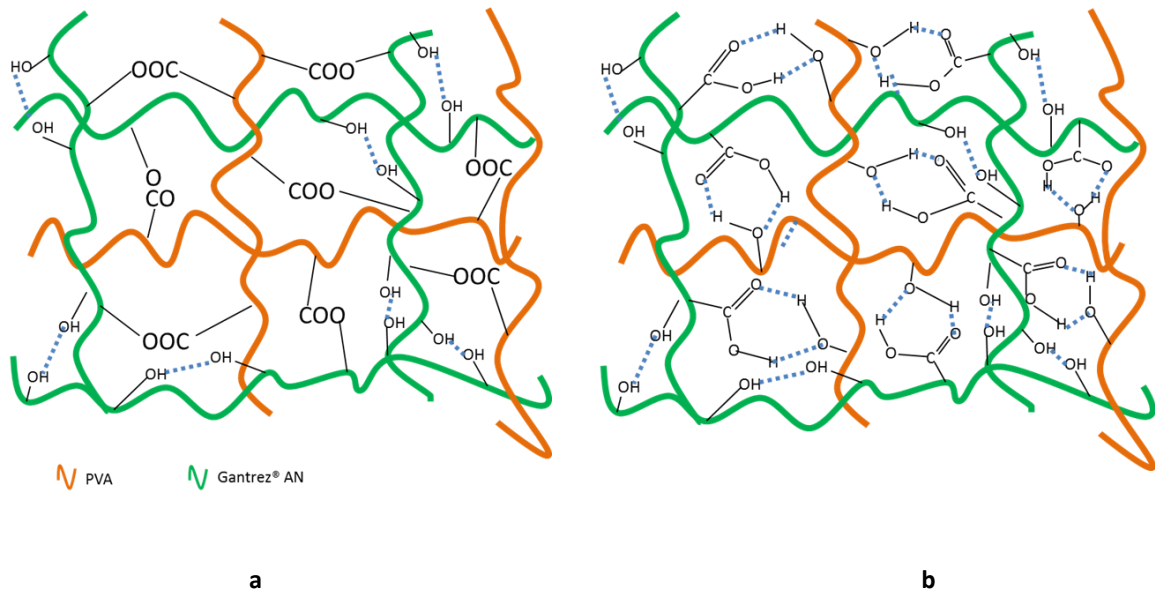


Figure 2.4 Proposed structure of chemically cross-linked autoclaved hydrogels (a) and physically cross-linked freeze-thaw hydrogels (b) based on PVA-Gant mixtures.

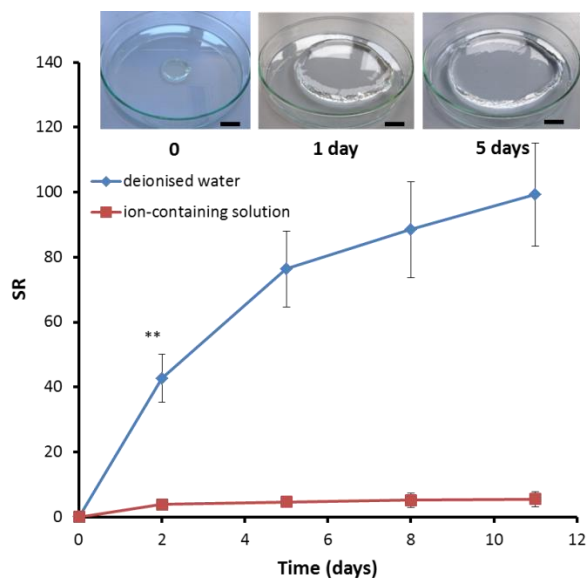


Figure 2.5 Swelling kinetics for PVAm-Gant (1:2) in deionized water and in an ion-containing solution at room temperature. Insert: photograph of a hydrogel undergoing swelling in deionized water. The scale bar shown is 2 cm.

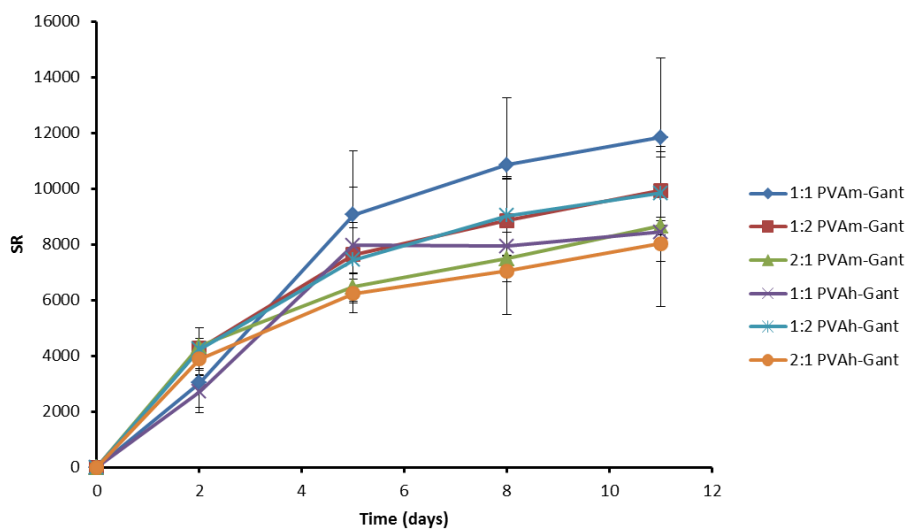


Figure 2.6 Swelling kinetics for PVAm-Gant and PVAh-Gant autoclaved hydrogels in deionized water.

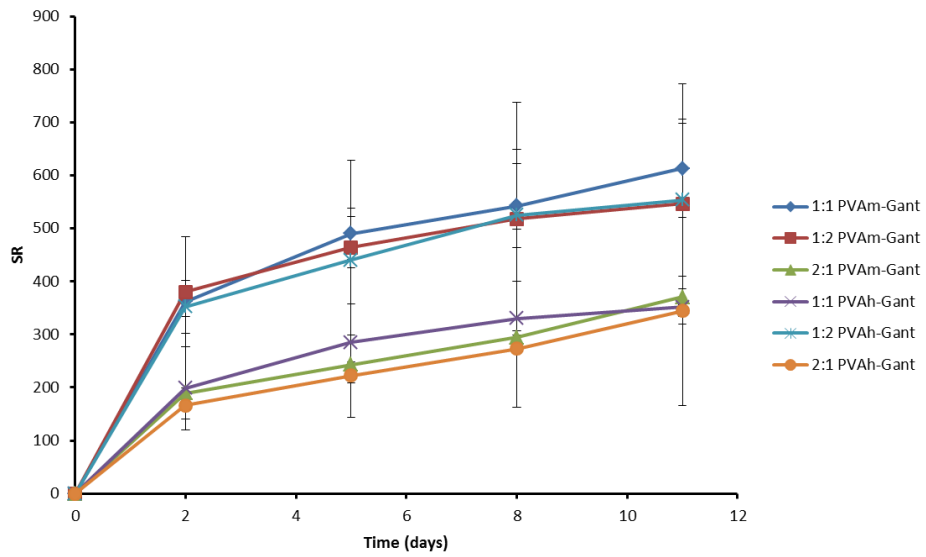


Figure 2.7 Swelling kinetics for PVAm-Gant and PVAh-Gant autoclaved hydrogels in simulated wound fluid.

No statistically significant effects of the polymer ratios of PVA molecular weights on the swelling ratios were observed ( $p < 0.05$ ). It should be noted that all the values have high standard deviations because of the high amount of surface water present which was impossible to fully remove during the gravimetric measurements.

The mechanical behaviour of the autoclaved hydrogels was evaluated using a tensile test (Table 2.1).

The exemplary strength-strain curves are shown in Figure 2.8.

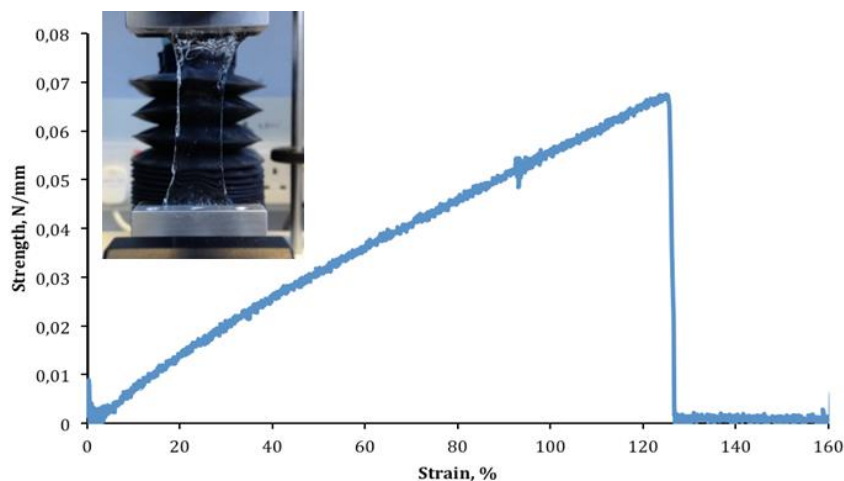


Figure 2.8 Strength vs strain curve exemplary and image of PVA-Gantrez<sup>®</sup> AN hydrogel stretching from the tensile test conducted using a Texture Analyzer (Stable Micro Systems Ltd, UK).

Young's modulus is commonly defined as the slope of stress (the force applied) versus strain (the deformation occurred in the material) curve. It is considered as the most relevant mechanical property of biomaterials.<sup>[24]</sup> The ultimate strength is the maximum stress that a material can withstand. The elongation to break is the deformation (strain) measured at fracture.<sup>[25]</sup>

Table 2.1 Mechanical properties of hydrogel samples. Data are shown as mean  $\pm$  standard deviation. Statistical significance ( $p < 0.05$ ) was calculated for the data related to Young's modulus, tensile stress at break, ultimate strength and elongation to break of the hydrogel compositions (a-f). Letters after each value indicate which other composition the sample is significantly different from.

Composition (mol/mol)	Young's Modulus (N/mm <sup>2</sup> )	Tensile stress at break (N/mm <sup>2</sup> )	Ultimate strength (N/mm)	Elongation to Break (%)
<sup>a</sup> PVAm-Gant (1:1)	$(4.6 \pm 1.2) \times 10^{-5}$	$(3.1 \pm 0.3) \times 10^{-3}$	$(6.4 \pm 0.8) \times 10^{-2}$ <sup>(e)</sup>	110 $\pm$ 17
<sup>b</sup> PVAm-Gant (1:2)	$(1.3 \pm 1.5) \times 10^{-4}$	$(2.6 \pm 0.2) \times 10^{-3}$	$(5.1 \pm 0.5) \times 10^{-2}$ <sup>(d)</sup>	79 $\pm$ 13 <sup>(d)</sup>
<sup>c</sup> PVAm-Gant (2:1)	$(1.2 \pm 1.5) \times 10^{-4}$	$(9.7 \pm 12.0) \times 10^{-3}$	$(4.9 \pm 1.3) \times 10^{-2}$ <sup>(d)</sup>	96 $\pm$ 32
<sup>d</sup> PVAh-Gant (1:1)	$(3 \pm 2.0) \times 10^{-5}$	$(4.7 \pm 0.1) \times 10^{-3}$	$(9.3 \pm 2.0) \times 10^{-2}$ <sup>(e)</sup>	159 $\pm$ 6 <sup>(e)</sup>

<sup>e</sup> <b>PVAh-Gant (1:2)</b>	$(2.3 \pm 1.1) \times 10^{-5}$	$(1.4 \pm 1.0) \times 10^{-3}$	$(2.8 \pm 2.0) \times 10^{-2}$ <sup>(f)</sup>	$87 \pm 44$
<sup>f</sup> <b>PVAh-Gant (2:1)</b>	$(4.3 \pm 0.5) \times 10^{-5}$	$(3.3 \pm 0.2) \times 10^{-3}$	$(6.8 \pm 0.4) \times 10^{-2}$	$101 \pm 2$

The autoclaved hydrogels exhibited suitable mechanical properties for the proposed application. Similar materials designed for wound dressing or other biomedical applications, already described in the literature, exhibit higher stiffness compared with these hydrogels.<sup>[26,27]</sup> For instance, the Young's modulus of the bacterial cellulose (nf-BC)-PVA composite hydrogels proposed by Qiao et al.<sup>[26]</sup> are between 0.5 and 1 MPa. Hydrogels have often been compared to living biological tissue for their elasticity and flexibility and are considered for this reason as a very good temporary scaffold for tissue repair.<sup>[28,29]</sup> All samples tested, showed more desirable values of elongation to break than the normal human skin itself, which goes from 75% at birth to 60% in the elderly.<sup>[30]</sup> Young's modulus and tensile stress at break data are similar for all samples tested (Table 2.1). 1 to 1 molar ratio compositions exhibited higher strength values, with the gel containing high Mw PVA being the most elastic among the formulations considered (with an elongation to break of  $159 \pm 6$  %).

The porous structure of freeze-dried hydrogels was studied using SEM (Fig 2.9). We observed highly porous structures with different pore sizes, wall thickness and distribution. Both large and small pores are clearly visible in these samples, indicating their inhomogeneous nature.

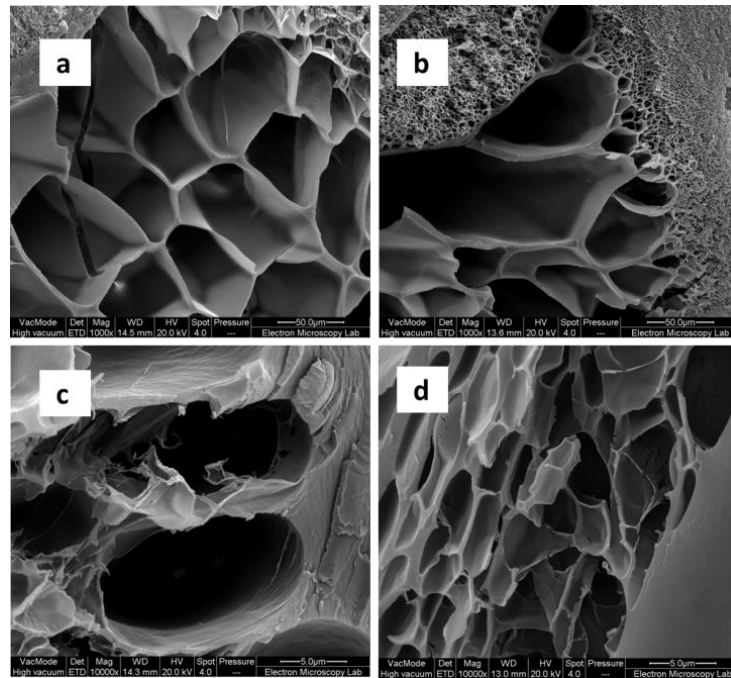


Figure 2.9. SEM images of freeze-dried hydrogels: a,c) PVAh-Gant (1:1); b,d) PVAh-Gant (1:2). Scale bar is 50 $\mu$ m in a,b); 5 $\mu$ m in c,d).

### 2.3.3 Adhesion to skin

Commercial wound dressings are often classified as adherent, low-adherent and non-adherent materials.<sup>[31]</sup> In fact, the level of adhesion represents an important characteristic of the product and it may significantly affect the healing process.<sup>[32]</sup> Depending on the type of the wound and stage of the healing a different degree of adherence could be required. The newly formed tissue and peri-wound skin is fragile and an extremely adherent dressing could cause further trauma and pain upon its removal.<sup>[31,33,34]</sup> At the same time, it is convenient to have a dressing capable of remaining in place for a number of days in order to protect the wound from bacteria and other pathogens. Porcine skin was used as a model to test the adhesion of the autoclaved hydrogels. Typical detachment profile can be seen in Figure 2.10.

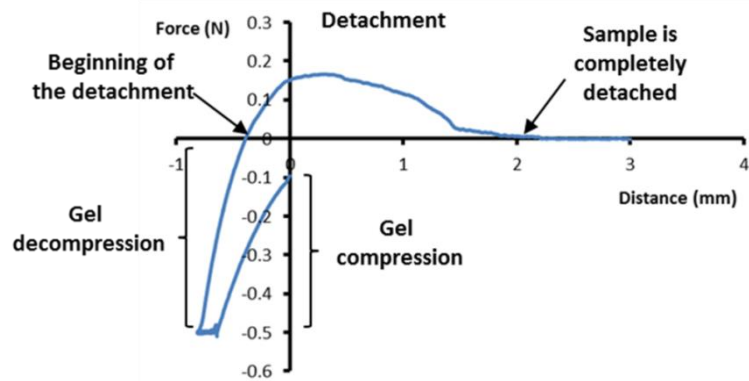


Figure 2.10 Exemplary profile of the detachment of autoclaved PVA-Gantrez® AN hydrogel sample from porcine skin during the adhesion test performed using a Texture Analyzer (Stable Micro Systems Ltd, UK).

The study of hydrogel detachment from skin surface can provide several characteristics of adhesion, including the force of detachment, the work of adhesion and cohesiveness (Figure 2.11).<sup>[14]</sup>

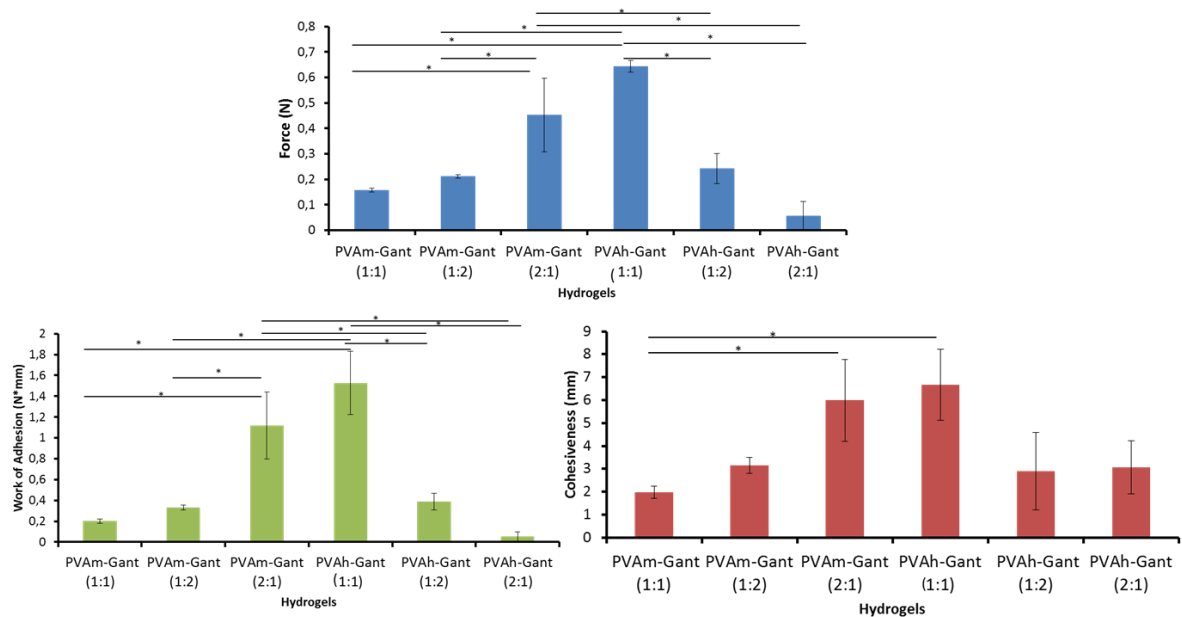


Figure 2.11 Adhesion of hydrogels to porcine skin: force of detachment values (a), work of adhesion value (b) and cohesiveness values (d) for autoclaved hydrogels. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

The samples prepared from lower molecular weight PVA (51.3 kDa) show greater adhesion when they contain an excess of PVA. On the contrary, the hydrogels composed of higher molecular weight PVA (66.3 kDa) showed the greatest adhesion when the 1:1 polymer ratio was used.

Adhesion is a complex phenomenon that is still not completely understood. It has been suggested that adhesion is directly related to the formation of non-covalent bonds, such as hydrogen bonds, between two surfaces in contact.<sup>[35]</sup> Additionally, it is believed that the diffusion of unbound or loosely bound macromolecules, such as free linear polymer present, into the outer layer of the epithelium could also contribute to the adhesion. Therefore by increasing the degree of cross-linking which results in fewer loose macromolecules with potential to diffuse should decrease the adhesion to skin. Skin condition is expected to substantially affect adhesion of hydrogels. In this work, a healthy intact porcine skin was used as a substrate to study adhesive properties of hydrogels. The measurements of hydrogel adhesion using intact skin provide better reproducibility of results; therefore, this model is more common in characterisation of wound care products. If the skin was damaged, completely different adhesion results are expected as the wound surface will have substantially different nature and the results are expected to be less reproducible.

#### 2.3.4 Antimicrobial properties

It is very important to protect the wound from bacteria and pathogens that could cause further damage and delay the healing process; especially when the injury is chronic, large in size or the patient is particularly at risk, for example, in severe burns.<sup>[36,37]</sup> For this reason, many products containing antimicrobial agents are already available on the market, such as Aquacel Ag<sup>®</sup> (by ConvaTec Ltd.) and Acticoat<sup>™</sup> (by Smith&Nephew).<sup>[38,39]</sup> Ionic silver (Ag<sup>+</sup>) has become very popular as an antimicrobial since the late 1960's when it was often used in association with sulfadiazine (silver sulfadiazine SSD). Several mechanisms were proposed for its antimicrobial action, such as the disruption of hydrogen bonding in bacterial DNA resulting in denaturation and its well-known ability



to bind thiol groups (-SH) present on the surfaces of biological molecules such as enzymes, causing their inactivation.<sup>[40-42]</sup> Another antimicrobial agent frequently used in wound management is molecular iodine (I<sub>2</sub>), which damages the cell walls of microorganism leading to leakage of the cell contents and cessation of protein synthesis. Both effects result in cell death.<sup>[43,44]</sup>

The antimicrobial properties of the PVAm-Gant and PVAh-Gant hydrogels were tested in a 24 h growth inhibition assay against a suitable Gram positive strain, *Staphylococcus aureus*. This pathogen is widely present in the environment and is responsible for many skin infections.<sup>[45]</sup> Commercial wound dressing Aquacel® Ag was evaluated in parallel as a control. This method allows an easy and clear evaluation of antimicrobial activities of drugs or biomaterials and it is commonly used in microbiology, as it gives a correlation between concentration and bacterial inhibition.<sup>[14,46]</sup>

A clear bacteria growth inhibition zone (Figure 2.12) was observed for all gel samples, confirming their intrinsic antimicrobial properties. As expected, it was higher when the bacteria concentration was lower (10<sup>4</sup> CFU/mL). All hydrogel samples showed *Staphylococcus aureus* growth inhibition comparable to the control.

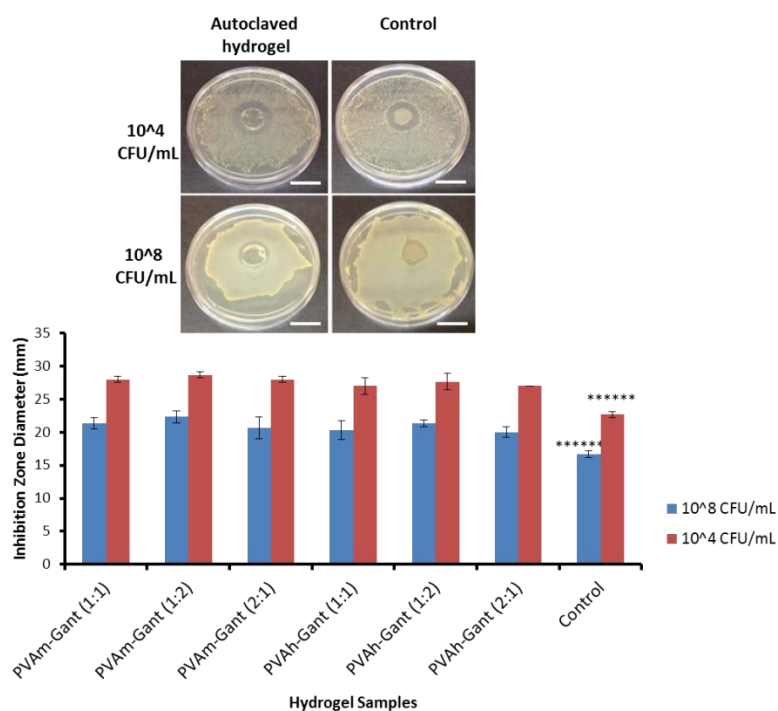


Figure 2.12. Antimicrobial activity of hydrogel samples (left) and control (right) against *S. aureus* ( $10^4$  and  $10^8$  CFU/mL). The images on the top were taken during the antimicrobial test. Aquacel® Ag (ConvaTec Ltd.) was used as control. Clear zones of growth inhibition were visible around the samples and the control. Scale bar is 2 cm. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

The antimicrobial activity of hydrogels containing PVA has not been reported previously. However, Gantrez® AN has some documented antimicrobial properties.<sup>[47]</sup> To establish the nature of the antimicrobial activity in autoclaved hydrogels we have prepared micronized dispersions of these samples and measured their pH. It was found that all samples exhibit pH of 3-4. This acidity may result from carboxylic groups formed by opening of maleic anhydride cycles and also from traces of acetic acid (>0.01% as estimated using <sup>1</sup>H NMR spectroscopy) present in autoclaved samples. Acidic pH is known to inhibit the growth of *Staphylococcus aureus*, which is likely to explain the antimicrobial properties of these hydrogels.<sup>[48]</sup>

### 2.3.5 Cytotoxicity analysis

An MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was performed to determine the indirect cytotoxicity of PVA-Gantrez® AN hydrogel samples. This colorimetric, quantitative method provides a rapid evaluation of cell viability and has now been considered as a standard technique to evaluate toxicological properties of various materials.<sup>[15]</sup>

The tetrazolium salt is positively charged and can penetrate viable eukaryotic cells where it is converted into a coloured product (formazan) with a maximum absorbance near 570 nm.<sup>[49]</sup> The test was carried out using extracts collected from the gels in sterile conditions (Figure 2.13). The MTT assay indicated that the extracts collected at 24 and 48 h from the hydrogels gave > 70% cell viability. These levels of cell viability indicate that the hydrogels could be considered biocompatible for the application as medical devices.<sup>[50]</sup>

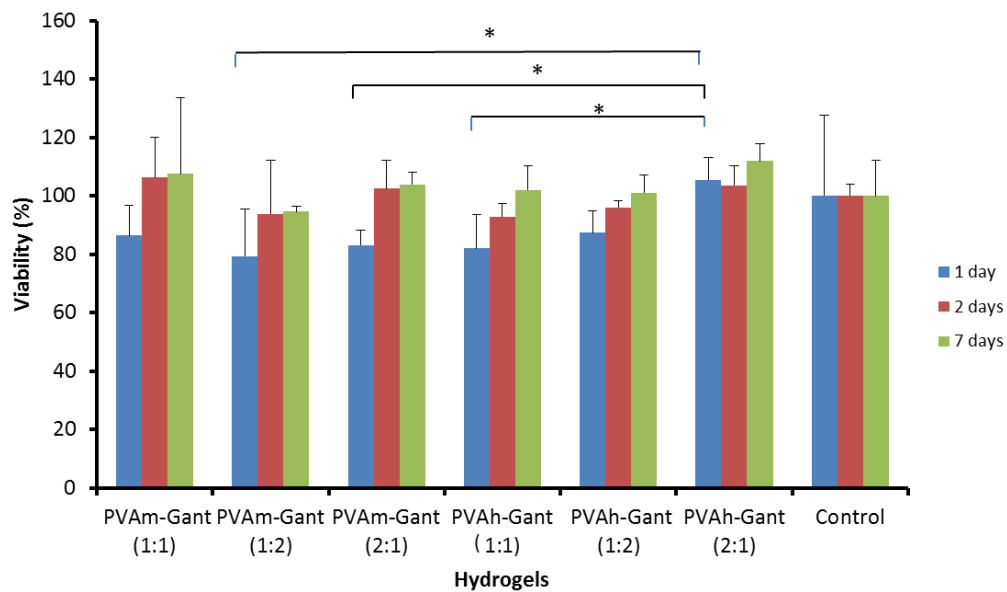


Figure 2.13 Indirect cytotoxicity assessment of hydrogels. Cell seeding density was  $8 \times 10^4$  cells/mL. Cell viability from MTT test performed using the hydrogels produced by autoclaving. Asterisk (\*) indicates a significant difference ( $p < 0.05$ ).

To assess the direct cytotoxicity human fibroblasts were seeded in the presence of the autoclaved hydrogels (Figure 2.14). The gel produced in the presence of 0.05% Triton<sup>®</sup> served as a positive control and killed all the cells as expected.

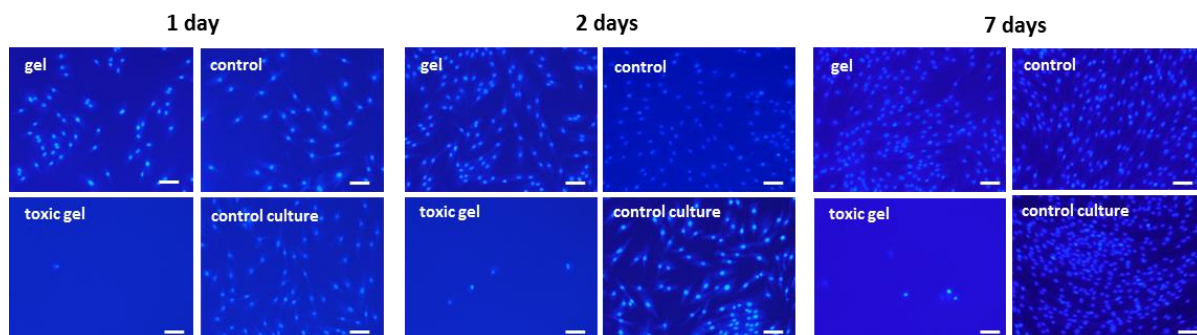


Figure 2.14 Images of cell cultures stained for nuclei fluorescence after 1, 2 and 7 days. The cells were seeded in the presence of autoclaved PVAm-Gant (1:1) hydrogel samples, a commercial product and a gel containing cytotoxic 0.05% of Triton<sup>®</sup>. Scale bars are 100  $\mu$ m. A control culture was monitored as well.

The cultures were stained for nuclei fluorescence and images were taken at 1, 2 and 7 days post-seeding. Cells growth and proliferation were comparable in the presence of the samples and the commercial product used as a control. Fibroblasts found favourable conditions to proliferate and did not show a modified behaviour when seeded in the presence of PVA-Gantrez<sup>®</sup> AN autoclaved hydrogels.

## 4 Conclusions

Autoclaving aqueous mixtures of PVA and Gantrez<sup>®</sup> AN is a novel method for the production of hydrogels, resulting in sterile, antimicrobial and biocompatible materials with very good swelling capability, excellent mechanical and adhesive properties. These hydrogels do not require any post purification because no monomers, toxic cross-linkers or initiators were used in their synthesis. This

method allows the rapid, straightforward synthesis of hydrogel samples having different shapes and volumes, depending on the final application required and on the capacity of the autoclave used. Cost-effective and relatively safe production steps in comparison with alternative cross-linking techniques provide significant advantages for the industrial production of these materials.

The materials produced are proposed for the development of novel wound dressings. The antimicrobial properties of the autoclaved hydrogels, which therefore do not require the addition of any antimicrobial agents such as silver or iodine, represent a great advantage for application in wound management.

The full transparency of the hydrogels is an additional advantage allowing visual monitoring of the wound without removing the dressing so that it can be left in place for a longer period of time, reducing the risk of contamination to the wound from the environment.

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### 3 On the adhesion of hydrogels to porcine skin: Effect of cross-linking, presence of linear polymer and sample hydration

Enrica Caló, Lucy Ballamy, Vitaliy V. Khutoryanskiy

The phenomenon of adhesion between synthetic and biological surfaces has been widely investigated and different theories on the mechanisms behind it were proposed. In this work, we investigate the adhesion of hydrogel dressings containing poly(vinyl alcohol) and Gantrez® AN produced by autoclaving to porcine skin. Several factors influencing the level of adhesion of these materials to skin, such as cross-linking degree, presence of free linear polymer within the network and sample hydration were considered and analysed. Their water content, gel fraction and swelling behavior were studied.

### 3.1 Introduction

Hydrogel-based wound care products have become very popular due to their unique physicochemical properties. Hydrogels are defined as three-dimensional networks that can be produced from hydrophilic natural or synthetic monomers or polymers, physically or chemically cross-linked.<sup>[1-3]</sup> They typically incorporate more than 95% of water, which makes them able to keep the wound always moist and hydrated.<sup>[4]</sup> Hydrogel products are usually applied on burns and wounds that produce medium or low level of exudate.<sup>[5, 6]</sup> They were shown to be able to promote autolytic debridement and accelerate the healing process.<sup>[7, 8]</sup> These materials can be found on the market as amorphous gels or flat sheets and they are typically transparent, allowing a possibility for monitoring the wound healing.<sup>[9, 10]</sup> They generally present high swelling degree and good mechanical properties. No 'dead space' is left between the wound bed and the hydrogel, where bacteria could accumulate thanks to their high conformability to the surface where they are applied. The right level of adhesion to skin is one of the several requirements that an ideal wound dressing must have.<sup>[11]</sup> It is possible to classify different types of wound dressing products as atraumatic, non-adherent, low-adherent and adherent materials. Each wound should be treated specifically choosing the appropriate dressing. A non-balanced adhesiveness can actually cause further damage to the wound and resulting pain.<sup>[11, 12]</sup>

Intact skin is composed of different sections (epidermis, dermis and subcutis) with the outermost layer represented by the stratum corneum.<sup>[13]</sup> This protects the body from external threats and prevents water loss, and it is considered a challenging barrier to be passed in topical drug delivery.<sup>[14, 15]</sup> In fact, approximately 10% of the stratum corneum is made of lipids (the other 90% is composed of keratin-filled corneocytes), such as ceramides, free fatty acids and cholesterol.<sup>[16]</sup> The skin surface lipids (SSL) represents a continuous hydrolipidic layer, whose composition is influenced by age, UV exposition, use of cosmetics and presence of skin disorders, such as dermatitis and

pytiasis.<sup>[17, 18]</sup> Upon wounding the skin structure could be disrupted to different extent (e.g. superficial and full-thickness wounds), but the stratum corneum is always involved. Therefore, the surface of a wound is completely dissimilar from the one of intact skin. For instance, the pH of chronic wounds is in the range of 7.15-8.9,<sup>[19]</sup> whilst in intact skin it is normally between 4.0 and 6.0.<sup>[13]</sup> The wound surfaces are also differed from the intact skin in the hydrophilicity of the tissues exposed, the presence of blood vessels, sebaceous glands, and different molecular species.<sup>[20]</sup> Additionally, the wound bed greatly varies from acute to chronic wounds.<sup>[21]</sup>

Thanks to their elasticity and similarities to biological tissues, hydrogels are currently considered as promising substitutes of pressure sensitive adhesives (PSAs) commonly used in the pharmaceutical industry.<sup>[2, 22, 23]</sup> Hydrogels structure and composition could be modified to make them more or less adhesive, and to prepare materials with different mechanical properties. It is in fact possible to modify the level of adhesion of these materials to skin or other biological surfaces by varying the cross-linking density, forming interpenetrating networks or loading them with nanoparticles.<sup>[23-25]</sup>

In the present work we have produced a series of hydrogels using combinations of PVA and Gantrez<sup>®</sup> AN, which were cross-linked by autoclaving their aqueous solutions. PVA is widely used in the industry as an adhesive, and in the pharmaceutical sector, it is often added to polymeric formulations in order to improve their rheological properties. PVA has been proposed in combination with polysaccharides (such as dextran, alginate or starch) or other natural polymers for wound care applications due to its excellent film-forming properties.<sup>[26]</sup> It is usually produced by the hydrolysis of polyvinyl acetate and PVAs with different degrees of deacetylation are available on the market.<sup>[27]</sup> Gantrez<sup>®</sup> AN is the trade name for the synthetic copolymer of methyl vinyl ether and maleic anhydride (poly(methyl-vinyl-ether-alt-maleic anhydride)). It is commonly used as an adhesive, thickening and suspending agent in the pharmaceutical industry; additionally it has been exploited in

the cosmetic and food sectors.<sup>[28,29]</sup> It has been shown to have antimicrobial properties which provides a great advantage to its application in wound care.<sup>[30]</sup>

The changes in autoclaving time provided an opportunity to produce hydrogels with different degree of cross-linking, presence of uncross-linked macromolecules within the network and different hydration levels, which was necessary to elucidate the role of these factors in the adhesion of these materials to intact and damaged porcine skin. To the best of our knowledge, this is the first study that systematically explored various factors involved in the adhesion of hydrogels to skin.

## 3.2 Materials and methods

### 3.2.1 Materials

PVA with a degree of deacetylation of 98.7%, medium Mw (51.3 kDa) and high Mw (66.3 kDa) was purchased from Alfa Aesar. Gantrez<sup>®</sup> AN and sodium hydroxide were purchased from Sigma Aldrich. The porcine skin used during the adhesion tests was supplied by P. & D. Jennings Butchers, Hurst (Reading, UK).

### 3.2.2 Hydrogels synthesis

Aqueous solutions of PVA (4.4% w/v) and Gantrez<sup>®</sup> AN (17.4% w/v) were mixed with 1.14:1, 0.58:1 and 2.33:1 volume ratios which provide 1:1.02, 1:1.92 and 2.09:1 molar ratios, respectively. Please note that we will refer to these samples as PVAm-Gant (1:1), (1:2) and (2:1) for samples containing medium Mw PVA, and as PVAh-Gant (1:1), (1:2) and (2:1) for samples made using high Mw PVA. The mixtures were stirred for approximately 3 hours at room temperature and after poured into glass petri dishes (8 cm in diameter). They were then autoclaved at 125°C, 1.4 bar for 60, 90, 120 and 150 minutes to produce samples with different cross-linking degree.

### 3.2.3 Thermogravimetric analysis (TGA)

The water content in the hydrogels produced was evaluated using TGA. The samples were heated up to 120°C and kept at this temperature for 25 minutes. The same analysis was carried out on hydrated samples at 5, 30 and 60 minutes after being immersed in deionised water at room temperature. The measurements were performed on a TGA Q50 (TA Instruments) in nitrogen atmosphere.

### 3.2.4 Gel fraction analysis

Gel and sol fractions (%) were determined for PVA-Gantrez® AN samples produced with different autoclaving times (60, 90, 120 and 150 min) and also for the hydrogels hydrated in excess of water for 5, 30 and 60 minutes. The samples were immersed for 48 h in 12mL of deionized water, which were then replaced with fresh water for additional 48 h. Each hydrogel sample and both portions of water were then freeze-dried and their dry weights (*Sol1*, *Sol2* and *Wd*, respectively) were recorded. Control samples were not extracted with water and were directly freeze-dried after autoclaving to establish the 100% gel fraction (*Wi*). The following formulae were used to calculate gel and sol fractions:

$$\text{Gel fraction (\%)} = \frac{W_d}{W_i} \times 100 \quad (1)$$

$$\text{Sol fraction (\%)} = \frac{W_{sol1} + W_{sol2}}{W_i} \times 100 \quad (2),$$

For samples used in hydration experiments the formula applied does not include the term *Sol2*, since only the water used for sample immersion for 5, 30 and 60 minutes, respectively, was used for sol fraction analysis.

### 3.2.5 Swelling studies

The swelling behaviour of the hydrogels produced using 60, 90, 120 and 150 min autoclaving was evaluated in deionised water during 11 days. The samples were cut in 3 mm diameter discs and placed in small baskets made of metallic mesh (2 cm diameter, 3 cm height). The baskets were then placed into plastic vials (3.5 cm diameter, 6 cm height) in excess of water. The initial weight of the gel ( $W_i$ ) and the weight of the wet and wiped basket were recorded. The samples were weighed regularly by weighing the baskets containing the gel (the baskets were always wiped with a filter paper in the same manner by the same operator, before placing them on the scale). The swelling ratio (SR %) of the PVA-Gantrez<sup>®</sup>AN hydrogels was calculated using the following formula taking into consideration the weight of the metallic baskets:

$$SR (\%) = \frac{W_s - W_i}{W_i} \times 100 \quad (3)$$

where  $W_s$  is the weight of the swollen sample.

### 3.2.6 Advancing contact angles measurements

Advancing contact angles measurements were carried out using Theta Lite optical tensiometer (Biolin Scientific) following the guidelines reported by Drelich.<sup>[31]</sup> The analysis was performed using samples of Duoderm (ConvaTec Ltd.) and autoclaved hydrogels in the initial and swollen state, as well as the surfaces of intact and damaged porcine skin.

### 3.2.7 Adhesion test

For the adhesion tests 20 mm diameter hydrogel samples were attached to the probe of the Texture Analyser XT Plus (Stable Micro Systems Ltd, UK) and shaved porcine skin was placed on the platform below. The test was performed with the following settings: pre-speed test 1.0 mm/s; test-speed 0.5 mm/s; post-test speed 1.0 mm/s; applied force 0.5 N; return distance 10.0 mm; contact time 60.0 s; trigger type auto; trigger force 0.1 N; and return distance of 10.0 mm. Force versus distance curves were produced using the Texture Analyser software (T.A. Exponent). The maximum force necessary to detach the sample from the porcine skin represented its adhesive strength, the area under the curve was defined as the total work of adhesion and the maximum distance travelled by the gel until detached as cohesiveness. All experiments were carried out in triplicates. Porcine skin from the back of the animal was used for the tests. The test was also performed using skin treated with NaOH, and polystyrene petri dishes as substrates. Samples of damaged porcine skin were prepared by covering the skin sections with a filter paper sprayed with 2.5 M NaOH solution for 1 hour at room temperature. Then the solution of NaOH was gently wiped with clean filter paper.

### 3.2.8 Adhesion as a function of degree of hydration

The adhesion test on intact skin was performed with hydrogel samples that were prepared by autoclaving polymer solutions for 60, 90, 120 and 150 min and subsequent hydration by their immersion in excess of deionised water for 5, 30 and 60 minutes. The same test was then performed again on PVAm-Gantrez<sup>®</sup>AN (1:1) samples autoclaved for 90 min and subsequently hydrated, and then de-hydrated at 50°C for 15, 30 and 60 minutes in a drying oven.

### 3.2.9 Statistical analysis

All the tests were carried out in triplicate. One-way ANOVA with Bonferroni post-hoc test on Prism (Graphpad, USA) was used to perform all the statistical analysis.



### 3.3 Results and discussion

#### 3.3.1 Synthesis of hydrogels

Transparent, bubble-free and flat hydrogels were successfully produced by autoclaving the aqueous mixtures of Gantrez® AN and PVA using the methodology described by us previously.<sup>[32]</sup> This method has shown to be faster, more environmentally friendly and considerably more cost-effective than many other techniques used for the synthesis of hydrogels.<sup>[1,33]</sup> In our method, deionised water is used to dissolve ready-made polymers and no cross-linking agents or initiators are needed for the production of hydrogels. Therefore, no post purification step is required. This method of hydrogel production presents many advantages and it has shown to be very promising and reproducible.<sup>[32]</sup>

#### 3.3.2 Water content, sol-gel fractions and swelling behaviour

Hydrogels usually contain high amount of water and this greatly influences their unique physicochemical properties and their resemblance to biological tissues.<sup>[34]</sup> It was hypothesised that their adhesiveness to skin is affected by the moisture content in the samples. It is crucial then, to accurately measure the water content of the hydrogels produced, which could be affected by the autoclaving cycle duration (60, 90, 120 or 150 minutes) chosen for their synthesis. TGA was performed on all samples, heating them up to 120°C (isothermal for 25 minutes) in order to remove all the water present. However, the water content resulted not to be significantly different when the autoclaving of the liquid mixtures of the two polymers is prolonged from 60 up to 150 mins (Table 3.1).

Gel content is often measured for chemical hydrogels synthesised by irradiation techniques to assess the cross-linking yield and level of degradation (or chain scission) of the material produced.<sup>[35, 36]</sup> In this work, the analysis was performed to measure the cross-linked fraction of the autoclaved samples (Gel) and the soluble (Sol) fraction made of free macromolecules physically trapped in the gel matrix.

Table 3.1 Gel and Sol fraction (%), water content and SR% for PVA-Gantrez<sup>®</sup>AN (1:1) hydrogels prepared using different autoclaving time. Data are shown as mean  $\pm$  standard deviation. Statistical significance ( $p < 0.05$ ) was checked for all data (a-d). Letters after each value indicate which other composition the sample is significantly different from.

Autoclaving time	Gel fraction (%)	Sol fraction (%)	Water content (%)	SR (%)
<sup>a</sup> 60 min	65 $\pm$ 5 <sup>(d)</sup>	35 $\pm$ 13	84.0	98 $\pm$ 32
<sup>b</sup> 90 min	68 $\pm$ 2 <sup>(d)</sup>	32 $\pm$ 6	83.6	80 $\pm$ 38
<sup>c</sup> 120 min	73 $\pm$ 5	27 $\pm$ 3	83.6	91 $\pm$ 33
<sup>d</sup> 150 min	80 $\pm$ 2	20 $\pm$ 1	83.5	69 $\pm$ 37

From the values of gel and sol fractions shown in Table 1, the time of autoclaving significantly influenced the cross-linking. The gel fraction in the PVA-Gantrez<sup>®</sup>AN hydrogels presented in this work ranges from 65  $\pm$  5 to 80  $\pm$  2%. The high temperature (125°C) used for the autoclaving step promotes the formation of ester bonds between the -COOH of Gantrez<sup>®</sup> AN formed after the ring opening of the maleic ring in water, and the -OH groups of PVA. Therefore, when the mixtures are exposed to this heat for prolonged periods of time, there is increased potential for chemical cross-linking.

Hydrogels are well-known for their ability to absorb and retain large amount of aqueous fluids within their network.<sup>[37]</sup> The swelling behaviour of the hydrogel dressings produced was assessed in deionised water using gravimetric analysis for 11 days (Table 3.1).

The autoclaved hydrogels exhibit a very high swelling ratio (SR) in deionised water (up to 98%). However, due to the large errors experienced with this technique or because of the relatively small difference in the cross-linking degree among the samples, no significant difference was found in the swelling.

### 3.3.3 Advancing contact angles

Sessile drop advancing contact angles analysis allow to measure materials surface wettability, which is defined as the ‘tendency for a liquid to spread over a solid surface’.<sup>[38]</sup> Wettability represents a very important property for biomaterials. It gives us information about their chemical compositions and structures, and can greatly influence their biocompatibility and level of adhesion.<sup>[39]</sup> For instance, it can become very crucial for contact lenses which need specific levels of surface hydrophilicity to be comfortable to wear.<sup>[40]</sup> Contact angle greatly depends on water content of the materials. Hydrogels made of synthetic polymers as the materials presented in this work (Table 3.2), usually have very low contact angle values (0-40°) when fully swollen<sup>[39]</sup> with high levels of hysteresis, which is the difference between advancing (contact angle measured when additional drops are supplied to the first) and receding (contact angle measures when the liquid is picked up after deposition on solid surface) contact angles.<sup>[31, 38]</sup>

Table 3.2 Advancing contact angles data for Duoderm (ConvaTec Ltd), autoclaved hydrogel sample (PVAm-Gant 1:1 90 min autoclaving, in initial and swollen states), and skin (intact and chemically damaged skin).

<b>Sample</b>	<b>Advancing Contact Angle (°)</b>
<b>Duoderm</b>	105.5 ± 8.4
<b>Hydrogel</b>	89.3 ± 17.7
<b>Swollen hydrogel</b>	0 ± 0
<b>Intact skin</b>	36.2 ± 3.8
<b>Damaged skin</b>	15.6 ± 1.6

When a polymeric surface comes into contact with a liquid its configuration changes (depending on the nature of the liquid and the surface): the functional groups present migrate, rotate and rearrange achieving a new equilibrium. For instance, polar groups (such as -OH) of hydrophilic polymers (such as PVA) that are usually found far from the surface of dry films, redistribute closer to the outer region when the samples are immersed in water. This phenomenon does not influence the measurements of the advancing contact angles but can actually affect the receding one, which usually results less accurate for polymeric surfaces.<sup>[41]</sup>

#### 3.3.4 Adhesion to intact skin, damaged skin and polystyrene

As expected, the adhesiveness of the hydrogels decreases when the autoclaving time increases because the cross-linking degree is higher. In fact, one of the theories of the adhesion process attributes this phenomenon to the formation of hydrogen bonds between the two surfaces in contact.<sup>[27]</sup> If the cross-linking degree is higher less hydrogen bonds can be formed between PVA and the functional groups present on the skin surface (such as -OH or -NH<sub>2</sub> groups of ceramides or proteins). The force of detachment and the work of adhesion of the hydrogels are decreased with longer autoclaving time (Figure 3.1).

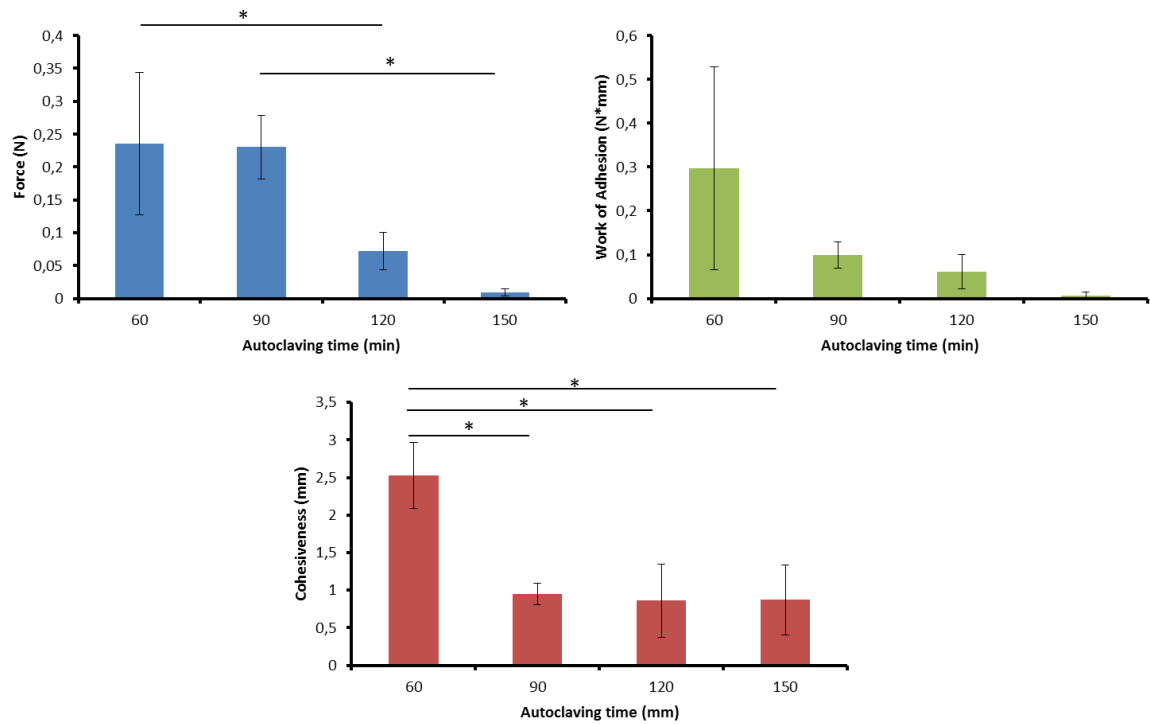


Figure 3.1 Force, work of adhesion and cohesiveness data for PVAm-Gant (1:1) tested on intact skin model. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

However, there seems to be an opposite trend when chemically damaged skin is used for testing (Figure 3.2). In this case the outer layers of the epidermis have been partially or completely disrupted with a significant change in the morphology of the skin surface.

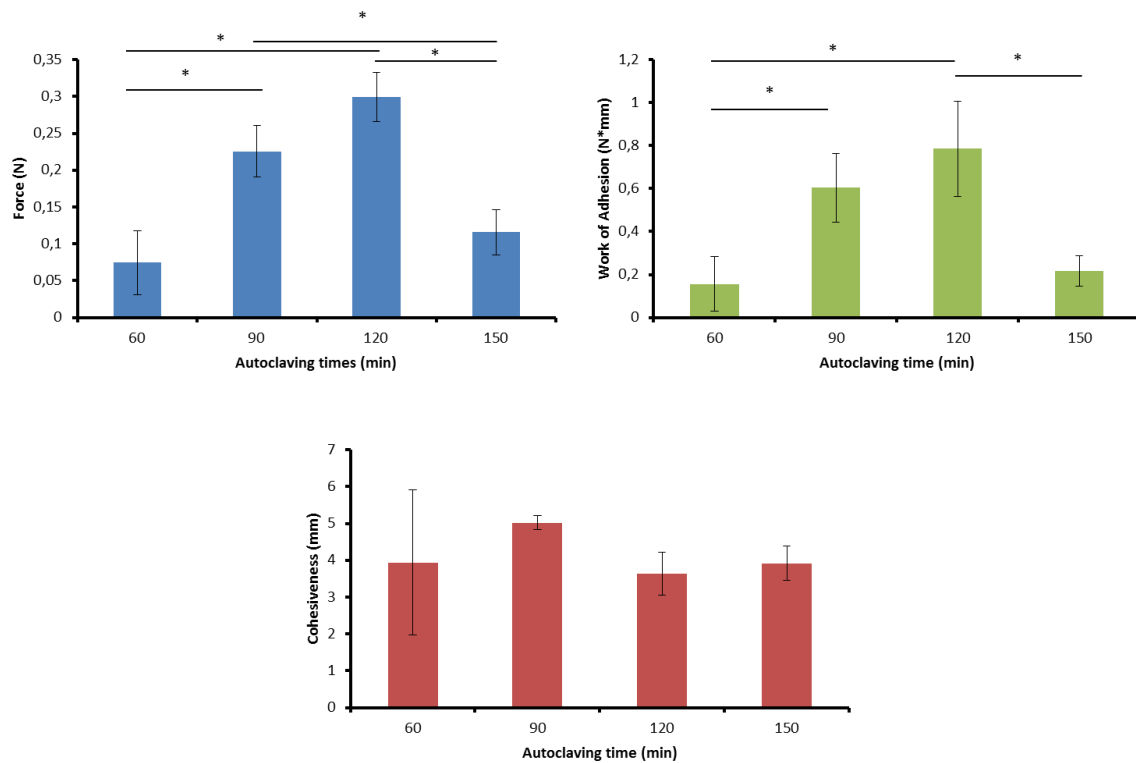


Figure 3.2 Force, work of adhesion and cohesiveness data for PVAm-Gant (1:1) tested on chemically damaged skin model. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

Many factors could be affecting the adhesion phenomenon, such as the surface energy in contact with the gel, different possible molecular interactions or the higher hydrophilicity of the inner layers of the skin now exposed. The roughness and the irregularities of the skin (intact and damaged) surface where the hydrogel is applied can also greatly influence its ability to adhere to it. Additionally, even the nature of the materials that adhere to each other represents an important parameter to be considered.<sup>[23]</sup>

We then compared the adhesion of the autoclaved hydrogels to skin and to polystyrene surfaces to observe how this changes when moving from a biological to a synthetic surface (Figure 3.3).

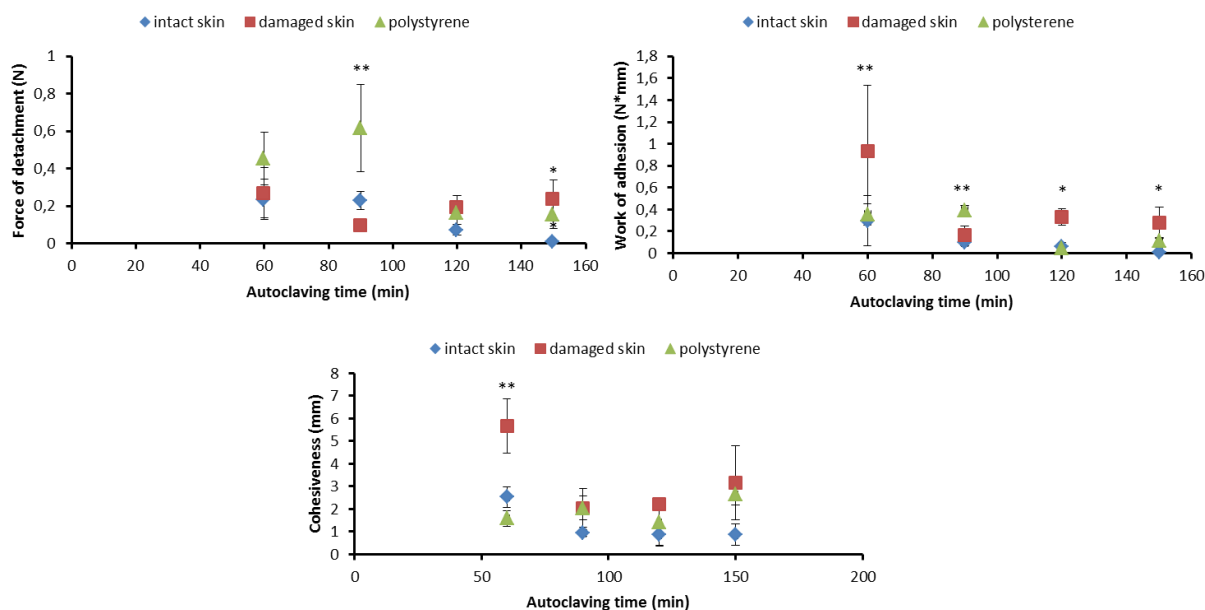


Figure 3.3 Adhesion to intact and damaged porcine skin, and polystyrene surface data for different autoclaving times PVAm-Gant (1:1) samples. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

The molecular interactions and adhesion mechanisms that take place between the polystyrene surface and the hydrogel samples are of course significantly different to what happens when these are applied to skin. Polystyrene is known to be a highly hydrophobic and neutral polymer. However, the adhesion of hydrogels to intact skin is more similar to their adhesion to polystyrene than to damaged skin (Figure 3.3). This can be due to the flat and regular surface of polystyrene used, where no air bubbles are formed at the interface with the gel,<sup>[23]</sup> and an easier adhesion and detachment can occur. Additionally the intact skin surface is also more hydrophobic similarly to polystyrene.

### 3.3.5 Effect of sample hydration on adhesion to skin

In the study of hydrogels swelling in water it was noticed that when the samples are fully swollen they no longer adhere to human skin. In order to establish the effect of hydrogel hydration on their adhesion, a series of samples were additionally hydrated by immersing them into excess of deionised

water for 5, 30 and 60 mins. Figure 3.4 shows how the adhesion of the hydrogels to intact porcine skin changes depending on their hydration. We plotted the force of detachment, work of adhesion and cohesiveness data obtained, as a function of SR (Figure 3.4).

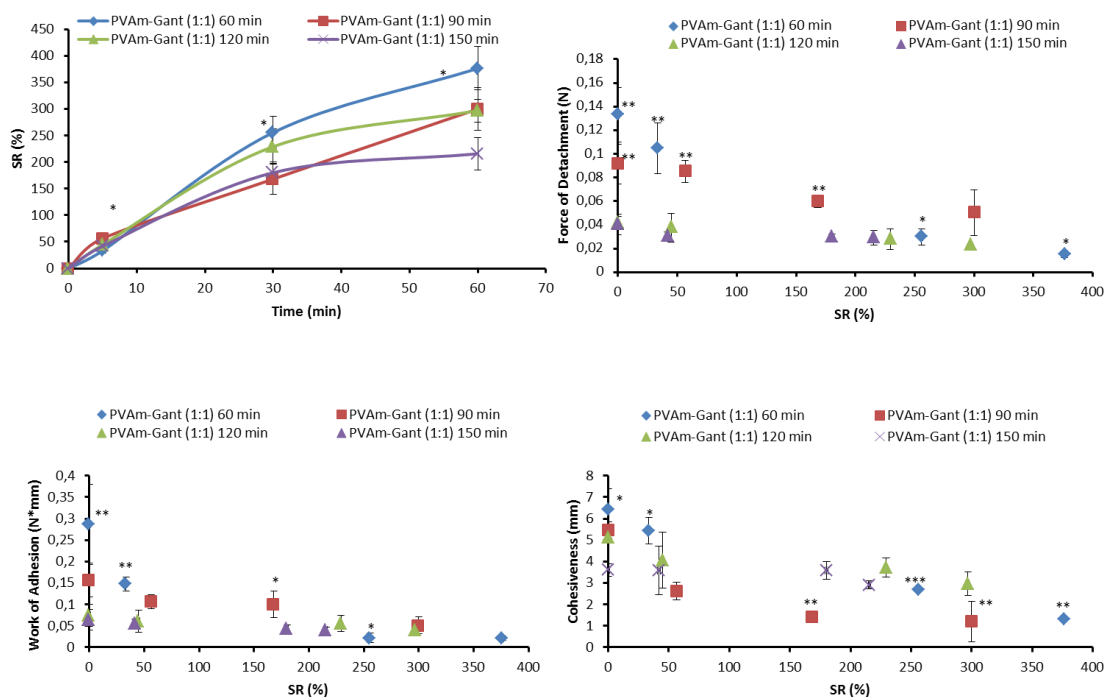


Figure 3.4 Adhesion test results on intact skin for hydrated PVAm-Gant (1:1) samples produced by autoclaving (after 60, 90, 120 and 150 min). The swelling ratios (SR) of the samples during the test are shown in the top left graph. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

As shown, the level of adhesion to skin clearly decreases when the hydrogels are hydrated, regardless of their cross-linking degree. When immersed in water, the hydrogels undergo substantial changes: their water content increases (Figure 3.5 shows TGA data for hydrated samples; please note that in 30 and 60 min samples, the final weight is 0.68 and 0.48 mg respectively, not 0), their pores are enlarged, and some of the free linear polymer chains entrapped in the network diffuse into the swelling medium.



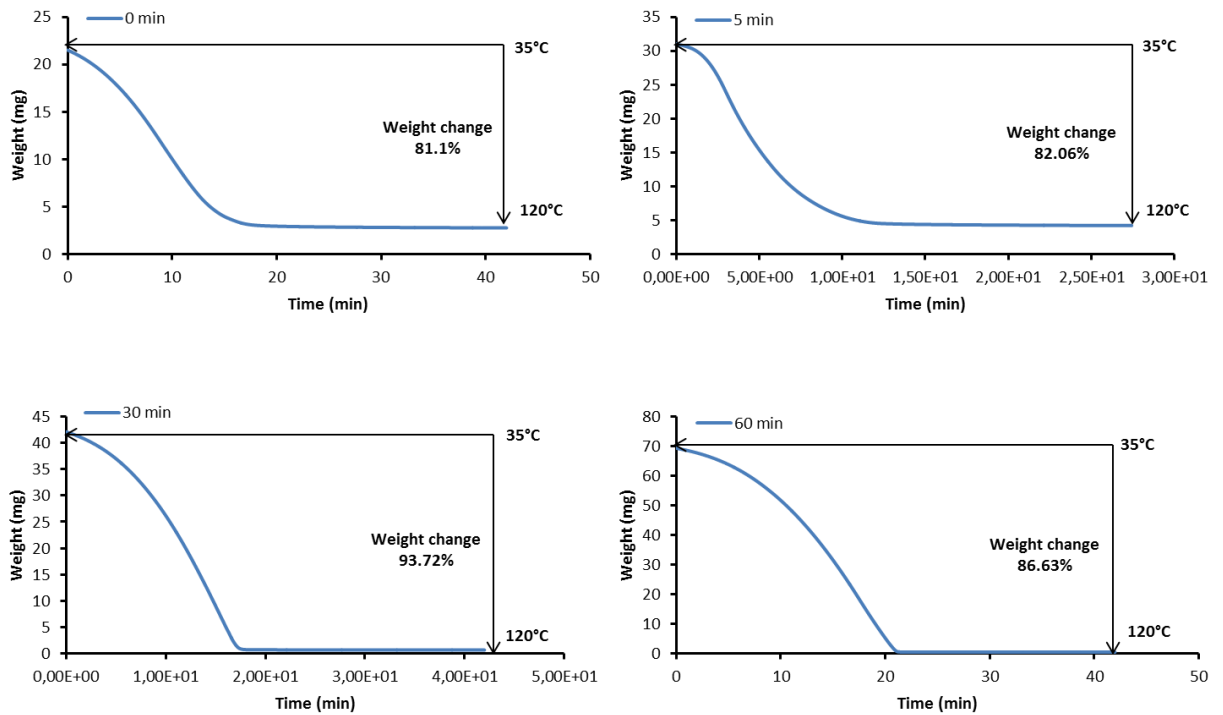


Figure 3.5 Water content as weight change measured by TGA for PVAm-Gant (1:1) hydrogel samples after 0, 5, 30 and 60 minutes in water.

Hydrogels adhesion to skin could then depend on: their water content, due to the different osmotic pressure present at the gel/skin interface, or on the free-linear polymer chains present, which could interact with the skin surface functional groups according to the adsorption theory, or even partially diffuse into the uneven areas of the outer layer of the epithelium.<sup>[2, 34, 42, 43]</sup> We then decided to dehydrate samples that were immersed in water for 1h (and therefore had lost the aforementioned free-macromolecules), by placing them for different periods of time (15, 30 and 60 minutes) in a drying oven at 50°C and test their adhesion (Figure 3.6). Percentage of dehydration of the samples was calculated using the following formula:

$$SR (\%) = \frac{W_s - W_d}{W_s} \times 100 \quad (4)$$

where  $W_s$  is the weight of the swollen sample and  $W_d$  is the weight of the de-hydrated sample.

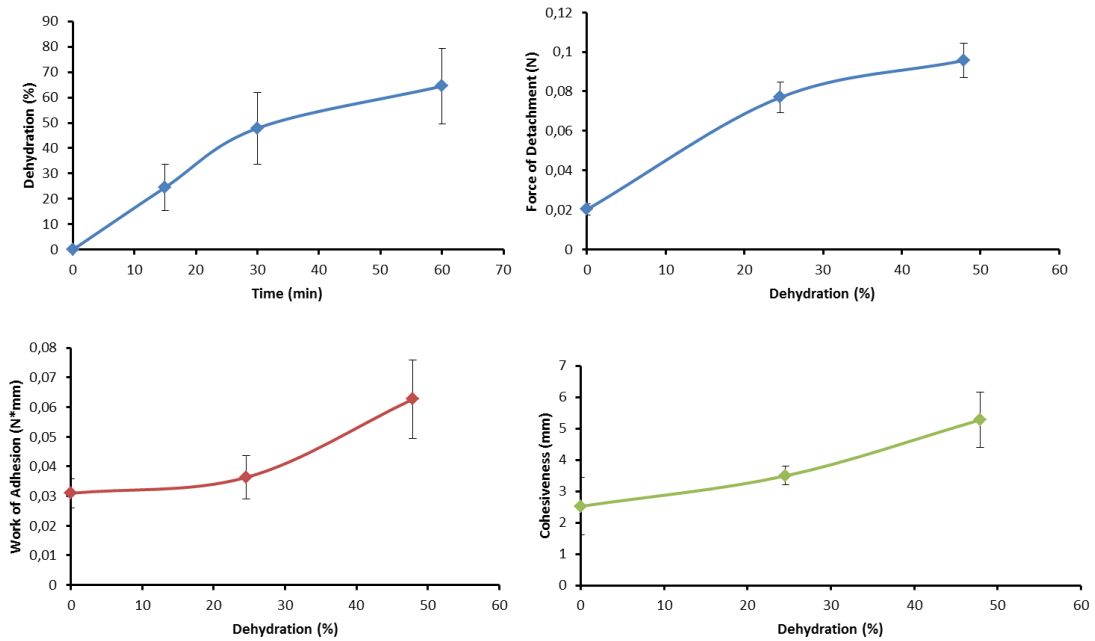


Figure 3.6 Adhesion of de-hydrated PVAm-Gant (1:1) 90 min autoclaving hydrogel samples to intact porcine skin.

The values of the force of detachment, work of adhesion and cohesiveness recorded for the hydrogels are plotted in Figure 3.6 as a function of their dehydration (Figure 5). It is clearly seen that the adhesiveness of the hydrogels to skin increases with gel dehydration. Hence, the water present in the hydrogels seems to play a crucial role in adhesion to skin. When the water at the interface between gel and skin is low (such as in the initial and in the de-hydrated state) adhesion is favoured, whereas when the water content is increased (the swollen state), the adhesiveness is reduced due to the hydrophobic nature of the surface of the skin repelling the gel.

## 4 Conclusions

According to the type and stage of the wound being treated, the dressing used must exhibit the right level of adhesion to skin to facilitate fast healing. The techniques used in this work, allowed us to achieve a better understanding of the adhesion process between polymeric hydrogel materials and skin. We took into consideration three important factors that might influence this phenomenon, such as the cross-linking degree, the presence of free linear polymers in the hydrogel matrix and the hydration of the dressings.

With the increase in the cross-linking density (which in this case can be modified changing the duration of the autoclaving cycle used to produce the samples) the adhesion of the hydrogels to intact skin decreased. On the other hand, a clear trend could not be observed when using chemically damaged skin.

The free macromolecules present in the materials do not seem to play a significant part in the adhesion mechanism, considering that the dressing adheres to skin even when these free polymers are likely to be lost (after the gel is immersed in water and then de-hydrated, gel fraction analysis results of the hydrated samples can be found in Table 3.3).

Table 3.3 Gel and Sol fraction (%) of hydrated PVA-Gantrez®AN (1:1) samples after 5, 30 and 60 mins in water. Data are shown as mean ± standard deviation. Statistical significance ( $p < 0.05$ ) was checked for all data (a-c). Letters after each value indicate which other composition the sample is significantly different from.

Time in water	Gel fraction (%)	Sol fraction (%)
<sup>a</sup> 5 min	105 ± 5	0 <sup>(c)</sup>
<sup>b</sup> 30 min	93 ± 9	0 <sup>(c)</sup>
<sup>c</sup> 60 min	92 ± 3	2 ± 0.6 <sup>(a, b)</sup>

The hydration of the dressing instead, seems to significantly affect the adhesion because of skin hydrophobicity, resulting in the highly swollen gels not adhering to the tissue.

With this study we clarified some of the events and features that could be influencing the adhesion of hydrogels to skin but more work is required in order to better explain this process.

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## 4 Superporous hydrogels produced by autoclaving for wound management

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Superporous PVA-based hydrogel foams were produced using a novel method of synthesis which involves autoclaving of aqueous solutions of water-soluble polymers with a small amount of sodium bicarbonate (used as blowing agent). No toxic monomers, initiators or cross-linkers are added to the reaction mixture. A simple, environmentally-friendly and fast new technique for superporous hydrogels production is presented. The resulting materials have shown to exhibit very high swelling ability due to their porous internal structure, good mechanical properties as well as good level of adhesion to skin. These hydrogels exhibit intrinsic antimicrobial activity against *Staphylococcus aureus*. The unique physicochemical properties of these novel foam-type hydrogels make them promising for wound management and care.



## 4.1 Introduction

Hydrogels have been extensively studied and used for the design of many biomedical products such as controlled-release drug delivery devices, engineered scaffolds for tissue or organs replacement, wound dressings, as well as contact lenses and personal hygiene items.<sup>[1]</sup> Chen et al.<sup>[2]</sup> introduced a new class of hydrogels with extremely fast and high swelling, called 'superporous' (SPHs) due to their internal open channel structure through which the swelling medium can easily flow by capillary effect.<sup>[2-4]</sup> The first SPHs were synthesised by gas blowing (or foaming) technique from a number of vinyl monomers, such as acrylamide (AM), 2-acrylamido-2-methylpropane sulfonic acid (AMPS), hydroxyethylmethacrylate (HEMA) or 3-sulfopropyl acrylate potassium salt (SPAK). Monomer, cross-linker (N,N'-methylene-bis-acrylamide), foam stabilizer (Pluronic F127, Pluronic F105 or Silwet L7605), water, acid (acetic acid (AA) or hydrochloric acid (HCl) to adjust the pH to 5-6), initiator (such as ammonium persulfate (APS)), catalyst (Tetramethylethylenediamine, TEMED) and NaHCO<sub>3</sub> suspension (as blowing agent), were added to a test tube, which was shaken after the addition of each ingredient. The gas bubbles formed after the addition of NaHCO<sub>3</sub> suspension were homogeneously distributed in the monomer solution using a spatula. The foams formed were allowed to swell in water to eliminating in this way the water-soluble compounds present, such as the foam stabilizer, and then dried for one day.<sup>[2]</sup> These novel materials, which were at first designed for the development of gastric retention devices, were then modified and improved into new generations of SPHs.<sup>[5,6]</sup> The earliest SPHs had some characteristics in common with the so-called 'superabsorbent' polymers (SAPs) that were reported thirty years before, and initially used in agriculture and diapers manufacturing. Both SPHs and SAPs, are cross-linked polymeric networks able to absorb aqueous fluids up to 1000 times their weight, but their internal structure differs (in SAPs the pores are not interconnected but closed or semi-open) and for this reason their swelling kinetics is different (size-dependent and -independent for SAPs and SPHs, respectively).<sup>[7,8]</sup> The second-generation of SPHs tried to overcome the mechanical weakness of conventional SPHs and

were called 'SPH composites' because of their structure.<sup>[9]</sup> In fact, they are composed of a matrix containing dispersed particles of a 'composite agent', which is a cross-linked hydrophilic polymer that can absorb the reaction liquid mixture, with all the compounds needed for SPHs synthesis (such as monomer, cross-linker and initiator).<sup>[3,10,11]</sup> SPH composites were proposed for intestinal and oral drug delivery but their response to mechanical stress was still not optimal.<sup>[7]</sup> The third generation was then represented by very innovating materials, to which was given the name of 'SPH hybrids'. These formulations contained a hybrid agent which is a water-soluble or dispersible polymer that can undergo physical or chemical cross-linking after the SPH network is formed, leading to a fully interpenetrating network (IPN) structure.<sup>[5,12]</sup> The SPH hybrids exhibit excellent elasticity and strength even in the swollen state and they are widely studied for proteins/peptides delivery.<sup>[5-7]</sup>

SPHs can be synthesised in four different ways: by the so-called 'cross-linking method', by porosigen technique, phase-separation technique, or by gas-blowing (or foaming) technique. The latter (as previously described by Chen et al.<sup>[2]</sup>) is certainly the most popular and it involves the addition of a chemical (e.g. sodium bicarbonate ( $\text{NaHCO}_3$ ) in acidic environment) or physical (e.g. nitrogen) foaming agent during the polymerization. The first reacts forming gas bubbles and the second just expands under pressure, but both leads to the characteristic structure of interconnected open cells throughout the hydrogel matrix.<sup>[13]</sup>

The method we propose for SPHs production involves the addition of a very small amount of  $\text{NaHCO}_3$  as foaming agent to the reaction mixture, which is exclusively represented by an aqueous solution of polymers, before a short (30 minutes) autoclaving at  $130^\circ\text{C}$ . The synthesis set-up is therefore very simple and time-saving, as well as low cost and environmentally friendly. No harmful initiators, cross-linkers or monomers are added to the formulations, so no post-purification steps are required. The SPHs we presented in this work are made from aqueous mixture of poly(vinyl alcohol) (PVA) and poly(methyl vinyl ether-*alt*-maleic anhydride) (PMVEMA). PVA is a neutral water-soluble polymer

which has been used for synthesis of hydrogels in combination with other natural or synthetic polymers for decades, since these materials made their first appearance in medicine in 1949.<sup>[14]</sup> PVA is used for many biomedical applications such as wound healing products manufacturing, thanks to its high biocompatibility and film-forming nature.<sup>[15,16]</sup> PMVEMA is better known by its trade name Gantrez<sup>®</sup> AN and it has shown to possess interesting intrinsic antimicrobial properties.<sup>[17]</sup> It represents a popular thickening and suspending agent in the pharmaceutical industry, and it is widely used in food and oral care products.<sup>[18,19]</sup> The PVA-Gantrez<sup>®</sup> AN hydrogels foams successfully produced by this new method are proposed as promising advanced materials for wound management.

## 4.2 Materials and methods

### 4.2.1 Materials

Medium molecular weight (51.3 kDa) PVA of 98-99% hydrolysis was purchased from Alfa Aesar (UK). PVA actual degree of deacetylation was found to be 98.7% by <sup>1</sup>H NMR. Nutrient agar used for the antimicrobial test was purchased from Oxoid Ltd UK. Gantrez<sup>®</sup> AN (Average Mw ~ 216 kDa) and all the other chemicals used were purchased from Sigma-Aldrich (UK). Aquacel<sup>®</sup> Ag dressing was kindly provided by ConvaTec Ltd, to be used as control during the antimicrobial studies.

### 4.2.2 Hydrogel foams synthesis

Aqueous solutions of PVA (16.6%w/v) and PMVEMA (14.8%w/v) were mixed together in 15 mL glass vials using a rotator mixer for 3 hours. Formulations with a final volume of 8mL and 1:1 volume ratio of the two polymers were prepared. Sodium bicarbonate (NaHCO<sub>3</sub>) powder was homogeneously spread using a spatula on the surface of glass petri dishes (8 cm diameter). The mixtures were then poured into the petri dishes. 0.5, 0.7 and 1 %w/v NaHCO<sub>3</sub> foams were produced using 40, 60 and

80mg of NaHCO<sub>3</sub> respectively. The petri dishes were then quickly sealed and autoclaved for 30 minutes at 130°C (CertoClav Multicontrol 12 L).

#### 4.2.3 Scanning electron microscopy (SEM) analysis

A FEI Quanta FEG 600 Environmental Scanning Electron Microscope (ESEM) was used to analyse the porous internal structure of PVA-Gantrez<sup>®</sup> AN hydrogel foams. Cross-sections of gold sputter coated freeze-dried samples were used. Liquid nitrogen (N<sub>2</sub>) was used to freeze the samples before their freeze-drying.

#### 4.2.4 Swelling studies

The swelling behavior of PVA-Gantrez<sup>®</sup> AN superporous hydrogels was assessed in deionized water and in simulated wound fluid (SWF), containing 8.298g of sodium chloride and 0.368g of calcium chloride per 1L of water. The initial weight of the samples ( $W_i$ ) was accurately recorded. The samples were kept in excess of water for 24h to study their fast swelling in this medium, whilst the duration of the experiment was extended to a longer period (9 days) when conducted in SWF. In both cases, the samples were weighed regularly, wiping away the excess of unbound medium using filter paper. The swelling ratio (SR in %) of the PVA- Gantrez<sup>®</sup> AN foams was calculated using the following formula:

$$SR (\%) = \frac{W_s - W_i}{W_i} \times 100 \quad (3),$$

where  $W_s$  is the weight of the swollen sample and  $W_i$  is the weight of the sample freshly prepared at the beginning of the experiment.

#### 4.2.5 Tensile test

The mechanical properties of the autoclaved hydrogel foams were studied using a Texture Analyser XT Plus (T.A., Stable Micro Systems Ltd, UK). 2 cm length rectangular samples (with 4 mm width) were cut from initial 8 cm diameter foams. An extension of 100 mm/min to the break was applied to all samples with an initial 'grip-to-grip' separation of 30 mm. Strength-strain curves were plotted during the test by T.A. software (T.A. Exponent).

#### 4.2.6 PVA-Gantrez® AN superporous hydrogels adhesion to skin

A T.A. (Stable Micro Systems Ltd, UK) was used to perform the adhesion test according to the method developed by us previously.<sup>[20]</sup> 20 mm diameter discs of superporous hydrogels were attached to the probe of the instrument and 5cm x 5cm porcine skin sections (from the shaved back of the animal, provided by P.& D. Jennings Butchers, Hurst, UK) were placed on the metallic platform below. The settings used for the analysis were the following: pre-speed test 1.0 mm/s; test-speed 0.5 mm/s; post-test speed 1.0 mm/s; applied force 0.5 N; return distance 10.0 mm; contact time 60.0 s; trigger type auto; trigger force 0.1 N; and return distance of 10.0 mm. Adhesive strength, total work of adhesion and cohesiveness data for the hydrogel foams produced by autoclaving were collected by the T.A. software (T.A. Exponent).

#### 4.2.7 Antimicrobial activity of hydrogel foams tablets

PVA-Gantrez® AN superporous hydrogel samples were freeze-dried and compressed in order to obtain disc-shaped tablets (10mm diameter and 3mm width), to perform a disc diffusion test.<sup>21,22</sup> *Staphylococcus aureus* NCTC 8532 (National Collection of Type Cultures) was used to test the

antimicrobial activity of the SPHs produced by autoclaving presented in this work. *Staphylococcus aureus* was grown overnight at 37°C (shaking at 200 rpm) in nutrient broth. Sterilized nutrient agar (prepared as indicated by the manufacturer) was used to prepare the plates, which were inoculated with two different concentrations of bacteria:  $10^8$  CFU/mL and  $10^4$  CFU/mL. The samples were placed exactly in the center of the agar plates. These were incubated for 24 hours at 37°C. The clear zone surrounding the tablets, related to bacteria growth inhibition, was measured using a ruler and recorded.

## 4.3 Results and discussion

### 4.3.1 Synthesis of superporous PVA-Gantrez®AN hydrogels

A new technique for SPHs production is presented in this work. Its novelty and advantage consist in the simplicity of its short steps of reaction. A cost-effective and eco-friendly procedure that involves the use of well-known ready-made macromolecules, such as PVA and Gantrez®AN, and water as a solvent is proposed. We have also recently reported the successful synthesis of non-superporous hydrogels from the same combination of polymers by autoclaving. In this case the method is modified to produce a superporous material: NaHCO<sub>3</sub> in powder (40, 60 and 80 mg) is introduced as foaming agent in the formulations and a shorter autoclaving cycle of just 30 minutes at 130°C is performed (Figure 4.1).

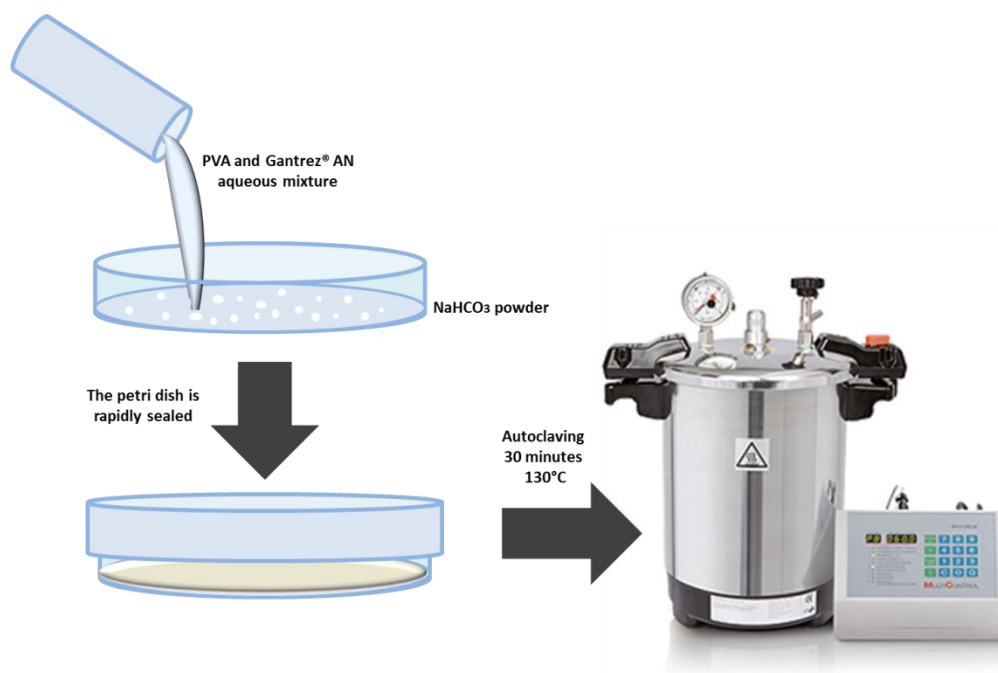


Figure 4.1 Technique used to synthesise PVA-Gantrez® AN superporous hydrogels.

Thanks to the acidic pH of the reaction mixture, NaHCO<sub>3</sub> rapidly generated CO<sub>2</sub> bubbles that move from the bottom of the petri dish towards the surface of the polymeric mixture. The high viscosity of the solution slows down the gas bubbles escape keeping them in the cross-linked hydrogel matrix that is forming at the same time during the autoclaving step. A sterile homogenous superporous sponge-like hydrogel is then formed. An interconnected structure of open pores can be observed looking at the SEM images of the cross-section of freeze-dried foam samples (Figure 4.2).

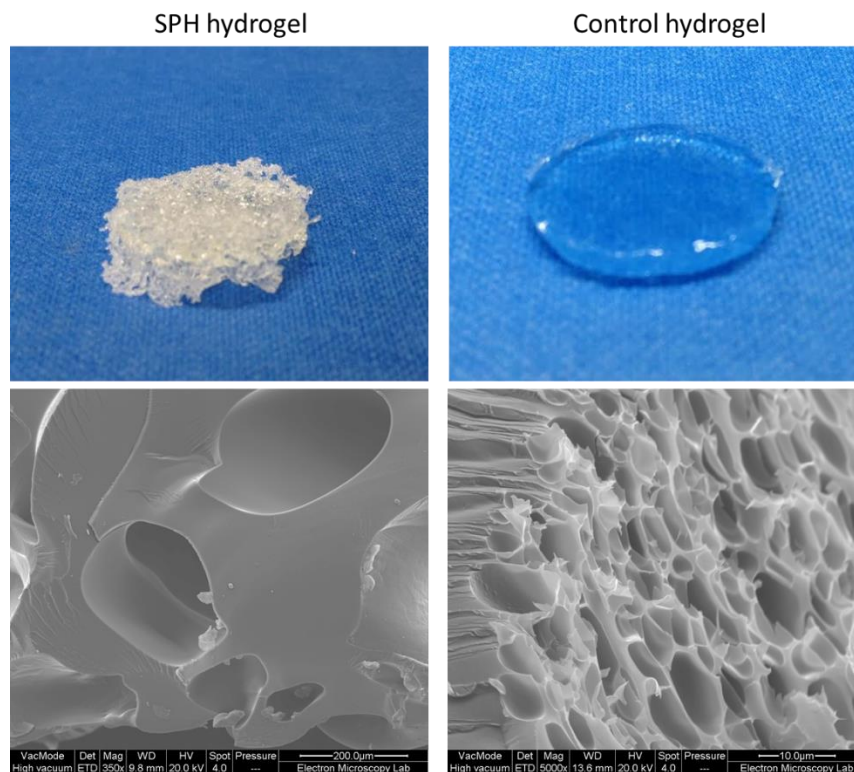


Figure 4.2 Macroscopic and SEM images of cross-sections of freeze-dried PVA-Gantrez®AN hydrogel foams (left), and control autoclaved hydrogel sample (right) non-containing NaHCO<sub>3</sub>.

The formation of the pores (~400 µm size) within the chemically and physically cross-linked hydrogel matrix is achieved without any complicated set-up, by addition of the viscous solution of the two polymers to NaHCO<sub>3</sub> powder before the autoclaving step. No great difference in the porosity is observed among the samples containing different amounts of NaHCO<sub>3</sub>. As shown in Figure 4.2, the absence of the porogen leads to the formation of porous materials where the size of the pores is significantly reduced (~10 µm).

#### 4.3.2 Swelling kinetics of autoclaved hydrogel foam samples in water and SWF

One of the reasons why researchers have moved from conventional hydrogels to SPHs, finding them more suitable for many biomedical applications is their incredibly fast size-independent ability to



swell in aqueous fluids.<sup>[2,23]</sup> The swelling behavior together with the foam-like consistency of these materials became in fact more desirable for specific applications such as the fast release of a pre-loaded drug, the management and dressing of wounds with high presence of exudate or the replacement of cartilage in areas subjected to frequent mechanical stresses.<sup>[6,24,25]</sup> A 24h swelling test in deionised water was performed to assess the profile of the initial swelling of autoclaved superporous PVA-Gantrez®AN hydrogels (Figure 4.3).

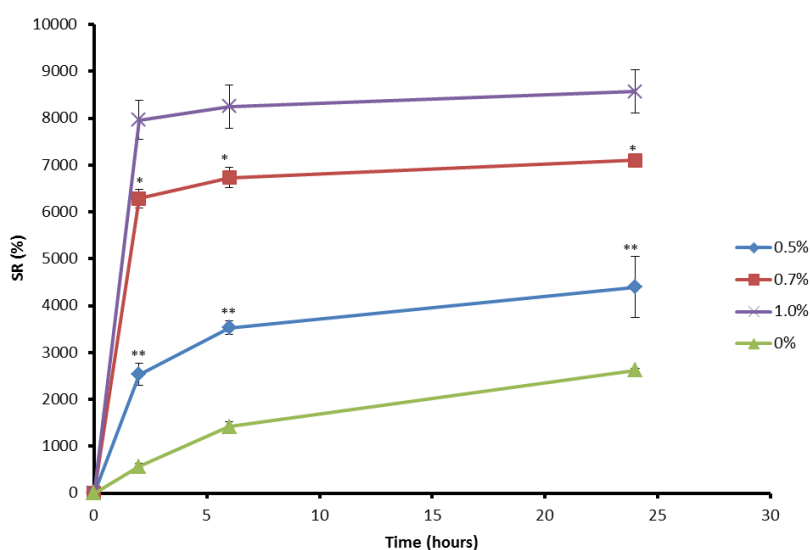


Figure 4.3 Swelling kinetics of 0.5, 0.7, 1%w/v NaHCO<sub>3</sub> PVA-Gantrez®AN foams and control (0%) in deionised water. Asterisk (\*) at each time point indicates significant statistical difference ( $p < 0.05$ ) among samples.

As expected, the materials exhibit very high and fast swelling in deionised water, which increases accordingly with the amount of NaHCO<sub>3</sub> contained in the formulation and consequently with the CO<sub>2</sub> spontaneously generated in the reaction mixture. The test using SWF was instead prolonged for 9 days to establish for how long a dressing made of the materials produced could remain stable on a highly exudating wound (Figure 4.4). Although the conditions of the test cannot exactly reflect the real wound environment, we could get an idea of the behaviour of these hydrogel foams in the presence of an excess of an ion-containing solution during a relatively long period of time.

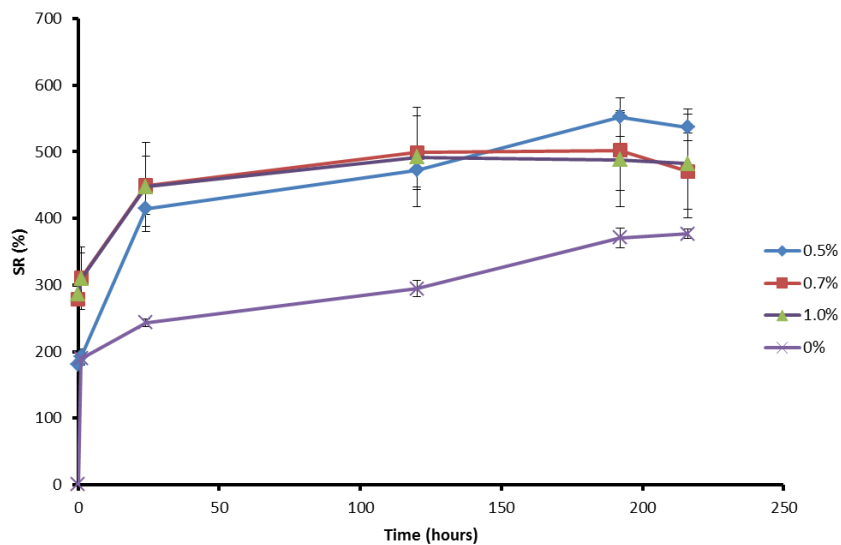


Figure 4.4 Swelling of autoclaved hydrogel foams and control in SWF during a period of 9 days.

The most commonly used wound dressings, such as plain gauze, need to be removed and changed on a daily basis, with associate pain and trauma for the fragile newly formed skin that often adheres to the products applied. Hydrogel dressings already allow a less frequent removal (up to 3 days) with the possibility of constantly monitoring the wound bed thanks to their transparency.<sup>[26]</sup> Therefore, hydrogel materials with enhanced swelling ability like SPHs, could improve this aspect even further. As shown in Figure 4.4, the SR of the autoclaved foams even if still very high is significantly reduced in SWF for the difference in the osmotic pressure between the gel matrix and the external medium containing high amount of mobile ions.<sup>[11,27]</sup> In this case, the different NaHCO<sub>3</sub> content does not affect the SR of the three formulations (no significant statistical difference was found at any time point,  $p < 0.05$ ).

#### 4.3.3 Mechanical properties

Several variables can affect the mechanical properties of SPHs prepared in the conventional methods, such as the monomer or the cross-linker used, the amount of foaming agent added or any post-synthesis step.<sup>[9,28]</sup> The SPHs presented in this work were produced by autoclaving without any

monomers or cross-linkers, and did not undergo any treatment after the gelation. We then compared the tensile performance of PVA-Gantrez<sup>®</sup> AN foam samples containing different amount of NaHCO<sub>3</sub> and a conventional hydrogel as a control (produced using the same technique but without addition of NaHCO<sub>3</sub>) using a T.A. in the ‘film stretching’ set-up (Table 4.1).

Table 4.1 Mechanical properties of PVA-Gantrez<sup>®</sup> AN SPHs. Data are shown as mean ± standard deviation. Statistical significance (p<0.05) was calculated for tensile stress at break, maximum strength and elongation to break and Young’s modulus values obtained for all hydrogel foams and control(a-d). Letters after each value indicate which other composition the sample is significantly different from.

Concentration of NaHCO <sub>3</sub> in the reaction mixture	Tensile stress at break (N/mm <sup>2</sup> )	Maximum strength (N/mm)	Elongation to Break (%)	Young’s Modulus (N/mm <sup>2</sup> )
<sup>a</sup> 0%	$(2.2 \pm 1.6) \times 10^{-4}$	$(6.3 \pm 0.9) \times 10^{-2}$	48.4 ± 10.2	$(1.0 \pm 0) \times 10^{-4}$
<sup>b</sup> 0.5%	$(4.7 \pm 2.6) \times 10^{-5}$	$(3.7 \pm 0.7) \times 10^{-2}$	58.7 ± 14.1	$(2.0 \pm 1.0) \times 10^{-5 (a)}$
<sup>c</sup> 0.7%	$(1.0 \pm 0.3) \times 10^{-4}$	$(4.5 \pm 0.7) \times 10^{-2}$	89.1 ± 7.4 <sup>(a)</sup>	$(3.3 \pm 0.4) \times 10^{-5 (a)}$
<sup>d</sup> 1.0%	$(1.1 \pm 0.8) \times 10^{-5}$	$(4.7 \pm 1.6) \times 10^{-2}$	82.8 ± 13.8 <sup>(a)</sup>	$(2.6 \pm 1.2) \times 10^{-5 (a)}$

As shown in Table 4.1, the resulting materials are less stiff and more flexible when the foaming agent is added and the superporous foam internal structure is then formed. The elongation to break, which is the deformation or strain that the material undergoes at fracture, is significantly improved in the

SPHs produced if compared with the control. The foam samples exhibit more desirable values of Young's modulus as well, which is defined as the slope of the stress vs strain curve.<sup>[29-31]</sup> However, the difference in the amount of NaHCO<sub>3</sub> contained in the foams did not lead to significant differences between the samples in Young's modulus values. No statistically significant difference was found for tensile stress at break and maximum strength data collected during the test ( $p>0.05$ ).

#### 4.3.4 Adhesion of PVA-Gantrez®AN foam dressing to skin

Moist dressings, such as alginates, hydrocolloids and hydrogels, have been shown accelerate the wound healing process more effectively, creating an optimal environment for the re-epithelization to take place, and protecting the newly formed skin not strongly adhering to it but at the same time not compromising its isolation.<sup>[32-34]</sup> Gauze as well as all most commonly used dry wound dressings, tends to adhere to a greater extent to the wound bed causing for this reason more pain and further damage upon its removal.<sup>[35]</sup> The level of adhesion to skin represents a very important aspect to be considered when designing a wound management product. We performed an adhesion test using a T.A. and shaved porcine skin as skin model to evaluate the adhesiveness of the hydrogel foams we produced by autoclaving (Figure 4.5).<sup>[36, 37]</sup>

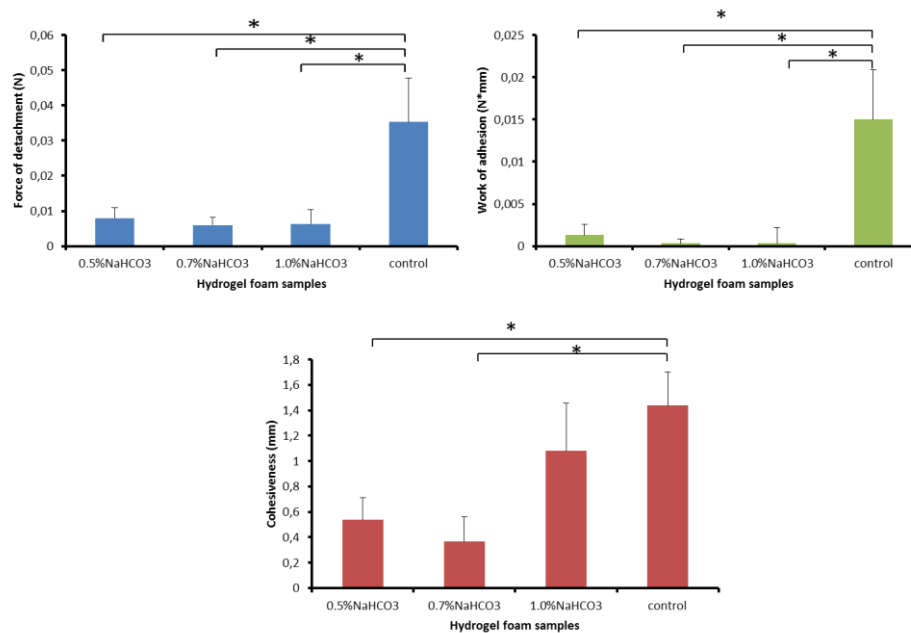


Figure 4.5 Evaluation of the level of adhesion to skin of PVA-Gantrez® AN SPHs and conventional hydrogel as a control. Force of detachment, work of adhesion and cohesiveness data are shown in the graphs. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

The parameters considered during the test were: the force required to detach the samples from the skin, the total work of adhesion, which is defined as the area under the force versus distance curve, and the cohesiveness, which represents the distance travelled by the samples until complete detachment.<sup>[38]</sup> As shown in Figure 4.5, the level of adhesion significantly decreases for the PVA-Gantrez® AN foam samples compared to the control. This could be explained with the more irregular surface presented by the foams and with the higher degree of physical and chemical cross-linking achieved in these materials, with a limited availability of groups capable of hydrogen bonding with the functional groups present on the skin (according to the adsorption theory of adhesion).<sup>[39,40]</sup> No statistically significant difference was found among the foam compositions ( $p > 0.05$ ).

#### 4.3.5 Disk diffusion test

Bacterial colonisation is a recurring reason for delays in the healing process and severe pain when it evolves into what is called 'critical colonisation' and finally, infection. The use of wound dressings containing antimicrobial agents, such as silver ions and iodine, is more frequently preferred or associated to the use of systemic antibiotics, especially when the patient is affected by circulatory pathologies that may prevent the drug from having the required therapeutic effect, and presents chronic infected wounds. However, their use is indicated only for compromised wounds and they should be carefully chosen depending on the type of wound treated and any possible allergies of the patient.<sup>[41-44]</sup> The antimicrobial activity of autoclaved PVA-Gantrez® AN foams was assessed using a disk diffusion test against *Staphylococcus aureus*, which is one of the opportunistic gram positive bacteria most commonly present in chronic wounds.<sup>[45-48]</sup> This test allows a rapid evaluation of bacteria growth inhibition and in this case, it was conducted using freeze-dried samples compressed in the form of disc-shaped tablets, to better visualize the results (Figure 4.6).<sup>[34]</sup>

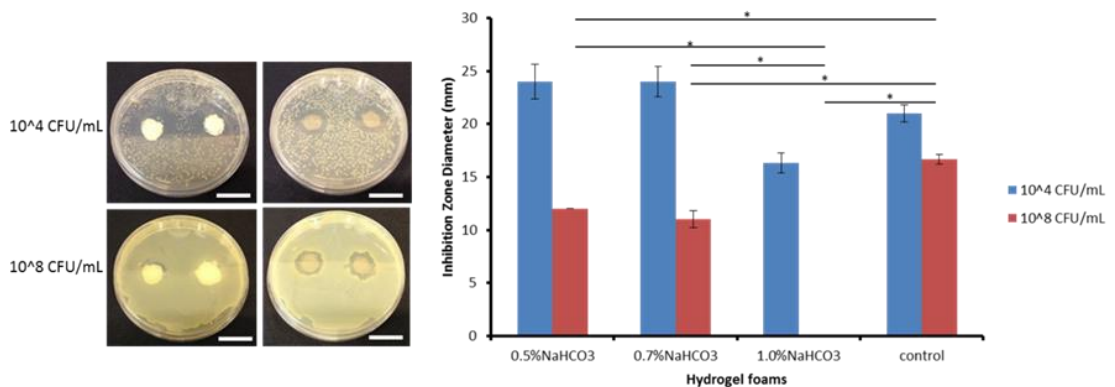


Figure 4.6. Antimicrobial activity assessment by disk diffusion method of PVA-Gantrez® AN hydrogel foams (left) and control (Aquacel® Ag, ConvaTec Ltd, right) against *Staphylococcus aureus* ( $10^4$  and  $10^8$  CFU/mL). Growth inhibition areas were measured. Scale bar is 2 cm. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

A clear zone with no bacteria proliferation is observed around the samples. As expected, the bacteria growth inhibition due to the presence of the SPH dressings was higher when the bacteria concentration was lower ( $10^4$  CFU/mL), and comparable with the control containing silver. The antimicrobial activity of the samples is related to the presence of Gantrez® AN, that in water is converted into its free acid form, and to their low pH (4-5), which creates a hostile environment for *Staphylococcus aureus*.<sup>[17]</sup> Therefore, the antimicrobial effect decreases with the increase in the pH, and samples containing 1%w/v NaHCO<sub>3</sub> (pH ~5) do not show this effect at bacteria concentration of  $10^8$  CFU/mL.

#### 4.4 Conclusion

A new technique of SPHs manufacturing is developed, which consists of several simple steps. The autoclaving of polymeric aqueous mixtures with the addition of NaHCO<sub>3</sub> for 30 minutes at 130°C leads to the successful formation of sterile hydrogel foam dressings. This method involves very low production costs and does not employ any toxic solvents or compounds, and thus represents a very promising alternative to the most common procedures used for SPHs synthesis. Different combinations of water-soluble polymers could be used to prepare SPHs using this method. The PVA-Gantrez® AN hydrogel foams described in this work have shown to possess a highly porous structure that enables them to rapidly swell in water and SWF, and could allow an easy loading and release of an active ingredient. Good mechanical properties and level of adhesion to skin were exhibited as well. Their intrinsic antimicrobial activity represents another relevant advantage for their applications as wound dressing materials. Further work would be required to optimize these materials for their development into final wound care products to be launched on the market.

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# 5 Novel Poly(vinyl alcohol)- Gantrez<sup>®</sup> AN cryogels for wound care applications

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Cryotropic gelation is a low cost, well-known technique that has been used for decades for the preparation of cryogels based on poly(vinyl alcohol). This technique does not require addition of any cross-linkers or initiators because a physical cross-linking takes place during the cryotropic gelation. Poly(vinyl alcohol)-Gantrez<sup>®</sup> AN cryogels with highly porous structure were successfully produced using only one freeze-thawing cycle. These cryogels exhibited excellent mechanical properties and high swelling ability in water. They exhibited intrinsic antimicrobial properties against *Staphylococcus aureus* without the addition of any antimicrobial agent due to the acidic nature of Gantrez<sup>®</sup> AN and related low pH of these cryogels. The *in vitro* biocompatibility of these cryogels was assessed using human dermal fibroblasts with very encouraging results. These cryogels are promising for applications in wound care.

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## 5.1 Introduction

Polymeric gels are widely used in the biomedical sector as drug delivery systems, matrices for cells immobilization, scaffolds for tissue engineering, chromatographic materials and wound care products.<sup>[1-2]</sup> Gels can be generally defined as three-dimensional swollen networks entrapping the solvent, which can be water (hydrogels) or an organic liquid (organogels). What crucially determines their physicochemical properties, and even their appearance, is the nature of their cross-linking and the method used for their synthesis. There are two main types of polymeric gels: chemically cross-linked gels, whose chains are linked with covalent bonds, resulting in irreversibly insoluble networks, and physical gels, which are formed by the physical cross-linking of the polymer chains and could potentially be re-dissolved upon changes in environmental conditions.<sup>[3]</sup> One of the most commonly used techniques for the synthesis of physically cross-linked hydrogels is the so-called 'cryotropic gelation' (from the Greek 'κρίός', frost and 'τρόπος', cause). In this method, the reaction mixture undergoes single or multiple freeze-thawing cycles, which leads to the formation of an internal structure of interconnected pores.<sup>[4-6]</sup> In fact, after reaching the crystallization temperature of the solvent, the system is represented by a heterogeneous block containing solvent crystals and the unfrozen liquid micro-phase (UFLMP), where the reagents quickly concentrate (this phenomenon is called 'cryoconcentration') leading to the gelation.<sup>[2,7]</sup> The solvent here acts as a pore-forming agent: when the solvent crystals melt down they leave a macro and micro-porous network inside the gel formed (cryogel). The size of the macro-pores present in the system as well as its mechanical properties such as the elastic modulus, directly depend on the concentration of the gel-forming reagents used and on the number and conditions of the freeze-thawing cycles.<sup>[8,9]</sup>

The most popular cryogels are based on poly(vinyl alcohol) (PVA) and are usually obtained after several (up to 8) repeated thermal cycles using water as a solvent (to produce opaque gels) or dimethyl sulfoxide (DMSO, to form transparent gels).<sup>[9]</sup> They have been proposed as materials

promising for cells entrapment and delivery, as well as cartilage and cardiac valves replacement and wound healing.<sup>[10-12]</sup> Wound dressings using PVA-cryogels have been shown to be particularly advantageous. It is well-known that hydrogels could accelerate wound healing thanks to their ability to absorb exudate, preventing the dehydration of the wound bed at the same time, and promoting the re-epithelization process.<sup>[13,14]</sup> Moreover, their cooling effect on the skin can provide some pain relief to the patients. Cryogels are better option to traditional dressings in particular when the injury is located in body parts that can be more frequently subjected to physical stress (such as joints) or compression (for example in the case of bed sores).<sup>[14,15]</sup> PVA is a neutral water-soluble synthetic polymer that has been used alone or in combination with other natural or synthetic polymers. It can form the basis of non-toxic, non-carcinogenic, and biocompatible materials with high water content.<sup>[16]</sup> In the present work, PVA was used in combination with poly(methyl vinyl ether-alt-maleic anhydride), another synthetic polymer used in food, pharmaceutical and oral care industries as a thickener and suspending agent, known by its trade name Gantrez® AN.<sup>[17-19]</sup> The two polymers were physically cross-linked by one freeze-thawing cycle to result in novel cryogels for wound care applications. To the best of our knowledge, this is the first study where Gantrez® AN is combined with PVA for cryogels synthesis. The formation of these materials was found to be highly dependent on the addition of NaOH in their aqueous mixture.

## 5.2 Experimental section

### 5.2.1 Materials

PVA of 98-99% hydrolysis and of Mw 57-66 kDa molecular weight was purchased from Alfa Aesar (UK). The analysis of PVA using <sup>1</sup>H NMR and gel permeation chromatography established the actual degree of deacetylation and Mw to be 98.7% and 51.3 kDa, respectively. Nutrient agar and nutrient

broth used to perform the antimicrobial properties test were purchased from Oxoid Ltd UK. Gantrez® AN (Mw ~ 216 kDa) as well as the other chemicals used for this work were purchased from Sigma-Aldrich (UK). AQUACEL® Ag dressing (used as a control for antimicrobial and cytotoxicity tests) was kindly provided by ConvaTec Ltd.

### 5.2.2 Cryogels synthesis

10 % w/v aqueous solution of PVA was prepared by dissolving the polymer in water at 92°C for approximately 7 h. This solution was mixed with 17.4 % w/v aqueous solution of Gantrez® AN, which was previously prepared by dissolving the polymer in water overnight at 40°C. Three mixtures with 1:1 volume ratio of the two polymers were produced with the addition of NaOH at different concentrations (0.4 %, 0.6 % and 0.8 % w/v), and poured into sealed glass petri-dishes (8 cm diameter). These were frozen at -18°C overnight (approximately 9 hours) and then thawed at room temperature (~25°C). An opaque, flat, stretchable and very easy to handle gel was formed as a result of this cryogelation.

### 5.2.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (1000x magnification) was carried out to investigate the porous structure of the materials produced. An FEI Quanta FEG 600 Environmental Scanning Electron Microscope (ESEM) was used to study gold sputter coated, freeze-dried PVA-Gantrez® AN cryogels, produced after one freeze-thawing cycle.

### 5.2.4 Sol-gel fractions analysis

Insoluble (Gel) and soluble (Sol) fractions (%) of PVA-Gantrez® AN cryogels were determined using a gravimetric method. The samples were immersed for 48 h in 12 mL of deionized water, which was then freeze-dried (sol 1) and then replaced with fresh water for another 48 h. The swollen gel and the soluble extract (sol 2) were also freeze-dried. Control samples were directly freeze-dried after

their synthesis (100 % Gel fraction). All analyses were conducted in triplicate. Gel and sol fractions were calculated using the following formulae:

$$\text{Gel fraction (\%)} = \frac{W_d}{W_i} \times 100 \quad (1)$$

$$\text{Sol fraction (\%)} = \frac{W_{sol1} + W_{sol2}}{W_i} \times 100 \quad (2),$$

Where  $W_d$  the swollen dry gel,  $W_i$  the control (a sample which was not immersed in water but directly freeze-dried), and  $W_{sol1}$  and  $W_{sol2}$  represent soluble fractions.

#### 5.2.5 Swelling behaviour of PVA- Gantrez® AN cryogels

The swelling properties of the cryogels were studied in deionized water and in a saline solution simulating the ionic strength of wound exudate (8.298g NaCl and 0.368g CaCl<sub>2</sub> dissolved in 1L of deionized water). The test was performed using metallic mesh baskets (2 cm diameter, 3 cm height) placed inside plastic vials (3.5 cm in diameter and 6 cm in height). The materials produced were cut into 3 mm diameter disc shaped flat samples and placed into the metallic baskets. The initial weight of the samples and the weight of each basket used (after being immersed into the medium used for the test and wiped always in the same manner) were accurately recorded.<sup>[20]</sup> The cryogels were kept in excess of medium for 8 days and weighed regularly, by weighing the baskets with the cryogel samples inside. The weights of the baskets were taken into consideration to calculate the weight of the swollen samples. The swelling ratio (SR in %) of the PVA- Gantrez® AN cryogels was calculated using the following formula:

$$SR (\%) = \frac{W_s - W_i}{W_i} \times 100 \quad (3),$$

Where  $W_i$  is the initial gel weight and  $W_s$  is the weight of the sample after additional swelling.

### 5.2.6 Mechanical properties

A Texture Analyser XT Plus (T.A., by Stable Micro System Ltd, UK) was used to perform the tensile test on the PVA-Gantrez® AN cryogels to assess their mechanical properties. The materials were cut into 20×3 mm samples. A 'grip-to-grip' separation of 30 mm was used and each sample was stretched, undergoing an extension of 100 mm/min to break. Strength-strain curves were recorded during the test.

### 5.2.7 Cryogels adhesion to porcine skin model

The adhesion of the cryogels to porcine skin was tested using Texture Analyser (Stable Micro System Ltd, UK) in a different set up: cryogel discs (20 mm in diameter) were fixed to the probe of the T.A. and shaved porcine skin (from the back of the animal, provided by P. & D. Jennings Butchers, Hurst, UK) 5cm×5cm specimens were placed on the area below. The following settings were used for the test: pre-speed test 1.0 mm/s; test-speed 0.5 mm/s; post-test speed 1.0 mm/s; applied force 0.5 N; return distance 10.0 mm; contact time 60.0 s; trigger type auto; trigger force 0.1 N; and return distance of 10.0 mm. The adhesive strength, the total work of adhesion and the cohesiveness of the materials produced were calculated from the data collected during this test by the T.A. software (T.A. Exponent) according to the methodology described by Boateng et al.<sup>[21]</sup>

### 5.2.8 Antimicrobial properties

The disk diffusion test<sup>[22]</sup> was used to investigate the antimicrobial properties of PVA-Gantrez® AN cryogels against *Staphylococcus aureus* NCTC 8532 (National Collection of Type Cultures). *Staphylococcus aureus* was grown overnight at 37 °C (shaking at 200 rpm) in nutrient broth. Sterilized nutrient agar was prepared as indicated by the manufacturer and used to produce the plates to be inoculated with two bacterial suspensions ( $10^8$  CFU/mL and  $10^4$  CFU/mL, respectively). Cryogel discs (1 cm in diameter) were placed in the centre of the agar plates and incubated for 24 hours at 37°C.



The clear zone around the samples was measured using a ruler to assess bacteria growth inhibition and proliferation.

#### 5.2.9 Biocompatibility of PVA-Gantrez®AN cryogels

The biocompatibility of the cryogels was evaluated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.<sup>[20,23,24]</sup> The extracts of the cryogels and control (AQUACEL® Ag, ConvaTec Ltd) were used for the test. 5 mg of each sample were incubated at 37°C in 8 mL of Dulbecco's modified Eagle's medium (DMEM) containing 2% v/v L-glutamine and 50 µg/mL gentamicin. The medium was collected after 1, 2 and 7 days and used to perform the MTT assay. Human dermal fibroblasts were seeded at a density of  $8 \times 10^4$  cells/mL in complete medium (containing 10 % Fetal bovine serum (FBS), 2% L-Glutamine and 50 µg/mL of gentamicin) in a sterile 96-wells culture plate and cultured to reach confluence. The extracts collected from the materials produced were used to replace the medium. The plates were incubated at 37 °C and 5% CO<sub>2</sub> for 24 h. MTT solution was prepared in warm PBS (0.5 mg/mL) following the manufacturers specification. 100 µL per well of this solution was added to the plates, which were then incubated at 37°C for 3 h. After removal of MTT and medium, DMSO (100 µL per well) was used to solubilize the formazan present in the cells. A Biotek Synergy HT detector was used to read the absorbance at 570/630 nm.

#### 5.2.10 Sterilization of cryogels by gamma irradiation

The materials produced were sterilized using gamma irradiation<sup>[25]</sup> by Bradford Synergy Health PLC (UK) in accordance with EN ISO 11137-1:2015 requirements for health care products, EN ISO 9001:2008 for quality management system, and EN ISO 13485:2012 for quality system-medical devices. The absorbed radiation dose was 33.3 kGy. The influence of the gamma irradiation on their swelling ability in deionized water and mechanical properties were assessed as described above.

### 5.2.11 Statistical analysis

The statistical analysis was performed using one-way ANOVA with Bonferroni post-hoc test on Prism (Graphpad software, USA) comparing different groups of data.

## 5.3 Results and discussion

### 5.3.1 Formation of PVA-Gantrez<sup>®</sup>AN cryogels

Flat cryogel samples were successfully produced from mixtures of aqueous solutions of PVA and Gantrez<sup>®</sup>AN, (with the addition of NaOH) using the freeze-thawing technique, which involves the cryotropic gelation process and leads to inter- and intra-chains physical cross-linking. Only one thermal cycle is required to form the materials presented in this work. The proposed method is less-time consuming and more cost-effective compared to many techniques commonly used to produce physical hydrogels.<sup>[3,26,27]</sup> It should be noted that PVA-Gantrez<sup>®</sup>AN cryogels did not form in the absence of NaOH within solutions under the freeze-thaw conditions used in this work. However, an aqueous solution of PVA alone did form cryogels in the absence of NaOH. The synthesis of PVA-based with the addition of strong bases such as NaOH and KOH has previously been reported; it was demonstrated that PVA-based gels prepared with the addition of NaOH have improved mechanical properties due to increased sample crystallinity.<sup>[28-31]</sup>

It could be hypothesized that the addition of NaOH to PVA-Gantrez<sup>®</sup> AN aqueous mixtures prevents the formation of weak intermolecular hydrogen bonds between –COOH of Gantrez<sup>®</sup> AN and –OH groups of PVA due to dissociation of carboxylic groups. The formation of more regular –OH---HO- hydrogen bonds between PVA chains is then facilitated under these conditions. Additionally, the effect of NaOH on PVA reported in refs<sup>[28-31]</sup> could also not be fully ruled out. Moreover, the addition

of NaOH to polymer solutions gives a more desirable pH range, 4.4-5.3, for wound care applications.<sup>[32-34]</sup>

In the conditions described above, the intra- and inter-chain hydrogen bonding is promoted and takes place more rapidly in the unfrozen liquid micro-phases (UFLMPs). In these areas the reagents mixture is highly concentrated because of the formation and expansion of solvent crystals during the freezing step at  $-18^{\circ}\text{C}$ . These expand in the mixture forming a structure that will generate the network of interconnected pores once the crystals melt down during the thawing step at room temperature.<sup>[4,11]</sup> This characteristic internal structure significantly influences the physical and mechanical properties of cryogels, which became very popular materials for different biomedical applications.<sup>[1,35]</sup> SEM analysis allowed us to observe pores morphology and distribution in the PVA-Gantrez<sup>®</sup>AN cryogels (Figure 5.1).

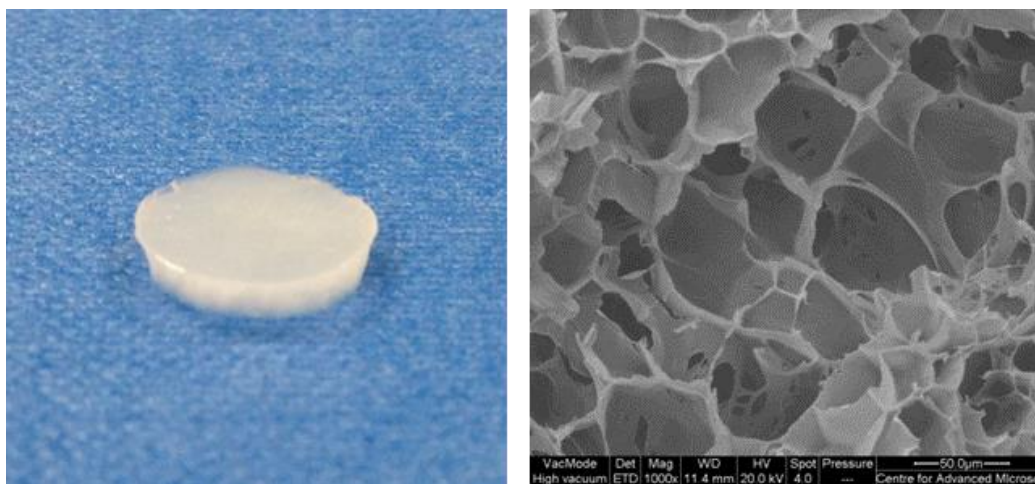


Figure 5.1. Macroscopic image of the swollen cryogel and SEM image (1000x magnification) of freeze-dried gold sputter coated sample of PVA-Gantrez<sup>®</sup>AN formed in the presence of 0.25 %NaOH.

All the PVA-Gantrez<sup>®</sup>AN cryogels present a highly porous structure with different pore sizes and thin gel walls throughout the matrix. The largest pores (10-60  $\mu\text{m}$ ) observed in these materials are

interconnected, and can be then attributed to the formation of large ice crystals and to the consequent water flow through the cryogel matrix during thawing.

It is well known that a second polymer added to PVA solutions in a common solvent may lead to liquid-liquid phase separation due to thermodynamic incompatibility between these materials, which affects the formation of pores.<sup>[36-38]</sup> It should be noted that PVA-Gantrez®AN forms completely transparent aqueous mixtures without addition of NaOH (Figure 5.2). However, the addition of NaOH to PVA-Gantrez®AN mixtures results in formation of slightly cloudy solutions, indicating phase separation.

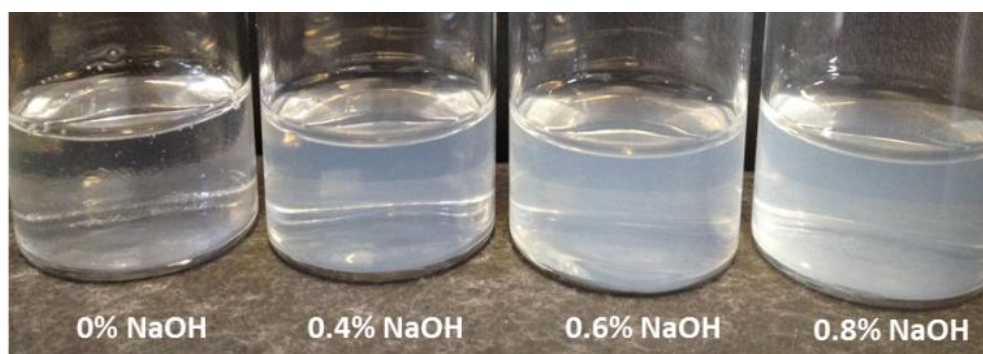


Figure 5.2 PVA-Gantrez® liquid mixture with no NaOH added and PVA-Gantrez®AN plus NaOH (0.4, 0.6 and 0.8%w/v) mixtures before freezing.

It is likely that the increase in pH of solution causes dissociation of COOH groups of Gantrez® AN, which prevents weak intermolecular  $-\text{COOH}\cdots\text{HO}-$  bonds formation and reduces its thermodynamic compatibility with PVA. It should also be noted that these cloudy solutions are colloiddally stable and even centrifugation at 60000 rpm for 1h did not result in any liquid-liquid separation. Perhaps, a phase separation in to PVA-Gantrez®AN mixtures in the presence of NaOH may affect the formation and morphology of pores; however, more detailed studies of this phenomenon are outside of the scope of this work.

### 5.3.2 Sol-gel fraction analysis

Formation of hydrogels from ready-made polymers often results in materials containing large quantities of loosely bound, non-cross-linked macromolecules. To quantify the efficiency of cross-linking and the content of loosely bound macromolecules a sol-gel analysis is usually performed.<sup>[39]</sup> This involves the extraction of a soluble fraction and gravimetric determination of soluble/insoluble fractions. In fact, gel content significantly influences the mechanical properties and the swelling behaviour of these materials.<sup>[12]</sup> Gel and Sol fractions of the cryogels produced were measured by gravimetric analysis of freeze-dried samples previously immersed in deionized water and their soluble residues (Table 5.1). Control samples directly freeze-dried after production were used to establish the average total weight of dry gel.

Table 5.1 Gel and Sol fractions (%) for PVA-Gantrez®AN cryogels. Data is shown as mean  $\pm$  standard deviation. Statistical significance ( $p < 0.05$ ) was calculated for all data (a-c). Letters after each value indicate which other composition the sample is significantly different from.

NaOH added to polymer mixture	Gel fraction (%)	Sol fraction (%)
<sup>a</sup> 0.4%	21 $\pm$ 1 <sup>(b,c)</sup>	79 $\pm$ 1
<sup>b</sup> 0.6%	40 $\pm$ 3	60 $\pm$ 14
<sup>c</sup> 0.8%	44 $\pm$ 4	56 $\pm$ 5

A significant increase in gel fraction of the materials is observed when the percentage of NaOH

added to the liquid mixture of the two polymers is raised from 0.4 % to 0.6 %w/v. Higher concentrations of NaOH during the synthesis of cryogels are likely to lead to higher degrees of PVA - OH...HO- inter- and intra-chain hydrogen bonding, resulting in more cross-linked cryogels with a lower soluble fraction, and ultimately in mechanically stronger gel.<sup>[12]</sup> In fact at higher pH values, when the -COOH groups of Gantrez<sup>®</sup>AN are ionized, the formation of weak hydrogen bonding between the two polymers chains (-COOH...HO-) is prevented and OH- groups in PVA macromolecules will have better opportunities to form -OH...HO- bonds.<sup>[36]</sup> As a result, the sol fraction is more likely to consist of hydrolysed Gantrez<sup>®</sup>AN, and the gel is enriched with PVA.

### 5.3.3 Swelling studies

The swelling behaviour of the cryogels in deionized water and in an ion-containing solution (simulating the wound fluid) were monitored for 8 days (Figures 5.3a and 5.3b). Moist dressings, such as hydrocolloids are usually left on the wound site from 3 up to a maximum of 7 days.<sup>[40]</sup> Due to the high degree of physical cross-linking achieved using the freeze-thawing technique, their swelling ratio (SR) values are substantially lower than reported for chemically-crosslinked autoclaved hydrogels made from the same combination of polymers.<sup>[41]</sup> In fact, these hydrogels can absorb more than 40 g of deionized water per 1 g of initial gel within 48 h. However, the water uptake of the cryogels is reduced in the ionic solution for the presence of mobile ions in the medium. This reduction in the cryogels swelling is related to their polyelectrolyte nature and the charge screening effects in the presence of inorganic ions.<sup>[42,43]</sup>

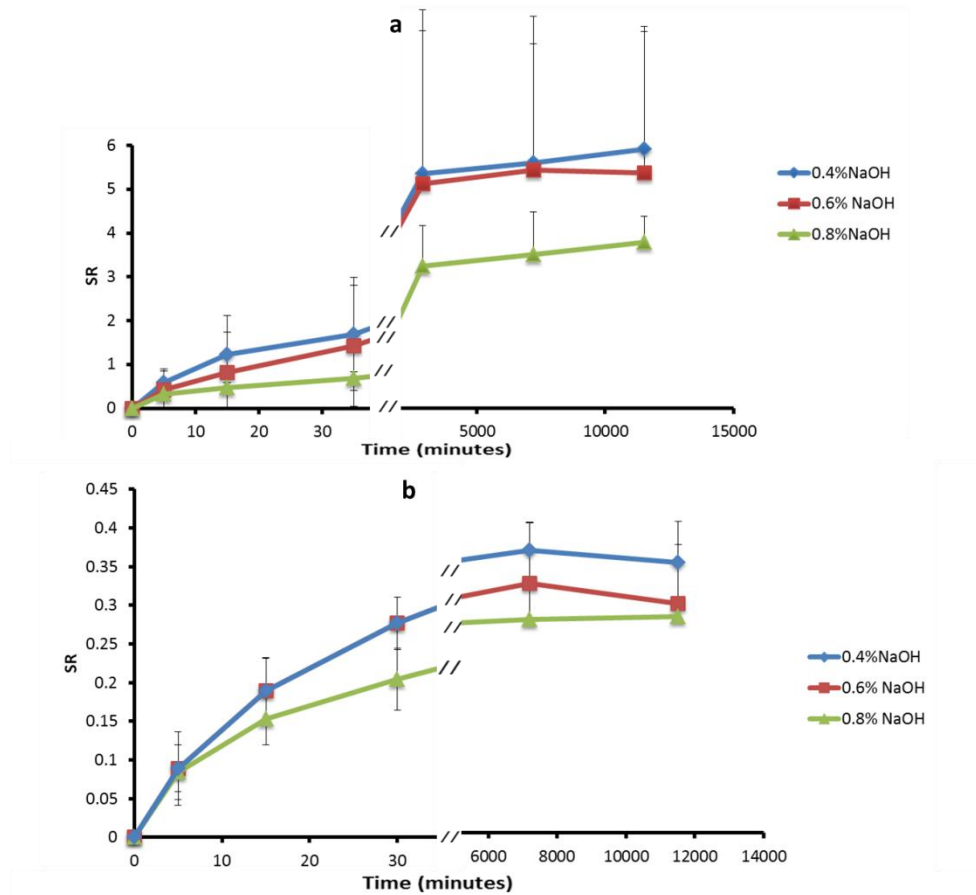


Figure 5.3 Swelling kinetics of PVA-Gantrez®AN cryogels in water (a) and simulated wound fluid (b).

The swelling ratios in both media are not affected by the amount of NaOH added to the liquid mixture of the two polymers to produce the cryogels (no statistical difference was found at any time point comparing all the samples). The samples slowly swell during the first 48 h, without the initial fast swelling exhibited for instance by the cryogels synthesised by Ahuja et al.<sup>[44]</sup> from the combination of PVA and carboxymethyl tamarind kernel polysaccharide (CMTKP) aqueous solutions. They tend to reach the equilibrium after approximately 4 days, similarly to what reported in the literature for example for cryogels prepared from mixtures of PVA with silk.<sup>[45]</sup> In simulated wound fluid the swelling is decreased by the presence of Cl<sup>-</sup> which act as kosmotropes (defined as ions able to cause changes in the structure of bound and free water present in polymeric solutions),

destabilizing the hydrogen-bonding hydration of PVA and Gantrez®AN through ionic hydration.<sup>[46-48]</sup>

The swelling of cryogels is also reduced by Ca<sup>2+</sup> cations, which interacting with negatively charged oxygens of –OH groups, and –COO– groups form complexes, increasing the cross-linking density. At the end of the experiment (after 8 days) the cryogels seem to start degrading and losing weight in the ion-containing solution, whereas in water they continue to absorb small volumes of medium.

### 5.3.4 Mechanical properties of cryogels

The mechanical properties of the PVA-Gantrez®AN cryogels produced were tested using a texture analyzer in the ‘film stretching’ mode and compared with those of PVA-only cryogels synthesised using the same cryotropic gelation method (Table 5.2).

Table 5.2 Mechanical properties of PVA-Gantrez® AN and PVA-only cryogel samples. Data is shown as mean ± standard deviation. Statistical significance (p<0.05) was calculated for the data related to tensile stress at break, maximum strength, elongation to break and Young’s modulus of the (a-d). Letters after each value indicate which other composition the sample is significantly different from.

Cryogels	Tensile stress at break (N/mm <sup>2</sup> )	Maximum strength (N/mm)	Elongation to Break (%)	Young’s Modulus (N/mm <sup>2</sup> )
PVA-Gant (a)	$(2.3 \pm 1.0) \times 10^{-4} (d)$	0.14 ± 0.04	158 ± 7 <sup>(c,d)</sup>	$(4.7 \pm 3.2) \times 10^{-5} (d)$
PVA-Gant (b)	$(1.2 \pm 0.7) \times 10^{-4} (d)$	0.24 ± 0.04	190 ± 2 <sup>(d)</sup>	$(8.3 \pm 1.2) \times 10^{-5} (d)$
PVA-Gant (c)	$(3.8 \pm 1.0) \times 10^{-5} (d)$	0.29 ± 0.04	218 ± 13 <sup>(d)</sup>	$(7.6 \pm 3.3) \times 10^{-5} (d)$
PVA only (d)	$(3.4 \pm 0.4) \times 10^{-2} (a,b,c)$	0.53 ± 0.31	304 ± 21 <sup>(a,b,c)</sup>	$(6.7 \pm 0) \times 10^{-4} (a,b,c)$



The presence of Gantrez® AN in the polymer mixture leads to the formation of more flexible materials compared to PVA-only cryogels. PVA macromolecules have a stronger tendency to form intra- and inter-chain hydrogen bonds compared to Gantrez® AN resulting in denser cross-linking, higher PVA crystallization and stiffer gels in case of pure PVA system. The addition of NaOH does not significantly influence the flexibility of the cryogels produced. Only the elongation to break, which is the deformation or strain that the material undergoes at fracture, increases with NaOH addition to the reaction mixture from 0.4% to 0.8% (Table 5.2). Note that strict comparison between the mechanical properties of PVA-Gantrez® AN cryogels prepared in the presence of NaOH and the properties of PVA only cryogel should be conducted when PVA only sample was also prepared with addition of NaOH. This was not done in the present work as PVA is not able to neutralize NaOH and would result in basic and biologically-incompatible samples.

#### 5.3.5 Adhesion to skin

The adhesion to skin is an important aspect when considering new biomaterials as wound dressing candidates. During the adhesion tests, PVA-Gantrez®AN cryogel samples were applied to sections of porcine skin and then detached. The force of detachment, work of adhesion and cohesiveness values for the cryogels tested were collected and evaluated (Figure 5.4).

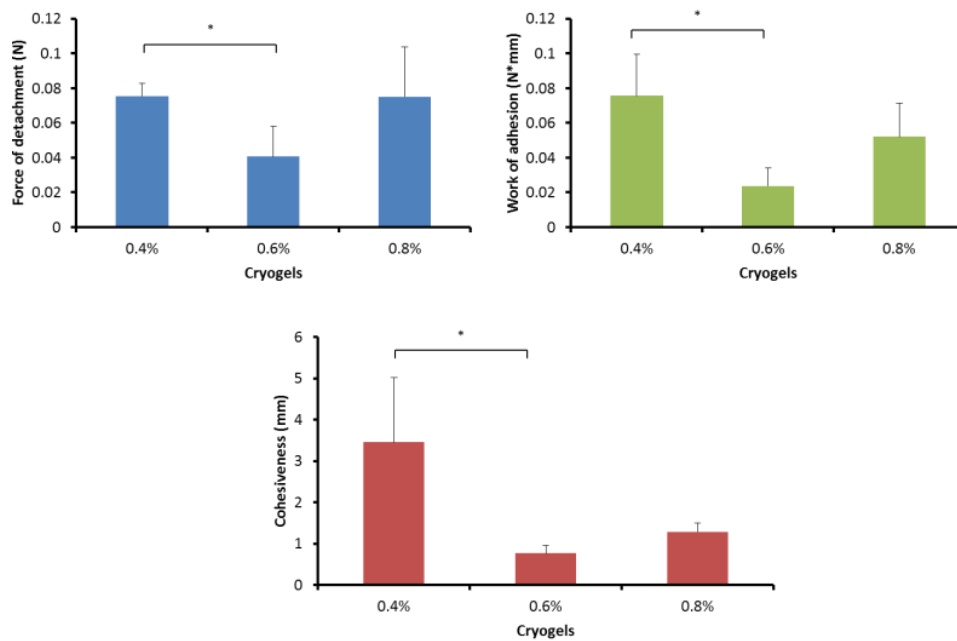


Figure 5.4 Force of detachment, work of adhesion and cohesiveness data recorded for PVA-Gantrez®AN cryogels during their application to porcine skin and detachment using a T.A. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

The cryogels become less adhesive when the percentage of NaOH added to the mixture of polymers is increased from 0.4% to 0.6%. The effect of NaOH on the adhesiveness of the cryogels to skin may be related to several factors. First, the increase in the pH disrupts the hydrogen bonding between -COOH groups of Gantrez® AN and -OH groups of PVA, promoting the formation of inter- and intra-chains of -OH---HO- hydrogen bonds. This leads to greater physical cross-linking and fewer loosely bound macromolecules present in the cryogel. This could reduce the contribution of their diffusion into the crevices of the skin lowering the adhesion. On the contrary, according to the adsorption theory, two materials can adhere to each other due to the formation of non-covalent bonds, such as hydrogen bonds, between the two surfaces in contact.<sup>[49]</sup> The cryogels formed in the presence of

greater quantities of NaOH will have more carboxylic groups ionized, making them less able to form hydrogen bonds with -OH groups present on the skin surface.

### 5.3.6 Antimicrobial activity

A clear *S. aureus* growth inhibition zone was observed around the PVA-Gantrez®AN cryogel samples that had been placed on the agar plates inoculated with the bacteria (Figure 5.5). The antimicrobial activity is higher when the bacteria concentration is lower ( $10^4$  CFU/mL), as expected, and comparable to the control containing silver and other silver loaded PVA-based cryogels reported in the literature.<sup>[50]</sup>

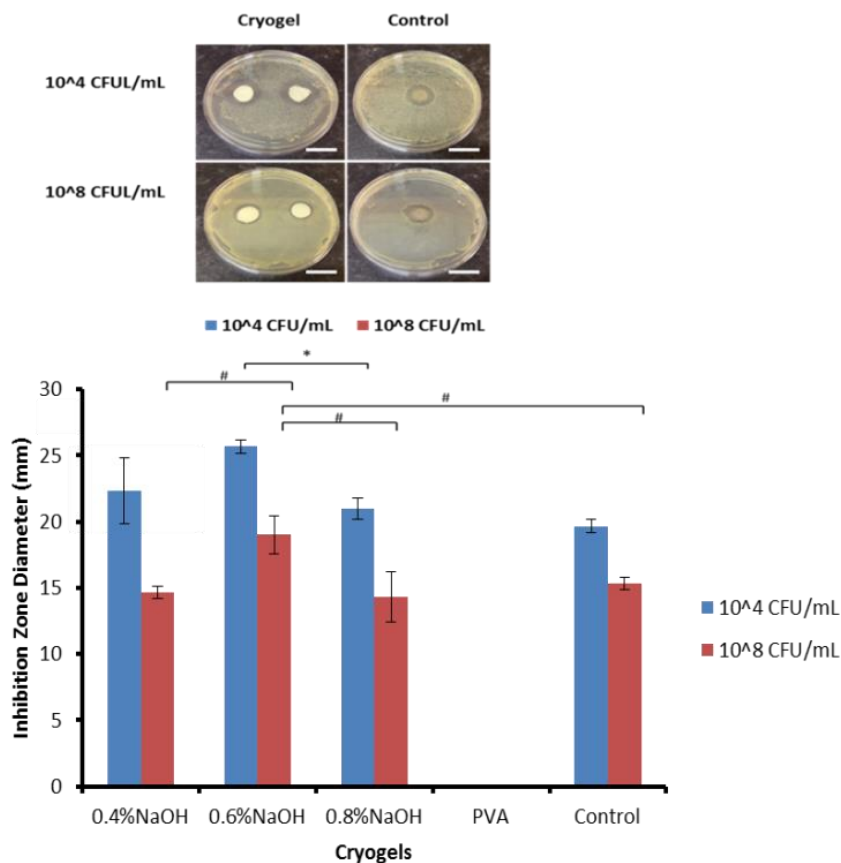


Figure 5.5 Antimicrobial activity of PVA-Gantrez®AN cryogels evaluated using a disk diffusion method performed with *S. aureus*. PVA-only cryogels and Aquacel®Ag were used as a negative and positive controls, respectively. Asterisk (\*) and hash mark (#) indicate significant statistical difference ( $p < 0.05$ ).

No bacteria growth inhibition was found for the PVA-only cryogels, whose presence did not affect *S. aureus* proliferation at all. The results obtained for the cryogels containing Gantrez® AN can be explained by the presence of this polymer, which has shown an intrinsic antimicrobial activity,<sup>[25]</sup> as well as with the low pH (from 4.3 to 5.2) of the materials produced. A leaching of Gantrez® AN or H<sup>+</sup> ions from the cryogels could be the reason for their antimicrobial properties. Mild acidic pH (~5.5) prevents *Staphylococcus aureus* proliferation, interfering with its gene expression and creating a hostile environment for its growth.<sup>[51]</sup>

#### 5.3.7 In vitro cytotoxicity studies

The biocompatibility of the materials produced was assessed in vitro performing an MTT assay using human dermal fibroblasts (Figure 5.6). This well-known colorimetric test allows a rapid and clear cytotoxicity evaluation of drugs and biomaterials.<sup>[52]</sup> The positively charged MTT reagent is promptly internalised by viable cells and converted into a coloured product (called 'formazan'), which exhibits a maximum absorbance at 570 nm.<sup>[23]</sup> PVA-Gantrez® AN cryogel extracts collected in sterile conditions were used for the test.

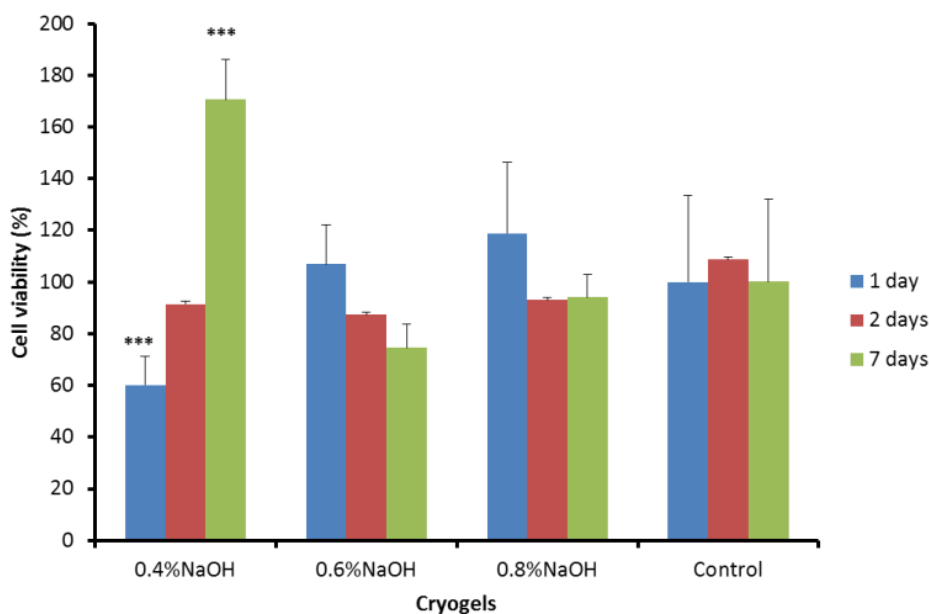


Figure 5.6 Cell viability assessment by MTT assay at 1, 2 and 7 days post-seeding for PVA-Gantrez®AN cryogels and control (Aquacel® Ag) extracts. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ).

The cryogel compositions, with the only exception of the samples where 0.4% of NaOH was initially added for the 1 day extract, showed good cell viability (above 70%). Thus, it is possible to confirm the biocompatibility of the materials produced. No difference was found in the viability of cell cultures placed in contact with extracts collected from the cryogel samples (and control) after 2 days. The gel formed with the addition of 0.4% of NaOH to the PVA-Gantrez®AN mixture, which showed the highest cytotoxicity for the 1 day gel extracts, exhibited the highest viability for the 7 days extracts. The pH plays a very important role in maintaining a favourable environment for the cells to grow and proliferate. In fact, the samples formed in the presence of 0.6% and 0.8% NaOH having the highest pH (~5), showed more desirable cell viability for the extracts collected after 1 day, with no difference compared to the control.

The sterility is necessary for most wound management products. The PVA-Gantrez® AN cryogels developed in this work were sterilized using gamma irradiation, which is commonly employed for the sterilisation of medical devices.<sup>[25]</sup> The swelling of the gamma-sterilized samples in deionized water is

slightly reduced due to additional cross-linking of the polymers induced by the gamma irradiation (Figure 5.7).

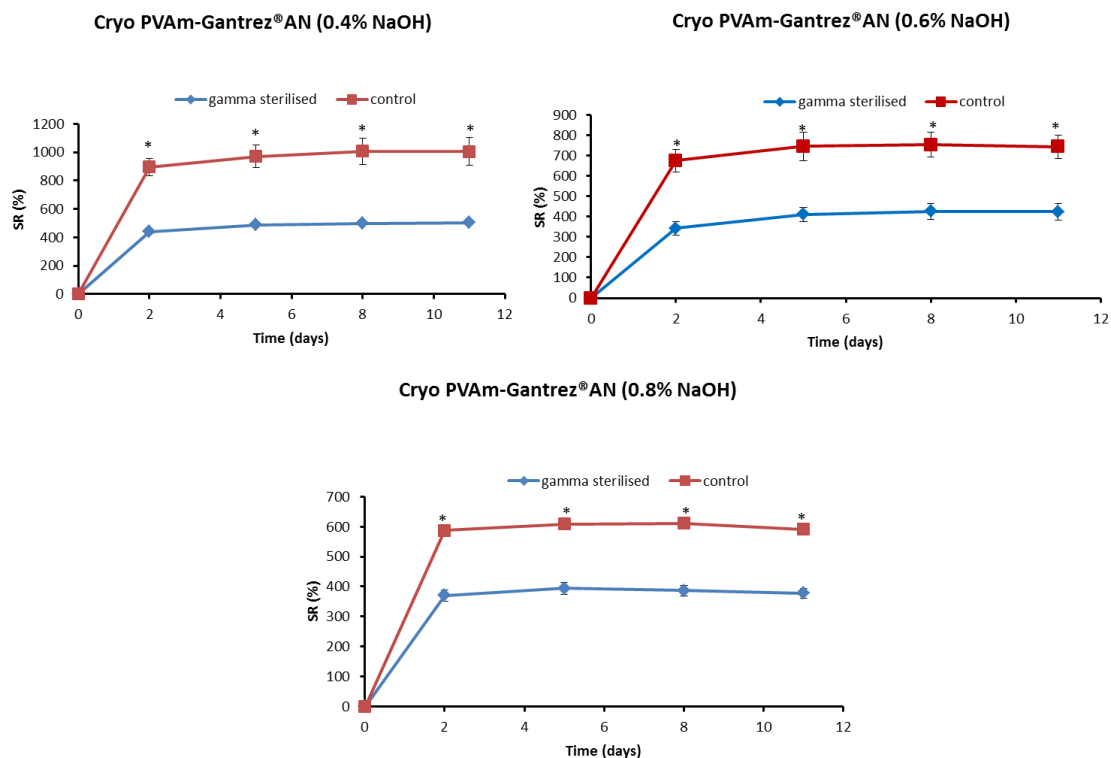


Figure 5.7 Swelling kinetics in deionised water for gamma-sterilised PVA-Gantrez® AN cryogels during a period of 11 days. Non-irradiated samples are used as control. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ ). Please note the test was performed on non-freshly prepared samples due to the irradiation process being conducted by Synergy Health plc. not in the house.

PVA and its blends with other polymers are known to undergo cross-linking reactions upon their radiation treatment.<sup>[39,40,53]</sup> However, the mechanical properties of PVA-Gantrez® AN hydrogels do not seem to be affected by the radiation treatment (Tables 5.3 and 5.4 no statistical difference was found among the data analysed).

Table 5.3 Mechanical properties of gamma-irradiated PVA-Gantrez®AN cryogels. Please note the test was performed on non-freshly prepared samples due to the irradiation process being conducted by Synergy Health plc. not in the house.

<b>Γ-irradiated cryogels</b>	<b>Tensile stress at break (N/mm<sup>2</sup>)</b>	<b>Maximum strength (N/mm)</b>	<b>Elongation to Break (%)</b>	<b>Young's Modulus (N/mm<sup>2</sup>)</b>
0.4% NaOH	$(6.0 \pm 4.4) \times 10^{-5}$	$0.19 \pm 0.1$	$36 \pm 4$	$(1.0 \pm 0.2) \times 10^{-2}$
0.6% NaOH	$(8.4 \pm 6.5) \times 10^{-4}$	$(8.0 \pm 0.3) \times 10^{-2}$	$43.2 \pm 2.2$	$(7.3 \pm 2.3) \times 10^{-3}$
0.8% NaOH	$(2.5 \pm 0.8) \times 10^{-5}$	$(5.7 \pm 4.3) \times 10^{-2}$	$46.1 \pm 1.7$	$(7.6 \pm 3.2) \times 10^{-3}$

Table 5.4 Mechanical properties of non-gamma irradiated PVA-Gantrez®AN cryogels. Please note the test was performed on non-freshly prepared samples due to the irradiation process being conducted by Synergy Health plc. not in the house.

<b>Non-γ irradiated cryogels</b>	<b>Tensile stress at break (N/mm<sup>2</sup>)</b>	<b>Maximum strength (N/mm)</b>	<b>Elongation to Break (%)</b>	<b>Young's Modulus (N/mm<sup>2</sup>)</b>
0.4% NaOH	$(2.1 \pm 1.6) \times 10^{-5}$	$0.2 \pm 0$	$78.2 \pm 7.5$	$(4.3 \pm 1.2) \times 10^{-3}$
0.6% NaOH	$(5.5 \pm 1.7) \times 10^{-5}$	$0.1 \pm 4 \times 10^{-2}$	$78.0 \pm 3.6$	$(5.6 \pm 1.2) \times 10^{-3}$
0.8% NaOH	$(3.9 \pm 1.7) \times 10^{-5}$	$(9.6 \pm 4.7) \times 10^{-2}$	$72.3 \pm 10.1$	$(9.6 \pm 3.7) \times 10^{-3}$

## 5.4 Conclusions

Highly porous PVA-Gantrez® AN cryogels were successfully prepared using a freeze-thawing method. The cryogels presented in this work were produced from mixtures of aqueous solutions of PVA and Gantrez® AN, after only one freeze-thawing cycle, with the addition of small quantities NaOH to adjust their final pH. The materials described in this study have shown good swelling capacity, as well as excellent mechanical properties and level of adhesion to skin. Their soft consistency and high moisture content could provide a comfortable feeling on traumatized and inflamed skin, which may improve patient compliance with the treatment. An intrinsic antimicrobial activity was confirmed due to the presence of Gantrez® AN, and the low pH, which is a significant advantage of these cryogels over PVA-only gels and many commercial products that require the addition of antimicrobial agents, such as iodine or silver.<sup>[41,42]</sup> The cryogels also demonstrated very good biocompatibility when tested using human dermal fibroblasts which make them promising materials for wound dressings.

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## 6 Conclusions

A large portion of the UK's health care system expenditure is devoted to the treatment and care of patients (hospitalized and non-hospitalized) presenting severe wounds.<sup>[1]</sup> Wound management and healing represents an ongoing challenge with some traditional products, such as plain gauze, in certain cases being ineffective, or even damaging to the tissue. Hydrogels have been shown to be very effective in accelerating the healing process of burns, dry and low- to medium- exudate wounds.<sup>[2, 3]</sup> A number of hydrogel products are already present on the market in the form of amorphous gels, such as Aquaform™ (Aspen Medical)<sup>[4]</sup> and Granugel® (ConvaTec)<sup>[5]</sup>, or as flat sheets, such as Hydrosorb® (Hartmann)<sup>[3]</sup> and Aquaflo® (Covidien).<sup>[6]</sup> However, the manufacturing of hydrogel materials can involve the use of expensive irradiation techniques.<sup>[7]</sup> Our work has shown that it is possible to make advanced biomaterials for wound care application using simple, low-cost and eco-friendly methods. We successfully created a small library of different dressing prototypes during this project, namely; hydrogels, SPHs and cryogels produced without the addition of a cross-linker or initiator, whilst using water as a solvent.

A new method of hydrogels production, involving the autoclaving of the aqueous mixtures of PVA and Gantrez® AN was developed and optimised. This technique does not present limitations in terms of volumes or containers used, and allows hydrogels from a combination of two or more water-soluble polymers, such as hydroxyethyl cellulose (HEC), poly(acrylic acid) (PAA), poly(vinyl pyrrolidone) (PVP) or polyacrylamide (PAAM) to be prepared.<sup>[8]</sup> This method allowed the production of bubble-free, transparent flat dressings with a heterogeneous internal structure, high swelling ratio, and good mechanical and antimicrobial properties.<sup>[9]</sup>

The second type of materials we developed were a modification of the autoclaved samples, containing NaHCO<sub>3</sub>. NaHCO<sub>3</sub> was added to the bottom of the petri dish before pouring in the reaction

mixture, this produced completely different hydrogels named SPHs. SPH gels require a shorter autoclaving step and a reduction in autoclave temperature, resulting in a new and simple method for production of novel SPHs. These foam-like hydrogel materials presented a much more porous structure than the hydrogel previously obtained. The same antimicrobial activity was exhibited, but their mechanical properties were poorer compared to bubble-free autoclaved hydrogels.

In both hydrogels and SPHs formed by autoclaving, PVA and Gantrez® AN were chemically cross-linked into a three-dimensional polymeric network. In contrast, physical cross-linking leads to very different materials as observed when studying PVA-Gantrez® AN cryogels produced by freeze-thawing. The non-covalent bonds present in these gels can be disrupted at high temperatures and generates a dense matrix which possesses a lower swelling ability (Figure 6.1).

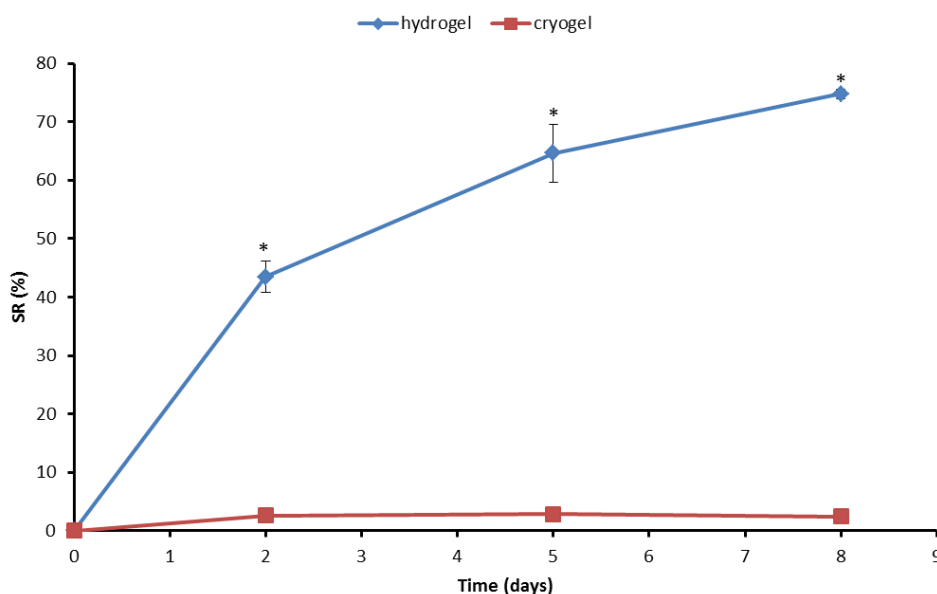


Figure 6.1 Swelling kinetics of cryogel and hydrogel samples with similar PVA-Gantrez® AN molar ratio (2:1) in deionised water. Asterisk (\*) indicates significant statistical difference ( $p < 0.05$ )

The nature of the cross-linking significantly influenced the swelling behavior of these two materials.

In the cryogel matrix, a more regular hydrogen bonding network was present, providing a denser

cross-linked structure. Under these conditions it is more difficult for the solvent to penetrate into the gel mesh and hence be absorbed and retained within the polymer chains, compared to the autoclaved hydrogel. The hydrogel exhibits a very high swelling ratio of more than 40% in the first 48h, and did not reach equilibrium after one week, whereas the cryogel showed much lower swelling (less than 3%), which starts to decrease after approximately 4 days (Figure 6.1). Cryogels showed good antimicrobial activity, as well as excellent biocompatibility, though recorded reduced mechanical strength.

The process of adhesion of synthetic materials to skin represents a very fascinating mechanism, of which we tried to get a better understanding of. We assessed the level of adhesion of the three dressings to skin using the Texture Analyser in bioadhesive test mode and porcine skin as a skin model (Table 6.1).<sup>[10, 11]</sup>

Table 6.1 Adhesion to intact skin test results for hydrogel, SPH and cryogel. Data are shown as mean  $\pm$  standard deviation. Statistical significance ( $p < 0.05$ ) was calculated for the data related to force of detachment, work of adhesion and cohesiveness (a-f). Letters after each value indicate which other material the sample is significantly different from.

	<b>Force of detachment (N)</b>	<b>Work of adhesion (N*mm)</b>	<b>Cohesiveness (mm)</b>
Hydrogel <sup>a</sup>	0.452 $\pm$ 0.140 <sup>b,c</sup>	1.117 $\pm$ 0.320 <sup>b,c</sup>	6 $\pm$ 2 <sup>b,c</sup>
SPH <sup>b</sup>	0.006 $\pm$ 0.002 <sup>a</sup>	0.0003 $\pm$ 0.0004 <sup>a</sup>	0.4 $\pm$ 0.2 <sup>a</sup>
Cryogel <sup>c</sup>	0.075 $\pm$ 0.030 <sup>a</sup>	0.052 $\pm$ 0.020 <sup>a</sup>	1.3 $\pm$ 0.2 <sup>a</sup>

The hydrogel samples were shown to be the most adhesive to intact porcine skin requiring 0.452  $\pm$  0.140 N to be completely detached whereas the SPH can be easily removed from the skin specimen (a force of only 0.006  $\pm$  0.002 N was required). This difference is probably due to the irregular surface of SPHs resulting from macroscopic pores evenly distributed in the matrix, giving a characteristic

sponge-like consistency. For these materials, the area in contact with the skin is reduced, and therefore fewer interactions with the skin surface are taking place lowering the adhesion between the two surfaces. In comparison cryogels have shown to be more adhesive than SPHs thanks to the smoothness of their outer layer. However, in contrast to the hydrogels the cryogels have a higher cross-linking degree, with less -OH groups available for hydrogen bond formation with the functional group present on the stratum corneum of the skin.

Each wound present a different challenge, and needs to be treated differently depending on the type, stage of the injury and the condition of a patient. Therefore, the choice of product needs to be made very carefully. The same materials could in fact result in fast and easy healing when applied to a certain wound, or conversely delay or inflict further trauma and so must be evaluated.

Our first aim was to the design novel techniques for the production of viable wound management materials. As a result, the methods we developed open new perspectives in the field, and can be used to prepare a much broader range of hydrogels and SPHs for biomedical applications, starting from different hydrophilic polymers. Secondly, we succeeded in the manufacturing of advanced prototype dressings, which were shown to be very promising with importantly, low cost.

However, our study could not cover all the aspects that must be considered when moving a product further towards its approval and final commercialization. For instance, more work is needed to determine the limitations of the intrinsic antimicrobial activity exhibited by the dressings against *Staphylococcus aureus*, to consider whether the addition of an antimicrobial agent, such as polyhexamethylene biguanide (PHMB) to the formulations ( of which no resistance has been reported yet)<sup>[12]</sup>, would be advantageous. It would also be interesting to carry out more experiments on the adhesion these materials to skin to fully understand this process, and to be able to modify and adjust their adhesiveness properties accordingly to the skin/wound type. It would be worthwhile investigating the *in vivo* biocompatibility of the materials produced using a mouse or rabbit model as



commonly found in the literature,<sup>[13-16]</sup> or pigs, which have shown similarities to human skin.<sup>[11]</sup> Finally, testing their efficacy on different types of wounds or burns would allow the establishment of their best indication for use.

Other considerations for the later stages of product development would be their combination with other materials such as cotton, gauze or fabrics, as well as the packaging to make a complete product.

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