Novel Nanocomposite Biomaterials to Control Cell Adhesion

Constança Caldeira Rodrigues Júnior

Thesis to obtain the Master of Science Degree in

Biomedical Engineering

Supervisor(s): Prof. Dr. Marga Cornelia Lensen
Prof. Frederico Castelo Alves Ferreira

Examination Committee
Chairperson: Prof. José Paulo Sequeira Farinha
Supervisor: Prof. Frederico Castelo Alves Ferreira
Member of the Committee: Dr. Carla Sofia Monteiro de Moura

June 2017
“It's no use going back to yesterday, because I was a different person then.”

*Lewis Carroll*
Acknowledgments

Firstly, I would like to thank professor Marga Lensen, for giving me the opportunity to join her research group at the Lensen Lab, TU Berlin, and guiding me through the work developed over the last months, and to professor Frederico Ferreira, for accepting to overview the project from IST. A special thank you has to be addressed to Çügdem Yeşildag, for all the guidance provided since the beginning, for the patience, availability and will to help and guide me these past months. Thanks to everyone at the Lensen Lab, for the support and companionship.

To my friends, who have taken their time to help, motivate and come along with me through these past years. Thank you for not hating me for always being late to our meetings, for your friendship and for your constant presence.

To Philipp, Kenn and Firas, for making my stay in Berlin as easy and wonderful as it could possibly be, for the late talks and walks to the supermarket, thank you.

Last but not least, a very big thank you to my family for the support along the way, especially to my mother, who never gave up on me. Thank you.
**Resumo**

Hidrogéis são materiais que possuem diversas propriedades que têm despertado um grande interesse na comunidade científica devido ao seu potencial para serem usados em várias aplicações biomédicas, como encapsulamento de medicamentos e células, ou matriz para aplicações em engenharia de tecidos. Estes materiais possuem a capacidade de absorver até mil vezes o seu peso em água, que juntamente com a sua estrutura porosa tridimensional, confere-lhes a capacidade de simular o microambiente a que as células estão naturalmente expostas. Algumas destas propriedades são facilmente ajustáveis, permitindo assim a manipulação de determinadas características. O poli(etileno glicol) (PEG) é um hidrogel intrinsecamente não imunogênico, não adesivo, que tem sido amplamente utilizado como biomaterial. Ao submetê-los a modificações químicas e físicas, é possível então conferir a estes materiais novas propriedades que permitem ampliar o seu espectro de aplicações em engenharia de tecidos e medicina regenerativa. Uma dessas modificações pode ser a adição de diferentes tipos de nanopartículas ao seu volume.

Neste trabalho são reportadas diversas abordagens que visam a combinação de nanopartículas esféricas de ouro e nanopartículas magnéticas de ouro com hidrogéis baseados em PEG. Estes nanocompósitos têm como propósito controlar a adesão e proliferação de fibroblastos à sua superfície. A caracterização topográfica e mecânica dos materiais mostrou que a presença de nanopartículas no volume do hidrogel não melhora a sua rigidez, mas influencia a rugosidade da sua superfície e o seu comportamento em água. Houve pouca adesão celular aos nanocompósitos, devido à baixa exposição de nanopartículas de ouro à superfície dos filmes.

**Palavras-chave:** hidrogel, PEG, nanocompósito, nanopartículas de ouro, adesão celular
Abstract

The diverse properties that hydrogels possess have been arousing great interest for many years due to their several biomedical applications, such as drug and cell carriers or as tissue engineering matrices. They possess the capacity of retaining up to a thousand times their dry weight in water, which added to their three-dimensional porous structure, confers them the ability to mimic native tissue microenvironment. Some of these properties are easily tunable, which makes it possible for researchers to manipulate certain characteristics. Poly(ethylene glycol) (PEG) is an intrinsically non-immunogenic, non-adhesive hydrogel, that has been widely used as a biomaterial. By undergoing chemical and physical modifications, it is possible to confer PEG-based hydrogels new properties that allow to widen their spectrum of applications in tissue engineering and regenerative medicine. One of those modifications can be the addition of different types of nanoparticles.

In this work, approaches to combine spherical gold nanoparticles and gold magnetic nanoparticles with PEG-based hydrogels are reported. These nanocomposites aim to control the adhesion, proliferation and viability of mouse fibroblasts by allowing cells to interact with the gold of the nanoparticles. The topographical and mechanical characterization of the materials showed that the presence of nanoparticles in the volume of the hydrogel does not improve its stiffness, but influences the roughness of its surface and swelling behaviour. Cells showed little adhesion to the nanocomposites, due to the lack of ability to expose the nanoparticles to the surface of the films.

Keywords: hydrogel, PEG, nanocomposite, gold nanoparticles, cell adhesion
# Contents

Acknowledgments ................................................................. v  
Resumo .................................................................................. vii  
Abstract ................................................................................ ix  
List of Tables .......................................................................... xiii  
List of Figures .......................................................................... xv  
Nomenclature ........................................................................... xvii  

1 Introduction  
1.1 Thesis Outline .............................................................. 1  
1.2 Motivation ......................................................................... 2  
1.3 State of the art ................................................................. 2  
1.4 Objectives .......................................................................... 5  

2 Theoretical Background  
2.1 Hydrogels ........................................................................... 7  
2.2 Gold Nanoparticles .......................................................... 10  
2.3 Nanocomposite Hydrogels ................................................ 12  
  2.3.1 Nanocomposite Hydrogels from Carbon-Based Nanomaterials .... 12  
  2.3.2 Nanocomposite Hydrogels from Polymeric Nanoparticles ......... 12  
  2.3.3 Nanocomposite Hydrogels from Inorganic Nanoparticles .......... 13  
  2.3.4 Nanocomposite Hydrogels from Metal and Metal-Oxide Nanoparticles .......... 13  
2.4 Cell Adhesion Mechanisms ................................................ 13  

3 Materials and Methods  
3.1 Poly(ethylene glycol) (PEG) ............................................. 17  
  3.1.1 UV-curing of PEG-based polymers .................................. 19  
  3.1.2 Acrylation of PEG-based Polymers .................................. 20  
3.2 Gold Nanoparticles Synthesis and Seeded Growth .................. 20  
  3.2.1 Synthesis of Au NPs Seeds .......................................... 20  
  3.2.2 Seeded Growth of Au NPs .......................................... 20  
3.3 Gold Magnetic Nanoparticles ............................................. 21  
3.4 Hydrogel Films Preparation .............................................. 22
3.4.1 PEG_{575} Hydrogel Film ........................................... 22
3.4.2 8PEG Hydrogel Film .............................................. 22
3.4.3 8PEG-PEG_{575} Blend Hydrogel Film ........................... 23
3.5 Nanocomposite Hydrogel Films Preparation .......................... 23
  3.5.1 PEG_{575} -based Nanocomposite Hydrogel ......................... 23
  3.5.2 8PEG-based Nanocomposite Hydrogel .............................. 23
  3.5.3 8PEG-PEG_{575} Blend-based Nanocomposite Hydrogel ............ 23
3.6 Cell Culture .................................................................... 24
3.7 Analytical Methods .......................................................... 24
  3.7.1 Swelling Experiments .................................................. 24
  3.7.2 Atomic Force Microscopy ............................................ 25
  3.7.3 Transmission Electron Microscopy ................................. 28
  3.7.4 Scanning Electron Microscopy ...................................... 29

4 Results ........................................................................... 33
  4.1 Gold Nanoparticles Synthesis ........................................... 33
  4.2 Surface Topography ........................................................ 34
    4.2.1 Gold Nanoparticles Nanocomposite Hydrogel Films ............ 35
    4.2.2 Gold Magnetic Nanoparticles Nanocomposite Hydrogel Films 37
  4.3 Swelling Behaviour .......................................................... 41
  4.4 Force Mapping ............................................................... 42
  4.5 Cell Adhesion to the Nanocomposite Hydrogel Films ............. 44

5 Conclusions .................................................................... 47
  5.1 Future Work ................................................................. 48

References ........................................................................ 51
List of Tables

4.1 Percentage of swelling ($\% S$), swelling ratio ($Q_M$), volume swelling ratio ($Q_V$) and mesh size ($\xi$) from blank, AuNP NC and AuMNP NC hydrogel samples ................................. 42
4.2 Young’s Modulus ($E$) and respective standard deviation ($SD$) from hydrogel samples in dry state ........................................................................................................... 43
4.3 Young’s Modulus ($E$) and respective standard deviation ($SD$) from hydrogel samples in swollen state ........................................................................................................... 43
List of Figures

1.1 Engineering of hydrogel for biomedical applications ................................................. 4
1.2 Number of publications related to hydrogels and hydrogels according to ISI Web of Science 4

2.1 Chemical structures for some common hydrogels ....................................................... 8
2.2 Schematic of methods for formation of hydrogels by chemical modification of hydrophobic polymers .................................................................................................................. 9
2.3 Example of different types of gold nanoparticles ......................................................... 11
2.4 Schematic of cell adhesion assembly ............................................................................. 15

3.1 Schematic structures of PEG hydrogels formed via different crosslinking reaction mecha-
nisms ................................................................................................................................. 18
3.2 Schematic representation of the PEG polymers used ..................................................... 18
3.3 Schematic representation of the radical reaction of polymerization .............................. 19
3.4 Gold magnetic nanoparticles Electron microscopy and optical characterization as pro-
vided by the product datasheet. A-SEM image, B-UV-Vis absorbance spectrum ............... 21
3.5 UV-Vis spectrum of the NiTmagold Cit particles measured at the Lensen Lab ............. 22
3.6 Schematic overview of hydrogel films preparation. ...................................................... 24
3.7 Schematic representation of the operating basis (a) and imaging modes (b) of the AFM. Image adapted from [62] ................................................................. 26
3.8 Typical interaction for an uncoated hydrophilic cantilever in air approaching a hard incom-
pressible hydrophilic surface. ......................................................................................... 27
3.9 Schematic layout of a TEM operated in diffraction contrast bright field imaging ........... 29
3.10 Schematic diagram of a scanning electron microscope ................................................. 30

4.1 TEM image of a sample of the 6.G4 batch of the synthesized AuNPs (a) and size distri-
bution of the particles present (b). ................................................................................. 33
4.2 UV-Vis spectrum of 6.G4 batch of AuNPs ................................................................. 34
4.3 Height image (top) and respective cross-section (bottom) of the lower surface of blank hydrogel films: A - PEG\textsubscript{575}, B - 8PEG, C - Blend ................................................ 35
4.4 Height image and respective cross-section of the top surface (A) and lower surface (B) of PEG\textsubscript{575} AuNP nanocomposite film ......................................................... 36
4.5 Height image and respective cross-section of the top surface (A) and lower surface (B) of 8PEG AuNP nanocomposite film ................................................. 36
4.6 Height image and respective cross-section of the top surface (A) and lower surface (B) of Blend AuNP nanocomposite film ................................................. 37
4.7 Height image and respective cross-section of the top surface (A) and lower surface (B) of PEG 575 AuMNP nanocomposite film ................................................. 38
4.8 Phase image of the lower surface of PEG 575 AuMNP nanocomposite film ............... 38
4.9 SEM Images of the PEG 575 AuMNP NC film from SE (a) and BSE (b) .................. 39
4.10 Height image and respective cross-section of the top surface (A) and lower surface (B) of 8PEG AuMNP nanocomposite film ................................................. 40
4.11 Phase image of the lower surface of 8PEG AuMNP nanocomposite film ................. 40
4.12 Height image and respective cross-section of the top surface (A) and lower surface (B) of Blend AuMNP nanocomposite film ................................................. 41
4.13 Force mapping of 8PEG AuMNP NC in swollen state diagram (a) and E distribution histogram (b) ................................................................. 44
4.14 Control cells cultured in 3 different culture plates (A, B and C) ............................ 44
4.15 L929 cells cultured in PEG 575 (A), 8PEG (B) and Blend (C) blank hydrogel samples . 45
4.16 L929 cells cultured in PEG 575 (A), 8PEG (B) and Blend (C) AuNP hydrogel samples 45
4.17 L929 cells cultured in PEG 575 (A), 8PEG (B) and Blend (C) AuMNP hydrogel samples 46
# Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>8PEG</td>
<td>8-arm Poly(ethylene)glycol</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic-force microscope</td>
</tr>
<tr>
<td>AuMNP</td>
<td>Gold Magnetic Nanoparticles</td>
</tr>
<tr>
<td>AuNP</td>
<td>Gold Nanoparticles</td>
</tr>
<tr>
<td>BSE</td>
<td>Backscattered Electrons</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane anhydrous</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
</tr>
<tr>
<td>FA</td>
<td>Focal Adhesions</td>
</tr>
<tr>
<td>NC</td>
<td>Nanocomposite</td>
</tr>
<tr>
<td>PEGDA</td>
<td>PEG Diacrylate</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene)glycol</td>
</tr>
<tr>
<td>PI</td>
<td>Photo-initiator</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean square</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SE</td>
<td>Secondary Electrons</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

This chapter starts with a brief outline of the scope of this thesis, followed an introduction on the topic of biomaterials and their applications. It then continues with a short explanation on the motivation underlying this work, followed by the state of the art on the use of hydrogels as biomaterials and the tuning of their properties in order to create patterns that set up ideal conditions for cell culture. A summary of the objectives aimed to be accomplished during the development of this work is then presented.

1.1 Thesis Outline

This thesis contains five main chapters. The first chapter introduces the work and the motivation underlying the development of the thesis. It gives a review of the state of the art and advances done in the area of hydrogels throughout the most recent years. Furthermore, it exposes the goals and objectives that were proposed in the beginning of the work that was carried out throughout the thesis.

The second chapter presents a brief overview about the theoretical background supporting the content of this thesis, and gives an insight on the reasons why the project was outlined this way. It starts with a general introduction on the main materials used in this thesis and their characteristics, namely hydrogels and gold nanoparticles. It is then followed by an overview on the types of hydrogels and their different characteristics depending on the materials chosen for their design. Furthermore, a short explanation on the mechanisms underlying cell adhesion phenomena is done, and the possibility of them being triggered by the presence of gold nanoparticles.

The third chapter consists on the description of the characteristics of PEG materials and their polymerization processes, as well as the acrylation process they had to undergo. The synthesis of the gold nanoparticles is also explained. Moreover, the techniques used to characterize the materials are exposed, as well as the methodology adopted to prepare the films.

The results from the experiments performed are presented in chapter four. The topographical and mechanical analysis of the films is presented, as well as its swelling behaviour and its performance upon cell culture. It is done an overview on what was the expected outcome, the results reached and their interpretation. It provides a deeper understanding on the characteristics that the materials expressed
and how they affected the adhesion of cells to their surface.

Chapter five concludes the work, providing a summary of the achievements accomplished and the main difficulties encountered throughout the project, and it finalizes with a short point of view on the different possible directions that may be followed up towards which this work may be continued.

1.2 Motivation

Generically, a biomaterial can be defined as a “material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” [1]. Biomaterials possess the ability to be integrated in a biological system without interacting negatively with it, such as inducing immunological responses, and therefore, they require to have certain mechanical, chemical and biological properties depending on their final application. These applications can range from drug delivery systems to scaffolds for tissue engineering [2]. Thus, when choosing or designing a biomaterial, it is important to take into consideration the function that is expected of the biomaterial and its biocompatibility, which may come as a challenge due to the complexity that is intrinsic to the human biological systems. The application of biomaterials in the field of tissue engineering is especially compelling, since it aims to regenerate damaged tissue by developing a way to maintain, restore or improve the biological function of the tissue, instead of replacing it [3].

Particularly, hydrogels have been of great interest to biomaterial scientists for many years, due to their hydrophilic character, chemical stability and ability to be biocompatible [4]. The use of poly(ethylene glycol) (PEG) as a biomaterial is especially interesting due to its ease to be manipulated. For several years, it has been vastly explored for pharmaceutical applications, and more recently researched about its ability to serve as a matrix for cell culture [5]. However, PEG itself is non-adhesive, which makes it necessary to adopt strategies that allow to provide them features that enable their successful application to control cell adhesion, migration, growth and to investigate fundamental cellular behaviour. Some of these strategies are based on the creation of topographical, chemical and elastic profiles [6, 7]. The incorporation of gold nanoparticles (AuNPs) in hydrogels has shown to be a promising way of creating materials that induce physiological responses in biological tissues [8]. As technology develops, more ways to engineer and confer hydrogels new characteristics arise and present themselves as a big contribution to the development of regenerative therapies.

Having taken into account the advantages these materials present, the main motivation underlying this thesis is to develop PEG based biomaterials micropatterned with AuNPs and AuNMPs in order to control cell adhesion. Moreover, this work proposes a possible solution to integrate these nanoparticles in the matrix of the hydrogels conferring them new characteristics.

1.3 State of the art

The challenge of mimicking or stimulating tissue functions has been addressed by researchers for several years, through the engineering of the physical, chemical and biological properties of biomaterials...
terials. These biomaterials aim to replicate the intrinsic complex nature of tissue and its surrounding environment, thus being a great promise in treating failures resulting from aging, injuries or diseases [9].

Among different types of biomaterials, hydrogels have shown a big potential in several different applications due to their unique characteristics which can be easily tuned in order to provide them certain properties that were otherwise not present. In addition, the development of several micro- and nanofabrication techniques allowed the addition of smaller and more precise features, which allowed to mimic the microenvironment to which cells are naturally exposed more accurately [10]. Furthermore, the advances made in polymer chemistry and biomolecular engineering over the last decade have widened the spectrum of applications of hydrogels as biomaterials, by allowing the engineering of more specific features that provide bigger similarities with the natural tissue, thus being an important step on the application of hydrogels as cell and drug carriers and as tissue engineering matrices.

In 1960 a publication by Wichterle and Lim reported the synthesis of hydrogels for biological use [11]. Since then, scientists have taken an increasing interest in exploring the potential of using hydrogels as biomaterials, initially focusing in their application on cell encapsulation and more recently as matrices for repairing and regenerating biological tissue [12, 2].

Although hydrogels present several advantages in their use for biomedical applications in comparison to other materials, they also possess some disadvantages. The addition of nanoparticles to the networks have shown big promises in surpassing these obstacles, by conferring them novel properties that were otherwise not possible to achieve. The diversity of particles, polymers and techniques available to engineer novel nanocomposite (NC) hydrogels has given these materials a whole set of applications in the biomedical field, gaining more visibility in the field (Figure 1.1). Moreover, AuNPs have shown to possess characteristics that are inherently interesting in the way they affect biological tissues, such as the interaction that they have with cells and proteins. An example is the phenomenon of protein adsorption to their surface, which can trigger a set of different physiological changes [13]. By incorporating them into hydrogels, AuNPs allow to add new characteristics that were otherwise not be present in the plain hydrogel. One of the earliest publications about nancomposite hydrogels was from the Willner group, where AuNPs were successfully incorporated in polyacrylamide (PAAm) [14]. Figure 1.2 gives an overview on the increase of publications related to hydrogels and hydrogel NCs over the years.

Despite its use dating back from the Roman times, it was only later that scientists started exploring the full potential of colloidal gold. Faraday was the first to observe that colloidal gold solutions had different properties than bulk gold, and since then, various methods to synthesize AuNPs with different sizes and shapes were developed, as well as surface modification methods and potential applications in biology and medicine were discovered [15]. Techniques to synthesize size-controlled monodispersed particles in an efficient way have thus been optimized for several years, being the method of Frens[16] and Turkevich[17] some of the most relevant and commonly known.

The present work follows the trend on optimizing techniques to conjugate the properties of AuNPs with PEG based hydrogels and develop biomaterials that aim to promote cell adhesion, with possible further applications in tissue engineering and regenerative medicine.
Figure 1.1: Engineering of hydrogels for biomedical applications. [13]

Figure 1.2: Number of publications related to (a) hydrogels and (b) hydrogels according to ISI Web of Science (data obtained November 2013). [13]
1.4 Objectives

Throughout this work, the main goal is to develop efficient strategies to synthesize novel NC hydrogel biomaterials which possess characteristics that induce and promote cell adhesion to inherently non-adhesive precursor hydrogels. By patterning the surface of these materials with AuNPs, which have shown to interact with adhesive cells and potentially promote their spread and migration, it is expected that the NCs formed will promote mouse fibroblasts L929 adhesion. Objectively, the goal is to expose the nanoparticles to the surface in order to allow direct contact between the gold and the cells and promote maximum interaction.

Proper characterization methodologies, such as topographical measurements and force mapping using the atomic force microscope are also intended to be performed. Furthermore, cell culture will be performed on the final samples in order to assess their effect on cell adhesion.
Chapter 2

Theoretical Background

Throughout this chapter, an insight about the theoretical background underlying this thesis is made. It is introduced the concept of soft lithographic methods, as well as the materials used, with an emphasis on hydrogels and their biomedical applications. The concept of nanocomposite is explored, as well as the use of gold nanoparticles as part of the composite materials and their effect on cell interactions. Furthermore, the mechanisms behind cell adhesion phenomena and how they can be controlled are briefly explained.

2.1 Hydrogels

Hydrogels are a group of polymeric materials, that due to their great hydrophilicity, have the ability to hold large amounts of water within their three-dimensional structures. They have the capacity of being highly biocompatible, which added to their easily tunable characteristics, allows the design and development of materials with tailored properties, such as biodegradation, mechanical stability, and ability to respond to stimuli [18]. Hydrogels can be synthetic, natural, or result from synthetic/natural polymer hybridization (Figure 2.1). [19]. There are four major types of natural polymers used to synthesize hydrogels for biomedical applications. These include proteins, such as collagen, gelatin and fibrin, polysaccharides, such as hyaluronic acid (HA), agarose and dextran, protein/polysaccharide hybrids, such as collagen/HA, laminin/cellulose and fibrin/alginate, and DNA. However, hydrogels with natural origins present some concerns when used as biological scaffolds due to the higher risk of triggering immunogenic reactions when compared to their synthetic peers. Synthetic hydrogels, on the other hand, are easy to tailor. Features such as biodegradability and the incorporation of certain structures and functionalities are easily reproducible, which are important characteristics for a material to possess [3].

Regarding their use as biomaterials, hydrogels can be categorized into three main groups: non-biodegradable, biodegradable and bioactive. Nonbiodegradable hydrogels, as the name indicates, are hydrogels that are able to keep their mechanical integrity over time. The biggest challenge to overcome when designing nonbiodegradable hydrogels is being able to create a material that is both mechanically strong and flexible. Most nonbiodegradable hydrogels result from the copolimerization of vinylated
Figure 2.1: Chemical structures for some common hydrogels: a-poly(ethylene glycol), also poly(ethylene oxide) (PEG/PEO); b-polyHEMA; c-poly(vinyl alcohol) (PVA); d-poly(acrylamide) (PAAm); e-an amino acid, the repeating unit of a protein; f-a disaccharide, the repeating unit of a polysaccharide. [20]
monomers or macromers, such as methoxyl poly(ethylene glycol) (PEG) monoacrylate (mPEGMA or PEGMA), acrylamide (AAm), acrylic acid (AAC), N-isopropylacrylamide (NIPAm), 2-hydroxyethyl methacrylate (HEMA), 2-hydroxypropyl methacrylate (HPMA), with crosslinkers, such as N,N’-methylenebis (acrylamide) (MBA) and ethylene glycol diacrylate (EGDA) [19]. It is also possible to create nonbiodegradable hydrogels by copolymerizing acrylated macromonomers, as is the example of using PEG-Diacrylate (PEGDA) and star-shaped macromonomers [21]. Regarding biodegradable synthetic hydrogels, polyesters are the most widely used polymers for scaffold fabrication, including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL) and their copolymers. Bioactive hydrogels are gels that are capable of mediating specific cell functions. By attaching certain bioactive elements to the polymeric network, it is possible to create materials that are able to more accurately mimic the environment in which the biological tissues naturally occur [2]. Hydrogels can also be categorized according to the nature of their cross-linking reaction as permanent or physical hydrogels. Permanent or chemical hydrogels have covalently-crosslinked permanent junctions, while physical hydrogels possess networks that result from either physical interactions, such as hydrogen bonds, ionic interactions, hydrophobic interactions, or from polymer chain entanglements (Figure 2.2) [22].

Thus, it is possible to generalize a hydrogel as being simply a hydrophilic polymeric network cross-linked in order to create an elastic structure. There are three general components that take part on hydrogel preparation: monomer, initiator and cross-linker; and various polymerization techniques can be used, such as bulk, by irradiation, solution and suspension polymerization [18].

When talking about cell culture and the application of hydrogels as matrices or scaffolds, it is important to address its water absorption capacity as its ability to permeate the nutrients into and cellular products out of the gel.

When inserted in an aqueous medium, the hydrogel will immediately start to absorb water within its matrix. The most hydrophilic groups will be the first to be hydrated, leading to the swelling of the network.
and exposing the hydrophobic groups which also interact with the water molecules. After this initial water intake, and after the hydrophobic and hydrophilic groups become saturated with water, also called total bound water, the network will imbibe additional water until it reaches an equilibrium swelling level. The additional imbibed water is called 'free water' or 'bulk water', and is assumed to fill in the spaces between network chains or larger pores. This happens due to the osmotic driving force of the network chains towards infinite dilution, and is opposed by the covalent or physical crosslinks, that culminates in an elastic network retraction force [2]. The amount of water imbibed within a hydrogel influences the diffusive properties of a solute through it. Thus, determining the amount of water imbibed is important to characterize the hydrogel, and is often represented in terms of percentage swelling ($%S$). The $%S$ can be defined by the following equation:

$$%S = \frac{W_S - W_d}{W_d} \times 100$$ (2.1)

where $W_S$ is the weight of the swollen gel and $W_d$ is the weight of the dry gel, which can be determined experimentally. The $%S$ of a hydrogel is directly proportional to the amount of water imbibed within it. So, a higher $%S$ signifies a higher amount of water imbibed and consequently, a higher diffusion rate of the solute. However, other factors may also have an important impact on the diffusive properties of the hydrogel, such as the micro-architecture of the polymer chain [22].

The mechanical properties of the hydrogel depend on several factors, such as the monomers used, the polymerization conditions, the crosslinking density, the degree of swelling, and the type of medium in which the material is swollen. The size of the polymer molecules of the hydrogel leads to a viscoelastic response. Hydrogels are not simply elastic materials, but behave viscoelastically [23].

### 2.2 Gold Nanoparticles

Gold nanoparticles (AuNPs) are materials that have several applications in various fields due to their physical and chemical characteristics. They possess unique optical properties which allow their application in techniques such as surface enhanced Raman scattering (SERS), surface plasmon resonance (SPR), and two-photon luminescence (TPL). They are also relatively simple to synthesize, and various methods allow to control their shape and size. Additionally, it is easy to modify their surface chemistry, making it possible to synthesize AuNPs with certain characteristics according to their application. Thus, AuNPs are widely used in biomedical and biological fields with different purposes, such as imaging, drug and gene delivery, targeted therapies and biosensors, among others [24].

When talking about biological applications of AuNPs, their shape, size and surface chemistry play an important role in determining their physiological behaviours and the way they will interact with proteins and cells. AuNPs can be manufactured into a variety of shapes, such as gold nanospheres, nanorods, nanobelts, nanocages, nanoprisms, and nanostar, as can be seen in Figure 2.3.

When proteins and other biomolecules are put in contact with AuNPs, they tend to be adsorbed to the surface of the nanoparticles and form a protein "corona" around them, which results in a reduction of
their surface free energy. This fact may induce different cellular responses which can be used to mediate cell adhesion, migration, differentiation and proliferation [26]. It is therefore important to optimize the technique used in the synthesis of these nanoparticles in order to obtain monodispersed particles with the desired size.

There are several factors that affect the interaction of AuNPs with proteins and cells. The modification of the surface chemistry of Au NPs with electrolytes such as citrate, hexadecyl trimethyl ammonium bromide (CTAB), sodium polystyrene sulfonate (PSS), among others, produces electrostatic attraction to the oppositely charged functional groups in proteins [27]. The size of the particles influences the amount of adsorbed protein on the surface due to the fact that different curvatures of the nanoparticles result in different protein binding constants. Smaller AuNPs have lower protein adsorption because of their larger curvature, which reduces the protein binding capacity [28, 29]. Hydrophobicity and hydrophilicity also influence the amount and composition of proteins adsorbed. For example, by modifying the surface of AuNPs with high densities of hydrophilic polyethylene glycols (PEGs), the nanoparticles acquire a high capacity to prevent the adsorption of plasma proteins [30].

There are two main approaches one can follow in order to prepare AuNPs, either by a top-down (physical) or bottom-up (chemical) approach. But most methodologies followed to produce AuNPs are still based in the reduction of chloroauric acid ($\text{H}[\text{AuCl}_4]$) in a liquid. In a generic overview, the process starts with the dissolution ($\text{H}[\text{AuCl}_4]$), followed by its fast stirring as a reducing agent is added. This causes the reduction of $\text{Au}^{3+}$ ions to neutral gold atoms. As the reaction continues, the number of particles formed increases until the solution reaches a point of supersaturation. At this stage, gold gradually starts precipitating in the form of sub-nanometer particles, and the remaining gold atoms that are formed attach to the existing particles. The size and shape of the AuNPs can be controlled through the type and concentration of the reduction agent used. To prevent them from aggregating, a stabilizing agent is added to the solution, which can be also used to functionalize the surface of the particles by adding, for example, organic ligands, originating particles with advanced functionalities [31].
2.3 Nanocomposite Hydrogels

In the field of biomaterials, a composite can be defined as a combination of two or more heterogeneous materials, in which the phases of the combined materials differ in form and composition, and are able to retain their properties and identities. Composites thus possess certain specific characteristics which are not present in any of the original phases alone. When one of the integrated components of a composite is on the nanoscale size spectrum, it can be called a nanocomposite (NC) [32].

Hydrogels, despite having easily tunable properties which make them suitable for various biomedical applications, have some constraints concerning their mechanical and physical properties to be used as tissue engineering scaffolds [3]. Several hydrogel preparation processes that aim to increase the stiffness of its networks have been developed, such as double network hydrogels, supramolecular hydrogels, microsphere cross-linked hydrogels and hybrid physically-chemically crosslinked hydrogels, presenting still, however, numerous limitations [33, 34]. Thus, the incorporation of nanoparticles in the hydrogel matrix has arisen as a possible solution to overcome the mechanical limitations previously encountered. Also, it provides added potential use in biomedical applications for presenting superior chemical, physical, and biological properties, owing it to the characteristics that are inherent to the added material and the interactions between the polymeric networks and the nanoparticles [35].

In comparison to conventional hydrogels, NC hydrogels possess generally significant improvements. For example, some NC hydrogels are capable of withstanding higher levels of stress and achieve higher equilibrium swelling ratios and rapid de-swelling rates with temperature changes. Furthermore, they are more prone to be stimulus-sensitive, which allows them to be responsive to certain environmental changes. This is especially useful in their application as drug delivery systems, where temperature changes may induce the release of components entrapped in the polymer network [36].

The materials composing the NC hydrogel can vary immensely, and the different possible combinations of matrix and nanoparticles influence the characteristics that are later observed.

2.3.1 Nanocomposite Hydrogels from Carbon-Based Nanomaterials

Carbon Nanotubes (CNTs) and graphene are materials that have recently emerged with numerous potential applications in various fields. Their incorporation in hydrogels provides them certain mechanical, electrical and optical properties, enhancing the potential of their use as conductive tapes, actuators, biosensors, tissue engineering scaffolds, among others. One of the biggest challenges about incorporating carbon-based materials in hydrogels is to functionalize their surfaces in order to make it possible for them to be embedded into the polymer matrix while maintaining an even dispersion [37, 38].

2.3.2 Nanocomposite Hydrogels from Polymeric Nanoparticles

Polymeric nanoparticles such as dendrimers, hyperbranched polymers, liposomes, polymeric micelles, nanogels, and core-shell polymeric particles have the ability to entrap hydrophobic or hydrophilic therapeutic agents. Particularly, dendrimers and hyperbranched polymeric nanoparticles show a higher
reactivity and loading efficiency when compared to polymeric hydrogels derived from linear polymers, due to the multitude of functional groups present at their periphery [39]. Moreover, bioactive agents such as drugs and proteins can be encapsulated within their branched structure, or conjugated on the periphery of the nanoparticles. Hydrogel networks reinforced with dendritic nanoparticles, via covalent or non-covalent interactions with the polymeric chains, can result in stiffer materials which may possess controlled bioactive agents release properties [40].

2.3.3 Nanocomposite Hydrogels from Inorganic Nanoparticles

Most of the inorganic nanoparticles used to synthesize NC hydrogels are minerals that are already present naturally in the human biological systems. Since they are necessary for the proper function of human tissues, NC hydrogels combined with these nanoparticles show favorable biological responsive behaviour. Thus, incorporating such nanoparticles within polymeric hydrogels is expected to introduce bioactive characteristics to the network. Some examples of particles used are hydroxyapatite (nHA), synthetic silicate nanoparticles, bioactive glasses, silica, calcium phosphate, glass ceramic, and β-wollastonite [41, 42, 43].

2.3.4 Nanocomposite Hydrogels from Metal and Metal-Oxide Nanoparticles

Metal and metal-oxide nanoparticles may possess properties such as electrical conductivity, magnetic properties and antimicrobial properties. These include gold (Au), silver (Ag), iron oxide (Fe$_3$O$_4$, Fe$_2$O$_3$), Titania (TiO$_2$), among others. Hence the application of NC hydrogels with metal and metal-oxide particles is mostly used as imaging agents, drug delivery systems, conductive scaffolds, switchable electronics, actuators and sensors [14, 35]. Although the interactions between the polymer and the metal/metal-oxide particles are weak, by functionalizing their surface it is possible to promote cross-linking with the polymeric network, thus enhancing this interaction and increasing the physical properties of the NC [44]. However, merely incorporating these particles into the hydrogel networks does not significantly enhance the mechanical properties of the hydrogel, but they can also be used to control biological mechanisms, like cell adhesion and migration phenomena [8]. Due to their electrical and thermal conductivity, embedding metallic nanoparticles within the hydrogel allows the design of scaffolds with enhanced electrical conductivity. Such scaffolds are useful to culture cells that require the propagation of electrical signals, such as cardiomyocytes [45]. Magnetic nanoparticles, on the other hand, allow the design of materials that can interact with external magnetic fields, making it possible to obtain stimuli-responsive hydrogels that can be used as controlled drug release systems or as tissue engineering matrices [46, 47].

2.4 Cell Adhesion Mechanisms

Adhesion to their surroundings and to the extracellular matrix (ECM) is essential for cells to maintain their tissue structure and mechanical integrity. The ECM is a complex network of proteins and
polysaccharides secreted and assembled by cells which provides them with structural and biochemical support. Transmembrane cell-cell and cell-matrix adhesion molecules provide a direct connection between neighbouring cells or ECM proteins and the cytoskeleton. These molecular networks are essential to assure architectural characteristics to cells and tissues, such as structure, shape and mechanical strength. Moreover, they allow the control of the orientation and localization of subcellular organelles, cell polarity and signal transduction. Physical properties of the cell micro-environment, such as matrix rigidity, topography and geometry, modulate biochemical cues mediated by these molecular networks. Therefore, it is necessary to understand the detailed mechanisms via which molecular interactions allow cells to sense such physical properties in order to create biomaterials to be applied in tissue engineering and regenerative medicine.

The self-assembly of molecular complexes, structured at the nano and microscale, is the underlying basis of the formation of cell adhesions, whether to synthetic biomaterials or to the natural ECM.

Adhesion to the ECM, in most eukaryotic cells, occurs via transmembrane integrin heterodimers, together with the binding of other cell membrane receptors, such as members of the immunoglobulin superfamily, non-integrin collagen and laminin receptors, glycolipids, glycosaminoglycans and glycosylphosphatidylinositol - linked receptors. Once integrins adhere to ECM proteins, such as collagen, fibronectin and laminin, they are activated and clustered into nascent adhesions \[48\]. In the beginning of the process, small focal complexes with around 0.5-1 \(\mu\)m diameter are formed. They are localised at cell protrusions - lamellipodia, which result from the polymerisation of an actin gel. Some of these complexes develop into mature, elongated structures known as focal contacts or focal adhesions (FAs). Conjugated with actin, they sustain the assembly of filament bundles and related molecules, such as myosins \[49\]. A number of proteins are recruited at FAs during the initial stages of integrin clustering, linking integrins and the cytoskeleton. These can be distinguished by proteins that directly associate with integrins (talin, \(\alpha\)-actinin and filamin) and those that bind indirectly. Amongst these proteins, those that also directly bind to the cytoskeleton play an important role in the regulation of FA assembly, cell adhesion and migration. Generally, adhesion complexes life cycle consist in formation, elongation, stabilization and merging with other adhesions, followed by disassembly at the rear of migrating cells or upon local disassembly of the actin network (Figure 2.4).

The dynamics and organisation of the cytoskeleton are closely associated with focal adhesion mechanisms. After the initial integrin activation and the recruitment of proteins at the adhesion site, actin filaments associate with the nascent adhesion. This enables the transmission of forces that are necessary to sustain further membrane protrusion and adhesion maturation, during which actin filaments are also assembled into thicker stress fibres. This event affects cell structure and polarity.

Molecular processes that physically connect the ECM to the cell cytoskeleton regulate the adhesion to the matrix. This is possible through the formation of self-assembled protein complexes. Moreover, cell adhesion to the ECM has a significant role in regulating important cell phenotypes such as proliferation, apoptosis, differentiation, endocytosis, motility, matrix degradation and remodelling. The processes controlling cellular sensing of the physical microenvironment depend on sensing of its nanoscale physical properties, such as nanoscale geometry, nanotopography and nanoscale mechanics \[50\].
Figure 2.4: Schematic of cell adhesion assembly: A - Cell spreading is associated with the assembly of focal adhesions and depends on the actin cytoskeleton and the development of forces along the basal cell surface, B - A simple diagram to illustrate how cycles of cell–ECM attachment and detachment participate in cell motility. [50]
Chapter 3

Materials and Methods

In the following chapter, a further explanation on the methods chosen to process the materials and engineer the samples is done, namely the polymerization and acrylation of PEG, the AuNPs synthesis and the NC hydrogels preparation. A short explanation on the properties of the materials used is also made. Furthermore, the theoretical background of the techniques used to characterize the materials is done, as well as how the instrumentation used works.

3.1 Poly(ethylene glycol) (PEG)

Poly(ethylene glycol) or PEG is a linear neutral polyether, and its chemical structure representation is \( H-(O-\text{CH}_2-\text{CH}_2)_n-\text{OH} \). Because of its biocompatibility, non-toxicity and non-immunogenicity, this hydrophilic hydrogel is widely used in biomedical applications. PEGs can be formed by macromonomers with a wide range of molecular weights and chain lengths. It is also possible to functionalize the terminal sites of macromonomers with various reactive end-groups, expanding their applications.

There are several different gelation methods that can be used to form PEG gels, including physical, ionic or covalent interaction. Chemically or covalently-crosslinking leads to relatively stable hydrogel structures with tunable physicochemical properties such as permeability, molecular diffusivity, equilibrium water content, elasticity, modulus, and degradation rate. According to the crosslinking reaction mechanism, the synthesis of covalently crosslinked PEG gels can be categorized as chain-growth, step-growth, or mixed-mode chain and step growth (Figure 3.1).

Networks formed by chain-growth polymerization usually derive from functional PEG molecules, such as PEG-di(meth)acrylate. Reactive centers, such as radicals, initiate the polymerization. They can be generated from thermal energy, redox reactions, or the photocleavage of initiator molecules. They propagate through unsaturated vinyl or allyl bonds on the PEG macromolecular monomers, resulting in chain polymerization. Photopolymerization is one of the preferable chain polymerization methods to create hydrogels, because it is fast and avoids long exposures to thermal energy. Step-growth gelation is based on the conjunct reaction of at least two multifunctional monomers with mutually reactive chemical groups in either stoichiometric balanced or imbalanced ratio, with the average monomer functionality
being greater than 2 [52]. Michael-type addition reactions and "Click" chemistry reactions are examples of step-growth methods used to create hydrogels [53]. Finally, PEG hydrogel networks can also be formed by mixed-mode mechanisms, that combine chain and step-growth polymerizations.

In this work, PEG Diacylate (PEGDA) and 8-arm PEG were used (Figure 3.2). The PEGDA, referred to as PEG$_{575}$ was purchased from Sigma-Aldrich, with a molecular weight (Mw) of 575 Da, provided as a liquid pre-polymer. The 8-arm PEG-OH with a Mw of 15 KDa, referred to as 8PEG, was purchased from Jenkem technology USA and put through an acrylation process prior to its use.

Figure 3.1: Schematic structures of PEG hydrogels formed via: A-chain-growth, B-step-growth, and C-mixed-mode step and chain growth polymerization. [51]

Figure 3.2: Schematic representation of the structure of PEG polymers used. Image adapted from [54]
3.1.1 UV-curing of PEG-based polymers

By UV-curing PEG-based hydrogels, it is possible to obtain time and space controlled polymerization. Because the presence of a radical is needed to react with the acrylate groups present in the terminal ends of the chains, the use of a photo-initiator (PI) is necessary. Some photo-initiators used in UV-cured systems generate free radicals and some others generate cations. The former can be used for curing acrylates, as is the aim in this work, and the latter can be used for curing cycloaliphatic epoxide-based systems [55].

The radical reaction of polymerization can be described as follows (Figure 3.3): initially, a radical is created on the PI by UV-radiation (initiation). This radical reacts with the acrylate group present at the end of the PEG chains, generating another radical. The second radical reacts with a second PEG chain, binding them chemically (propagation). The reaction terminates when the radical ending of one chain recombines with another radical (termination).

![Figure 3.3: Schematic representation of the radical reaction of polymerization: a-initiation, b-reaction, c-propagation, d-termination. [54]](image-url)
### 3.1.2 Acrylation of PEG-based Polymers

The addition of acrylate groups to the terminal ends of the polymer chains allows to obtain UV-curable polymers. In this work, this acrylation process was necessary to be performed with 8PEG, since PEGDA was purchased already in the acrylated state. All reagents used were purchased from Sigma-Aldrich, unless stated otherwise. The acrylation procedure was done following the method described by Z. Zhang at the Lensen Lab group \[56\]. It starts with the 8PEG and the catalyst K$_2$CO$_3$ being dried separately in a vacuum oven at 95°C for 4 hours. A reflux column was set up on the reaction flask. In order to avoid the presence of humidity throughout the reaction, air was removed by bubbling through a nitrogen (N$_2$) flow, followed by the addition of the catalyst, dichloromethane anhydrous (DCM), and acryloyl chloride to the reaction flask. The reaction was carried out at 51°C and in absence of light for at least 3 days to ensure maximal conversion. The resulting products went through a filtration process in order to eliminate the remaining catalyst. The solvent was evaporated using a N$_2$ stream.

The filtered polymer was dropped into a beaker containing cold petroleum ether, resulting in its precipitation. The remaining acryloyl chloride was dissolved in petroleum ether. The precipitate was re-suspended in DCM and poured into a sedimentation funnel with a small amount of a saturated solution of NaCl in distilled water. The organic phase dried over MgSO$_4$. After overnight drying, the MgSO$_4$ was filtered and a small amount of 4-methoxyphenol was dissolved into the filtrate, which acted as an inhibitor and avoided undesired polymerization during storage. Finally, the flask was placed on a rotary evaporator Hei-VAP Value (Heidolph Instruments GmbH & Co. KG, Germany) and left until the solvent was removed. The acrylated 8PEG was stored in a flask, under 6°C and kept away from the light.

### 3.2 Gold Nanoparticles Synthesis and Seeded Growth

All chemicals used were purchased from Sigma Aldrich and used as received unless stated otherwise.

#### 3.2.1 Synthesis of Au NPs Seeds

The synthesis of the citrate capped AuNP seeds was done following the method described by Bastus et al.[57]. Initially, a 2.2 mM solution of trisodium citrate in deionized water (150 ml) was heated in a three-necked round bottom flask for 15 minutes. The solution was kept under vigorous stirring until achieving its boiling point. A condenser was used to prevent evaporation of the solvent. Afterwards, 1 mL of a solution containing 25 mM of H[AuCl$_4$] · 3H$_2$O (precursor solution) was added to the solution of trisodium citrate. This resulted in a pink mixture that was kept stirring under reflux for an additional 10 minutes.

#### 3.2.2 Seeded Growth of Au NPs

Right after the conclusion of the synthesis of AuNP seeds, the resulting product was cooled down
until it reached a temperature of 90 °C. Afterwards, 1 mL of the 25 mM H[AuCl₄] · 3H₂O solution was injected into the reaction. The mixture was kept under stirring for 30 min. This process was repeated two times. After the third addition of the precursor, the AuNPs solution was diluted by extracting 55 mL of the AuNPs solution and adding 53 mL of deionized water and 2 mL of a solution of 60 mM trisodium citrate. This solution was then used as the seed for the subsequent growing step, having the whole process been repeated again. The reaction temperature was maintained at 90 °C during the growing steps. In that way, depending on the number of growing steps, spherical Au NPs with diameters from 20 up to 200 nm were possible to achieve. For this work, particles with a diameter of 30-40 nm were used, referred to as batch 6.G4.

### 3.3 Gold Magnetic Nanoparticles

The gold magnetic nanoparticles (AuMNPs) used in this work were NITmagold Cit 50nm particles purchased from nitparticles, Zaragoza, Spain. The surface of the particles is coated with citrate anions, and the average particle diameter is 51.8 ± 6.1 nm. The solution had a molar concentration of 0.05 nM, with a particle concentration of 3.2×10¹⁰ particles/mL and a peak SPR wavelength at 536 nm. The optical characterization and SEM image can be found in Figure 3.4 and were provided in the product datasheet.

![SEM image of AuMNPs](image)

**Figure 3.4:** Gold magnetic nanoparticles Electron microscopy and optical characterization as provided by the product datasheet. A-SEM image, B-UV-Vis absorbance spectrum

In order to confirm the viability of the purchased particles, the UV-Vis spectrum was measured again (Figure 3.5) at the Lensen Lab, showing an SPR wavelength at 538 nm, which is 2 nm higher than the one provided from the product datasheet. This small difference does not affect the results regarding the molar concentrations provided.
3.4 Hydrogel Films Preparation

A photo-initiator (PI) 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone, $M_W$ 224.26 $\text{g mol}^{-1}$, purchased from Sigma-Aldrich, was added to all PEG-based polymers. The concentration of PI is expressed in weight percentage with respect to the quantity of polymer. Figure 3.6 gives a general overview of the preparation of the blank hydrogel films.

3.4.1 PEG$_{575}$ Hydrogel Film

1 wt% of PI was added to a flask, together with 0.2 g of PEG$_{575}$. The flask was put under sonication for 10 minutes, to promote the dissolution of the PI in the hydrogel. Few drops were then put on a microscope glass and covered with a thin glass. The mixture was put to cure under UV light 366 nm, under a $N_2$ atmosphere in absence of $O_2$ for 18 minutes.

3.4.2 8PEG Hydrogel Film

1 wt% of PI was added to a flask, together with 0.2 g of 8PEG. As the 8PEG is in a solid state, the flask was put on a heating plate at 80°C until the hydrogel melted. The flask was then quickly put under sonication for 10 minutes, to promote the dissolution of the PI in the hydrogel. Few drops were then put on a microscope glass using a glass pipette, and covered with a thin glass. The microscope glass and the pipette were pre-heated at 80°C before handling. The mixture was put to cure under UV light 366 nm, under a $N_2$ atmosphere in absence of $O_2$ for 18 minutes.
3.4.3 8PEG-PEG\textsubscript{575} Blend Hydrogel Film

1\% wt of PI was added to a flask, together with 0.1 g of 8PEG and 0.1 g of PEG\textsubscript{575} (1:1 ratio). The flask was put on a heating plate at 80°C until the hydrogel melted, while being stirred for 15 minutes. Few drops were then put on a microscope glass using a glass pipette, and covered with a thin glass. The microscope glass and the pipette were pre-heated at 80°C before handling. The mixture was put to cure under UV light (366 nm) and under a N\textsubscript{2} atmosphere in absence of O\textsubscript{2} for 18 minutes.

3.5 Nanocomposite Hydrogel Films Preparation

A photo-initiatior (PI) 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone, $M_{\text{w}}$ 224.26 g mol$^{-1}$, purchased from Sigma-Aldrich, was added to all PEG-based polymers. The concentrations of PI, AuNPs and AuMNPs are expressed in weight percentage with respect to the quantity of polymer. The procedures for the nanocomposites containing AuNPs and AuMNPs are similar, differing only on the application of an external magnetic field to the AuMNPs nanocomposites. Figure 3.6 gives a general overview of the preparation of the NC films.

3.5.1 PEG\textsubscript{575} -based Nanocomposite Hydrogel

1\% wt of PI was added to a flask, together with 0.2 g of PEG\textsubscript{575} and 40\% wt of AuNPs or AuNMPs solution. The flask was put under sonication for 30 minutes, to promote the dissolution of the PI and the dispersion of the AuNPs or AuMNPs in the hydrogel. Few drops were then put on a microscope glass and covered with a thin glass. The AuNPs mixture was put to cure under UV light (366 nm) and under a N\textsubscript{2} atmosphere in absence of O\textsubscript{2} for 20 minutes. In the case of the mixture with the AuMNPs, it was left on top of a magnet for 15 minutes prior to the UV curing at a 366 nm light for 25 minutes.

3.5.2 8PEG-based Nanocomposite Hydrogel

1\% wt of PI was added to a flask, together with 0.2 g of 8PEG and 40\% wt of AuNPs or AuNMPs solution. As the 8PEG is in a solid state, the flask was put on a heating plate at 80°C until the hydrogel melted. The flask was then quickly put under sonication for 30 minutes, to promote the dissolution of the PI and the dispersion of the AuNPs or AuMNPs in the hydrogel. Few drops were then put on a microscope glass using a glass pipette, and covered with a thin glass. The microscope glass and the pipette were pre-heated at 80°C before handling. The AuNPs mixture was put to cure under UV light (366 nm) and under a N\textsubscript{2} atmosphere in absence of O\textsubscript{2} for 20 minutes. The AuMNPs mixture was UV cured in the presence of a magnet under the microscope glass under UV light (366 nm) for 25 minutes.

3.5.3 8PEG-PEG\textsubscript{575} Blend-based Nanocomposite Hydrogel

1\% wt of PI was added to a flask, together with 0.1 g of 8PEG and 0.1 g of PEG\textsubscript{575} (1:1 ratio) and 40\% wt of AuNPs or AuNMPs solution. The flask was then put on a heating plate at 80°C until the
hydrogel melted, while being stirred for 15 minutes. The flask was then quickly put under sonication for 15 minutes, to promote the dispersion of the AuNPs or AuMNPs on the hydrogel. Few drops were then put on a microscope glass using a glass pipette, and covered with a thin glass. The microscope glass and the pipette were pre-heated at 80°C before handling. The AuNPs mixture was put to cure under UV light 366 nm, under a N₂ atmosphere in absence of O₂ for 20 minutes. The AuMNPs mixture was UV cured in the presence of a magnet under the microscope glass at a 366 nm UV light for 25 minutes.

Figure 3.6: Schematic overview of hydrogel films preparation.

3.6 Cell Culture

Mouse fibroblasts L929 (provided by Dr. Lehmann, Fraunhofer Institute for Cell Therapy and Immunology, IZI, Leipzig, Germany) were cultured in RPMI 1640 medium with addition of 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (PS) in an incubator CB150 Series (Binder GmbH, Germany) at controlled temperature (37°C) and CO₂ atmosphere (5%). Medium, sera and reagents were provided by PAA Laboratories GmbH, Germany, unless stated otherwise.

3.7 Analytical Methods

3.7.1 Swelling Experiments

The following procedure was adapted from Zusiak et al. [58]. For each sample, the degree of swelling was measured to estimate certain structural parameters, namely: molecular weight between cross-links, effective cross-link density, and mesh size. Hydrogel swelling is a function of network structure, degree of cross-linking, as well as hydrophilicity. Firstly, the dry mass \( M_D \) of the hydrogels was measured. The hydrogel films were then incubated at 37°C in deionized water for 24 hours, and the weight after swelling \( M_S \) was measured. The swelling ratio based on the hydrogel mass \( Q_M \) was calculating using the following equation:
\[ Q_M = \frac{M_S}{M_D}. \]  

(3.1)

\( Q_M \) was then further used to calculate the volume swelling ratio \( Q_V \):

\[ Q_V = 1 + \frac{\rho_p}{\rho_s}(Q_M - 1), \]  

(3.2)

where \( \rho_p \) is the density of the hydrogel (1.12 g/cm\(^3\) for PEG [59]) and \( \rho_s \) is the density of the solvent (1 g/cm\(^3\) for water). To determine the hydrogel mesh size \( \xi \), Flory-Rehner calculations were used [60]. First, the molecular weight between cross-links \( M_c \) was calculated by:

\[
\frac{1}{M_c} = 2 \frac{M_n}{M_p} - \frac{\pi}{V_1} \left( \ln(1 - v_2) + v_2 + \chi_1 v_2^2 \right) v_2^{1/3} - \frac{\pi}{2},
\]  

(3.3)

where \( M_n \) is the number-average molecular weight of the un-cross-linked hydrogel (the molecular weight of the polymer), \( V_1 \) is the molar volume of the solvent (18 cm\(^3\)/mol for water), \( v_2 \) is the polymer volume fraction in the equilibrium swollen hydrogel, which is equal to the reciprocal of \( Q_V \), \( \pi \) is the specific volume of the polymer \( (\rho_p/\rho_s) \), and \( \chi_1 \) is the polymer-solvent interaction parameter (0.426 for PEG-water) and assumed constant. The mesh size of the network was then determined following the procedure described by Canal and Peppas [61], where the root-mean-square end-to-end distance of the polymer chain in the unperturbed state \( \langle r_0^2 \rangle^{1/2} \) was calculated as follows:

\[ \langle r_0^2 \rangle^{1/2} = l C_n^{1/2} n^{1/2}, \]  

(3.4)

where \( l \) is the average bond length (0.146 nm), \( C_n \) is the characteristic ratio of the polymer (typically 4.0 for PEG) and \( n \) is the number of bonds in the cross-link:

\[ n = 2 \frac{M_c}{M_r}, \]  

(3.5)

where \( M_r \) is the molecular weight of the repeat unit (44 for PEG), and the mesh size could then be calculated by:

\[ \xi = v_2^{-1/3} \langle r_0^2 \rangle^{1/2}. \]  

(3.6)

### 3.7.2 Atomic Force Microscopy

The atomic force microscope (AFM) is part of the scanning probe microscopes family. The operating process of the AFM is based on the measurement of the force between the tip of a cantilever and the sample. The cantilever works as a type of spring, which is attached to a rigid substrate that can be held fixed. Depending on whether the interaction at the tip is attractive or repulsive, the cantilever will deflect towards or away from the surface of the sample.

In order to produce the images, the deflection of the cantilever must be detected and converted into an electrical signal. This is done using a laser beam that is reflected from the back of the cantilever onto
a detector. It is used the optical lever principle, which means that a small change in the bending angle of the cantilever is converted to a measurably large deflection in the position of the reflected spot.

The change on the angle of the reflected laser beam results in a change of the position where the beam is being detected on the photodetector. Most AFMs use a photodiode that consists of four quadrants, so that the laser spot position can be calculated in two directions. The vertical deflection (measuring the interaction force) can be calculated by comparing the amount of signal from the “top” and “bottom” halves of the detector. The lateral twisting of the cantilever can also be calculated by comparing the “left” and “right” halves of the detector (Figure 3.7 (a)). The signals from the four quadrants of the detector are compared to calculate the deflection signal. In this work, the measurements were done using Nanowizard II, JPK instruments.

**Surface Topography**

There are different possible approaches to acquire the signal and translate it into an image. These imaging modes differ from the way the cantilever response is being monitored (Figure 3.7 (b)).

When the deflection of the cantilever is used as the feedback signal, this is known as contact mode imaging. In contact mode the tip never leaves the surface, and the lateral deflection can give information about the friction force between the tip and the sample. This allows to put in evidence areas that may have the same height, but different chemical properties. In contact mode, the setpoint value is the deflection of the cantilever, so a lower value of the setpoint gives a lower imaging force.

Dynamic modes are based on the vibration of the cantilever, and, rather than the static deflection of the tip, the oscillation of the cantilever is measured. In intermittent contact mode, the cantilever oscillates and the tip makes repulsive contact with the surface of the sample at the lowest point of the oscillation. The lateral forces can be much lower than the ones in contact mode, since the proportion of the time where the tip and sample are in contact is quite low. In intermittent contact mode, the setpoint value
is the amplitude of the oscillation, so a higher setpoint value means less damping by the sample and hence a lower imaging forces. In non-contact mode, the cantilever oscillates close to the sample surface, but without making contact with it. This imaging mode is not widely used, since there is a possibility of contact between the tip and the surface because of the attractive force between them. Force modulation mode is the dynamic form of contact mode, where the tip does not leave the surface at all during the oscillation cycle.

**Force Mapping**

Is is also possible to use the AFM to determine elastic properties, such as the elastic modulus, of samples. The vertical deflection of the cantilever can give a direct measure of the interaction force. The force curves are displayed as a x-y plot, where the height positions for the approach or retract of the cantilever are represented on the x-axis, and the cantilever property that is being measured on the y-axis.

![Figure 3.8: Typical interaction for an uncoated hydrophilic cantilever in air approaching a hard incompressible hydrophilic surface. Image adapted from [62].](image)

Initially, as the cantilever approaches the surface, the forces are too small to give a measurable deflection of the cantilever. Thus, it remains in its undisturbed position, until the attractive forces (usually Van der Waals and capillary forces) overcome the cantilever spring constant and the tip jumps into contact with the surface. When the tip gets in contact with the sample, it remains on the surface while the separation between the base and the sample decreases further. This causes a deflection of the tip and an increase in the repulsive contact force. Once the cantilever is retracted from the surface, the tip may remain in contact with the surface due to some adhesion, causing the cantilever to deflect downwards. As the force from the cantilever becomes enough to overcome the adhesion, the tip will break free of the surface (Figure 3.8).

There are several models that can be used to calculate the parameter of interest, but most of them are based on the Hertz model and are extended to match the experimental conditions concerning the indenters’ shape or the thickness of the sample. The Hertz model assumes the sample to be an isotropic and linear elastic solid occupying an infinitely extending half space. Moreover, it is assumed that the
indenter is not deformable and that there are no additional interactions between the indenter and the sample. If these conditions are met, the Young’s modulus \((E)\) of the sample can be fitted or calculated using the Hertzian model. Poisson’s ratio \((\nu)\) is the parameter describing the material, and is set to 0.5 (incompressible materials like rubber). The geometry of the indenter determines which equation is to be used, in this case a conical geometry was used and the equations can be defined as follows:

\[
F = \frac{E}{1 - \nu^2} \frac{2\tan\alpha}{\pi} \delta^2
\]

\[
a = \frac{2\tan\alpha}{\pi} \delta,
\]

where \(F\) is the force, \(\delta\) is the indentation, \(a\) is the radius of the contact circle and \(\alpha\) is the semi-opening angle of the cone. \(E\) can then be calculated by fitting the force indentation curves (\(F\)-\(\delta\)-curves) using \(E\) as a fit parameter.

\subsection*{3.7.3 Transmission Electron Microscopy}

Transmission electron microscopy is a microscopy technique in which a beam of electrons is used as an irradiation source to form an image. Transmission electron microscopes (TEM) are capable of imaging with a higher resolution than optical microscopes due to the smaller de Broglie wavelength \((\lambda)\) of the electrons, which can be translated as:

\[
\lambda = \frac{\hbar}{p},
\]

where \(\hbar\) is the Planck constant and \(p\) the momentum of the electron.

At lower magnifications, the TEM image contrast is based on the absorption of electrons by the material depending on the differences in composition or thickness of the material. At higher magnifications, the intensity of the image is modulated by the complex wave interactions. Alternate modes of use of the TEM allow for the observation of modulations in chemical identity, crystal orientation, electronic structure and sample induced electron phase shift as well as the regular absorption based imaging [63].

The TEM consists of an emission source, which may be a tungsten filament or needle, or a lanthanum hexaboride (LaB\(_6\)) single crystal source. The gun is connected to a high voltage source (typically 100–300 kV) and, given sufficient current, the gun will begin to emit electrons either by thermionic or field electron emission into the vacuum (Figure 3.9) [64]. The thermionic emission current density \((J)\) can be related to the work function of the emitting material through Richardson’s law:

\[
J = AT^2 \exp\left(\frac{-\phi}{kT}\right),
\]

where \(A\) is Richardson’s constant, \(\phi\) the work function and \(T\) the temperature of the material. The electron source is typically mounted on a Wehnelt cylinder to provide preliminary focus of the emitted electrons into a beam. The upper lenses of the TEM then further focus the electron beam to the desired...
size and location [65].

Figure 3.9: Schematic layout of a TEM operated in diffraction contrast bright field imaging. Image adapted from [66].

The electron beam can be manipulated by mainly resourcing to two physical effects. Electromagnets apply a magnetic field which interacts with the electrons. Electrostatic fields can cause the electrons to be deflected through a constant angle. Coupling of two deflections in opposing directions with a small intermediate gap allows for the formation of a shift in the beam path, this being used in TEM for beam shifting. Added to the use of an electron imaging system, it is possible to have sufficient control over the beam path. The optical configuration of a TEM can be rapidly changed, as lenses in the beam path can be enabled, have their strength changed, or be disabled entirely simply via rapid electrical switching, the speed of which is limited by effects such as the magnetic hysteresis of the lenses [64].

In this work, TEM images were acquired using a TECNAI G²20 S-TWIN microscope operating at 200 kV with a point of resolution of 0.24 nm.

3.7.4 Scanning Electron Microscopy

The scanning electron microscope (SEM) is a type of electron microscope that allows to produce images by scanning the surface of the sample using a focused beam of electrons. These electrons interact
with atoms at various depths within the sample, which translate into signals that contain information on the topography and composition of the sample's surface. These signals include secondary electrons, reflected or back-scattered electrons, characteristic X-rays and light (cathodoluminescence), absorbed current (specimen current) and transmitted electrons. The most common SEM operating mode is based on the detection of secondary electrons, although the detection of back-scattered electrons is often used [67].

The electron beam is generated in a hot cathode with hairpin-shaped tungsten or a LaB$_6$ single crystal source, similar to the TEM system. These electrons are accelerated in an electric field with a voltage of typically around 9 - 30 kV through an anode and are focused with magnetic lenses on the objects surface (Figure 3.10) [68].

Heavier elements appear brighter than lighter elements due to the fact that the latter back-scatter less intensively, thus allowing to retrieve images with good contrast. The measurements have to be performed in vacuum to avoid the interaction of electrons with other molecules present in the air. Samples also have to be treated prior to the measurement, to ensure their conductivity. Non-metallic samples should be sputtered with conductive materials like carbon or gold to induce conductivity, and all samples should be dried and water-free [68].

In this work, SEM imaging was performed on a LEO 982 offered by ZEISS Company, the optical parts
of the microscope being from GEMINI Optics. The samples were carbon-coated and the measurements were performed using an Inlens detector operating at 20.0 kV and 10 kV.
Chapter 4

Results

In this chapter, the results achieved after applying the previously described methodology are reported. The synthesized gold nanoparticles are characterized, as well as the 8-PEG after undergoing the acrylation process. A topographical analysis of the hydrogel films is done, followed by a comparison of certain behaviours between the different materials.

4.1 Gold Nanoparticles Synthesis

![TEM Image of the 6.G4 batch of AuNPs](image)

![Size distribution of the AuNPs](image)

Figure 4.1: TEM image of a sample of the 6.G4 batch of the synthesized AuNPs (a) and size distribution of the particles present (b).

Citrate capped AuNPs were synthesized using a seeded growth method, and characterized using TEM and UV-Vis spectroscopy. From the TEM imaging of a sample from 6.G4 batch (Figure 4.1 (a)), it is possible to see that the synthesized AuNPs are spherical. Using ImageJ program, the size distribution of the nanoparticles was calculated, presenting diameters between 25 and 40 nm, with a mean value of 30 nm and a standard deviation of 3.13 (Figure 4.1 (b)). The UV-Vis spectrum shows an SPR peak
at 527 nm corresponding to a maximum absorbance of 0.97 (Figure 4.2). Using the Lambert-Beer equation defined as $A = \varepsilon l c$, where $A$ is the maximum absorbance, $\varepsilon$ the extinction coefficient for AuNPs ($6.06 \times 10^9$ [70]), and $l$ the length of the cuvette (1 cm), it was possible to calculate a molar concentration ($c$) of 0.16 nM.

### 4.2 Surface Topography

As the hydrogel films were prepared, both surfaces (top and lower) were subjected to a topographical analysis. This analysis was made by AFM in intermittent contact mode. The images were processed using JPK Data Processing program. The RMS (root mean square) roughness presented is an average value of 10 measurements made in arbitrary directions with a length of 10 µm.

Initially, blank samples of the hydrogels were synthesized and analyzed in order to understand the topography of the surface when there were no nanoparticles present in their volume and allow the comparison between the hydrogel itself and the NC hydrogel. Figure 4.3 A-C shows the height images (top) and respective cross-section profile (bottom) of the lower surface of the blank hydrogel films. The three blank samples show a relatively smooth surface with an RMS roughness (Rq) of 840.4 pm for PEG$_{575}$, 554.5 pm for 8PEG and 860.2 pm for the Blend material. The small height deviations resembling particles in the PEG$_{575}$ and 8PEG samples may be due to impurities present in the instruments used to prepare the samples or present in the surrounding environment, that got transferred to the samples.
4.2.1 Gold Nanoparticles Nanocomposite Hydrogel Films

In an attempt to grant the PEG\textsubscript{575}, 8PEG and Blend hydrogels the ability to trigger cell adhesion mechanisms, nanoparticles were added to their volume upon their synthesis. The goal was to detect AuNPs on the surface of the hydrogel films, to ensure that cells could interact with the gold of the particles.

The top surface of the PEG\textsubscript{575} AuNP NC (Figure 4.4 - A) showed in its height image some spherical particles with different sizes. The size difference suggests the presence of some impurities together with AuNPs in the sample. Given the possibility of detecting AuNPs in the surface of the hydrogel film, these were expected to be more visible on the lower surface (4.4 - B), due to gravitational forces that could stimulate particles to deposit. However, the height profile of the lower surface of the hydrogel is not consistent with the presence of any particles, and showed an RMS roughness of 1.6 nm, which is only slightly higher when compared to the blank PEG\textsubscript{575} film but may indicate the presence of AuNPs in the volume of the hydrogel.

The top surface of the 8PEG AuNP NC (Figure 4.5 - A) shows a smooth profile, with a few spherical protrusions appearing in the image. Despite being distributed all over the area analyzed, they are very low, which may indicate these particles are indeed embedded in the gel. The lower surface (Figure 4.5 - B) however, shows the presence of elongated particles. It is not expected for these to be AuNPs, as they are supposed to show a spherical profile. These elongated particles are very similar to the ones found on the surface of the blank 8PEG sample, which may indicate that these are topographies created by the crystallization of 8PEG. The RMS roughness of the lower surface of the hydrogel film is 1.0 nm, being only slightly higher when comparing to the blank 8PEG film. This can be explained by the same reasons pointed out for the case of PEG\textsubscript{575} films.

For the 8PEG-PEG\textsubscript{575} Blend material, it is possible to observe small particles present in both top and lower surfaces of the hydrogel film. On the top side (Figure 4.6 - A), some of the particles detected are elongated, similarly to the ones detected in the 8PEG AuNP NC and blank samples. However, a few
Figure 4.4: Height image and respective cross-section of the top surface (A) and lower surface (B) of PEG$_{575}$ AuNP nanocomposite film

Figure 4.5: Height image and respective cross-section of the top surface (A) and lower surface (B) of 8PEG AuNP nanocomposite film
other spherical particles are detected, which can be gold. Due to the low height difference measured (4 nm), this may indicate that the particles are embedded or entrapped under the gel and thus just exposing very little gold at the surface or even none at all. The lower surface of the film (Figure 4.6 - B) also shows a few spherical particles present, with a slightly bigger height difference measured (7.06 nm). As it is in the case of the top surface, these may be AuNPs embedded or entrapped within the hydrogel matrix, and are unlikely to have their surface exposed. The RMS roughness of the lower surface of the 8PEG AuNP NC film is 646.6 pm, which is lower than the RMS roughness measured in the blank 8PEG film. This may be due to the effect that adding AuNPs in solution to the pre-curing mixture has in the retardation on the re-solidification of 8PEG after melting and handling, as it helps on the dissolution of the solid hydrogel.

Figure 4.6: Height image and respective cross-section of the top surface (A) and lower surface (B) of Blend AuNP nanocomposite film

4.2.2 Gold Magnetic Nanoparticles Nanocomposite Hydrogel Films

Since the density of particles being detected in the surface of the films using AuNPs was very low, a new strategy was thought of by introducing the use of AuMNPs. These particles would still have the gold necessary to promote cell adhesion to the films, and possessed magnetic properties that allowed the use of a magnet to force the particles to migrate to a specific area of the hydrogel.

In order to attempt to pull the AuMNPs to the lower surface of the hydrogel films, a magnet was placed under the pre-cured gels. In the case of the PEG 575 AuMNP NC, it was possible to leave the sample in contact with the magnet some time before the curing, as it is in liquid state at room temperature, with the goal of enhancing the migration of the nanoparticles to the bottom of the gel. In Figure 4.7 - B, it is possible to see several spherical particles that resemble the AuMNP that were expected to be detected.
However, the heights detected, considering the diameter of 50 nm of the nanoparticles, is quite lower than expected, being the highest peak detected in the cross-section around 12 nm. This can indicate that the particles are embedded in the gel. Figure 4.8 shows the phase image of the lower surface of the film, which indicates that the particles detected are composed of a different material from the surrounding matrix. The RMS roughness of this surface is 2.0 nm. On the other hand, it is not possible to detect particles on the top surface of the film (Figure 4.7 - A).

![Figure 4.7: Height image and respective cross-section of the top surface (A) and lower surface (B) of PEG\textsubscript{575} AuMNP nanocomposite film](image)

SEM surface characterization was performed in the PEG\textsubscript{575} AuMNP NC film. Figure 4.9 - a, shows...
the image from the Inlens secondary electrons (SE) detector, where it is possible to identify several different particles in that area. As these can be also impurities, imaging using backscattered electrons (BSE) was performed, to identify only particles with a higher atomic number. Figure 4.9 - b only shows the particles that are most probably gold, and comparing to the image obtained from AFM, the SEM images show a considerably lower amount of particles present. This can be explained by the fact that both techniques are local characterization techniques, and it can have happened that the areas analyzed did not match.

![SEM Image of the PEG575 AuMNP NC film from SE](image1)

![SEM Image of the PEG575 AuMNP NC film from BSE](image2)

Figure 4.9: SEM Images of the PEG575 AuMNP NC film from SE (a) and BSE (b).

Moving to the top surface of the 8PEG AuMNP NC film (Figure 4.10 - A), it is possible to see a few spherical particles. The cross-section that crosses one of the visible particles indicates a height of around 35 nm, which can indicate a AuMNP. On the other hand, on the lower surface of the film (Figure 4.10 - B), a bigger density of particles can be detected, with heights around 10 nm according to the cross-section. The presence of particles on the top of the film could be explained by the fact that the magnet has to be placed at the same time as the film is being cured, as it is not liquid at room temperature, and the fast polymerization of the film may not allow the particles to completely migrate to the bottom. However, the profile of the particles detected on the lower surface is similar to the one observed in the PEG575 AuMNP NC sample, which can indicate that these are indeed nanoparticles. Their elongated shape may be due to imaging artifacts. The lower surface of the 8PEG AuMNP NC film has a RMS roughness of 2.8 nm. The phase image of this surface does not indicate a significant difference on the materials present, which may indicate that the particles are embedded and covered by the hydrogel material (Figure 4.11).

Regarding the Blend sample, contrary to what was expected, it is not possible to detect a significant density of particles present in neither of the surfaces of the film. On the top surface of the film (Figure 4.12 - A), one small spherical body can be detected. On the lower surface of the film (Figure 4.12 - B), there are no visible particles present. The fact that no particles were detected may be associated by
Figure 4.10: Height image and respective cross-section of the top surface (A) and lower surface (B) of 8PEG AuMNP nanocomposite film

Figure 4.11: Phase image of the lower surface of 8PEG AuMNP nanocomposite film
the fact that this is a local characterization method, and it may have happened that the measurements were not made on areas where AuNPs were indeed present. This surface has an RMS roughness of 1.7 nm, which is higher than the blank Blend material. This may indicate the presence of particles embedded under the material that were not able to fully migrate to the bottom of the film due to the fast polymerization of the gel, since the challenge of having to manage the gel at 80°C to avoid resolidification was present.

Figure 4.12: Height image and respective cross-section of the top surface (A) and lower surface (B) of Blend AuMNP nanocomposite film

4.3 Swelling Behaviour

In order to calculate the swelling parameters of the hydrogel films, 3 individual samples of each film were weighed in dry state, incubated at 37°C in deionized water, and weighed again after 24 hours of incubation, in swollen state. Table 4.1 shows the parameters calculated through the methods described in section 3.7.1.

Looking first at the swelling ratios, both mass ($Q_M$) and volume ($Q_V$) based, it is possible to see that the PEG575 and Blend materials are the ones that have higher ratios. This means that these materials swell less than the 8PEG materials, as it can be also observed by the %S values. Comparing the blank samples with the NC samples, the materials in which it is seen a bigger difference in its swelling behaviour are the 8PEG, that has an increase in its swelling ratios when nanoparticles are present in its volume, whilst the opposite happens with the PEG575 and Blend materials. This may be due to the difference in the structure of the polymer, as the presence of nanoparticles may affect differently the crosslinkage of the polymers.

41
Table 4.1: Swelling ratio (\(Q_M\)), volume swelling ratio (\(Q_V\)) and mesh size (\(\zeta\)) from blank, AuNP NC and AuMNP NC hydrogel samples

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>AuNP</th>
<th>AuMNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG</td>
<td>8PEG</td>
<td>Blend</td>
</tr>
<tr>
<td>%S</td>
<td>40</td>
<td>313</td>
<td>57</td>
</tr>
<tr>
<td>(Q_M)</td>
<td>72±7</td>
<td>21±3</td>
<td>64±6</td>
</tr>
<tr>
<td>(Q_V)</td>
<td>69±8</td>
<td>15±4</td>
<td>60±7</td>
</tr>
<tr>
<td>(\zeta) ((\text{\AA}))</td>
<td>3.9±0.9</td>
<td>72.7±25.6</td>
<td>5.6±1.4</td>
</tr>
</tbody>
</table>

In the case of 8PEG, the presence of nanoparticles in its structure induces a decrease of water intake. As for the PEG\(_{575}\) and Blend materials, the presence of nanoparticles may decrease the level of crosslinkage, allowing the polymer to have a bigger water intake, as a higher degree of crosslinking of a polymer may increase its resistance to solvents [71].

The AuNP NC materials show a slightly higher swelling ratio than their AuMNP equivalents. This may be due to the fact that most of the AuMNP are concentrated in a portion of the hydrogel film, as they were forced to migrate to the bottom of the film, and the migration might affect the crosslinking process by decreasing its rate, which allows the hydrogel to swell more.

Regarding mesh size, as smaller fractional volume results in higher mesh size, looking at equation 3.3, it is expected that the mesh size of the swollen polymers will be higher for a lower \(Q_V\), as it shows.

It is also important to refer that the possible presence of impurities in the samples may affect the swelling behaviour of the hydrogels, as well as the human error associated to the measurements made.

### 4.4 Force Mapping

In order to determine the Young’s Modulus (\(E\)) of each of the hydrogel samples, force mapping was done on the lower surface of the films using AFM. The force mapping was performed in contact mode using CONTGD-G tips with a half-cone angle of 10° at the apex, on a \(10\times10\mu\text{m}\) area with \(8\times8\) pixels, which allows to determine 64 values of \(E\) for each sample, being the values presented a mean value of those. For the dry state force mapping, the calibration was done using a glass microscope slide and 3 different values for InvOLS and spring constant \(k\) were measured, being later used the mean values to fit the force-indentation curves to the Hertz model, having 67.32 nm/V for InvOLS and 0.5251 N/m for \(k\). InvOLS (Inverse Optical Lever Sensitivity) is a parameter that describes the amount of photodiode response (Volts) per nanometer of cantilever deflection.[72]

Regarding the dry measurements (Table 4.2), the materials based only on PEG\(_{575}\) showed to be the stiffest and 8PEG the softest. When analyzing their behaviour upon the presence of nanoparticles, the NC films all showed a slightly decrease in \(E\), on both AuNP and AuMNP cases. This can be explained due to the fact that in both cases, the nanoparticles were not functionalized with groups that allowed to cross-link with the acrylate groups of the gels. This may have inhibited the cross-linking of the gel, resulting in materials with lower stiffness.
For the underwater measurements (Table 4.3), the samples were fixed to the bottom of a small plate with the lower surface facing up, using a biocompatible glue provided by JPK Instruments. They were then submerged in deionized water, left to swell for 20 minutes and then measured. The measurements had to be made in a short time period of submersion due to the fact that it was not possible to keep the samples fixed after a long period of swelling. The calibration was done using a thin glass fixed in the bottom of a plate with water. The measurements were done with the same specifications used in the dry state, with calibration values of 66.04 nm/V for InvOLS and 0.4525 N/m for $k$. The values for $E$ measured in the swollen state follow a similar behaviour to the ones measured in the dry state, as the PEG$_{575}$ materials showed higher values, followed by the Blend and finally the 8PEG samples. Furthermore, the NC materials continue showing lower values for $E$ when comparing to the blank hydrogel films.

When comparing the values between the measurements done in the dry and wet state, the AuMNP NCs have slightly higher values for $E$ in the wet state than in the dry state. This can be due to the fact that, when the gel swells, the particles get closer to the surface, resulting in a stiffer profile. The PEG$_{575}$ and Blend AuNP NCs measured in the wet state have higher values than the ones measured in the dry state, which can be explained by the same reason presented for the AuMNP NCs. However, the values in both measurements do not differ too much, and this may be due to the fact the the gels are not being left underwater enough time to swell to a significant extent due to the limitations presented above.

The relatively high values observed for the standard deviations ($SD$), especially in the cases of the AuMNP NCs, are explained by the wide range of values measured for $E$ in each of the 64 points of measurement done in the sample. In the case of 8PEG AuMNP NC film, where the error is very high, it can be seen that the force map and histogram of force distribution show a few areas where the values measured are much higher, causing the very big deviation. Most of the values for $E$ in this case are concentrated between 0-1 GPa (Figure 4.13). This range may happen due the difference in stiffness between measuring a surface containing only gel and a surface containing a nanoparticle, resulting in a
set of values that may differ in orders of magnitude.

Figure 4.13: Force mapping of 8PEG AuMNP NC in swollen state diagram (a) and $E$ distribution histogram (b).

### 4.5 Cell Adhesion to the Nanocomposite Hydrogel Films

Cell culture was performed on the samples using L929 fibroblasts. After 24 hours of incubation with a concentration of 40 000 cells/mL at 37°C and in an atmosphere of 5% CO$_2$, the samples were analyzed under a ZEISS microscope and the images processed using the AxioVision (ZEISS) program. Figure 4.14 shows the control cells from 3 different wells in the tissue culture polystyrene (TCPS). It is possible to observe that after 24 hours of incubation, most of the cells are widely spread and adhered to the TCPS.

Figure 4.14: Control cells cultured in 3 different TCPS wells (A, B and C)

Figure 4.15 shows images of the cells cultured in the blank hydrogel films. As it is known that PEG materials are naturally anti-adhesive [7], the spherical morphology observed in the 3 different PEG materials was expected. This indicates that the cells are not adhering to the substrate, although it is possible
Figure 4.15: L929 cells cultured in PEG$_{575}$ (A), 8PEG (B) and Blend (C) blank hydrogel samples to see some initial spreading of the cells present on the Blend sample (4.15 - C). This fact may be due to the fact that combining the two different PEGs may have created some topographical inconsistencies on the film due to a non-completely homogeneous mix of the two materials, which stimulates cell adhesion.

Figure 4.16: L929 cells cultured in PEG$_{575}$ (A), 8PEG (B) and Blend (C) AuNP hydrogel samples

Observing the cell morphology that resulted from the cultures done on the AuNP NC samples, it is possible to see that in the PEG575 (Figure 4.16 - A) and Blend (Figure 4.16 - C) AuNP NC films, only a few cells show some initial spreading and most cells on the image present a spherical shape. On the 8PEG sample (Figure 4.16 - B), most cells are spherical, thus not showing a good adhesion to the material. This fact may be due to the fact that, according to the topographical measures, some AuNPs are detected on the PEG$_{575}$ and Blend AuNP NCs, but it was very difficult to detect any particles present at the 8PEG AuNP NC. The low adhesion to the two latter materials can also be explained by the fact that it is necessary for the cells to be directly in contact with the gold in order for the adhesion mechanisms to be enhanced, and the particles are mostly embedded inside the gel. Regarding the blend AuNP film, the fact that it has an apparent better adhesion than the PEG$_{575}$ may be due to the topographical inconsistencies that may be created due to a non homogeneous mix of the two different PEG materials, as it was referred before.

Regarding the cells cultured on the AuMNP films, these show the most spreaded morphology on the PEG$_{575}$ AuMNP NC film (Figure 4.17 - A), as it was expected due to the high number of gold particles detected in the AFM topographical measurements. However, comparing to the control, the number of cells that adhered to the material is not as big as it was expected, and this may be due to the fact that most of the AuMNP are embedded in the gel, thus only a small area of the particle would be exposed, resulting in very little gold being actually in contact with the cells. Moreover, the roughness of the material...
is higher, which also stimulated cells to adhere to areas with higher topographical inconsistencies, as it was mentioned before. There is also the fact that there might be a higher concentration of particles in that specific area of the gel, as they might not be homogeneously dispersed in the sample, which causes the area of the film to be more stiff than the rest. Cells respond to physical patterns and tend to migrate and adhere to surfaces with an increased rigidity, a phenomenon known as durotaxis [73]. In the 8PEG and Blend AuMNP NC films (Figure 4.17 - B, C), the cells are mostly round and do not show any signs of spreading. This is probably due to the fact that no AuMNPs were able to reach the surface of the hydrogel films and be exposed, as it is possible to see from the topographical measurements.
Chapter 5

Conclusions

This work has contributed for the further understanding of how mechanical and physical properties of PEG-based hydrogels can be manipulated and tailored in order to confer them certain characteristics that influence cell adhesion.

The incorporation of nanoparticles to different types of PEG-based hydrogels has shown to affect the mechanical properties and swelling behaviour of the materials, as well as their topographical and structural profile. These changes can influence the way the materials interact with cells, and by tuning their composition and optimizing their preparation process, it is possible to create hydrogel films that are suitable to be used for biological applications. In this work, the aim was to incorporate gold nanoparticles into a PEG-based hydrogel matrix in order control cell adhesion.

The methodology chosen to synthesize citrate capped AuNPs for further incorporation to the hydrogel materials was a seeded growth method. This approach has shown to be an efficient way of achieving spherical monodispersed particles that are suitable to be further used in the preparation of PEG-based nanocomposites.

The incorporation of the synthesized AuNPs into the hydrogel films showed to have different effects in the different types of matrices. The topographical profiles of the PEG575 and Blend gels showed some possible particles being close to the surface, although in a small number. However, it was harder to detect any particles present in the 8PEG AuNP NC films. Regarding the effect that the AuNPs have on the swelling behaviour of the hydrogel, these showed to slightly increase the capacity of the PEG575 and Blend materials to incorporate water in their volume. For the 8PEG gels, this capacity was decreased. Furthermore, the presence of AuNPs resulted in a decrease of the stiffness of the materials. These facts may be due to the influence that AuNPs have on the crosslinking efficiency of the gels, as the surface of the particles was not functionalized in order to allow them to crosslink with the acrylate terminal groups of the PEG gels. This resulted in very little or no impact in the adhesion of cells onto the materials, mainly because the gold was most probably not exposed to the surface and thus not triggering cell adhesion mechanisms, as it was intended.

The use of magnetic gold nanoparticles was thought of as a strategy to overcome the lack of particles present at the interface of the hydrogel films. By using a magnet to force the particles to migrate to the
bottom of the film, it was expected to find a higher number of particles at the interface of the hydrogel films, with a higher probability of the gold being exposed to the surface, enabling its interaction with cells. The topographical profile of the AuMNP gel samples showed positive results on the PEG\textsubscript{575} gels, with spherical particles being consistently detected at the lower surface of the films. However, for the 8PEG and Blend materials, the results were not as satisfactory. This situation was probably due to the methodology used to process the gels, as the 8PEG requires to be manipulated under high temperatures in order to avoid its re-crystallization. Regarding the swelling behaviour and stiffness of these materials, the results were quite similar to the ones observed in the AuNP NCs. Their stiffness showed no increase, with a decrease on the values of the Young’s modulus, and the water intake capacity was increased for the PEG\textsubscript{575} and Blend materials, but not for the 8PEG ones. The reasons behind these results are most probably the same as the ones pointed out for the AuNP NC cases. Moreover, the AuMNPs NCs showed an increase in the swelling capacity of the gels in comparison to the AuNP NCs. Regarding their performance upon cell adhesion studies, the PEG\textsubscript{575} Au MNP NC was the only one in which it was possible to detect a spread morphology on the cells, whilst on the other two materials, they kept their spherical shape, indicating that these were not able to trigger cell adhesion mechanisms. These results may once again be due to the effect AuMNPs have in the crosslinking efficiency of the gels, and the fact that it was not possible to successfully expose enough gold to the surface of the film, enabling its interaction with the cells.

Overall, it was possible to adopt simple and quick approaches to prepare NC hydrogel films by combining PEG with AuNPs and AuMNPs. Their characterization has helped to enhance the further understanding of the nature of the properties acquired by the hydrogels by incorporating AuNPs and AuMNPs, and their impact on triggering cell adhesion mechanisms.

5.1 Future Work

Regarding future developments and further continuity of this work, there are some material properties that can be further explored for various applications.

Considering the effect that the crosslinking density of the hydrogels has on their mechanical and physical properties, it would be interesting to further understand the impact that the incorporation of AuNPs and AuMNPs might have in it. The surface functionalization of the nanoparticles in order to allow them to crosslink with the terminal groups of the PEG materials and the study of its effects in the final characteristics would also be an interesting point to explore and further understand the importance of the interaction of the nanoparticles with the hydrogel matrix. Moreover, regarding the use of AuMNPs, it would be interesting to use higher concentrations of particles and measure the topographical profile of the films after being subjected to controlled magnetic fields.

Another interesting point to explore would be the use of fluorescent gold nanoparticles, such as pamoic acid capped gold nanoparticles, to understand the dispersity level of the particles within the hydrogel matrix, as it is not possible to clearly understand how efficient is the use of sonication to promote a homogeneous dispersion, or if the particles tend to aggregate when mixed with the gels.
It is known that AuNPs and AuMNPs possess electrical and thermal properties that can be taken advantage of in order to provide hydrogels the ability to respond to external stimuli, such as temperature changes or magnetic fields. It would be interesting to adapt the preparation methodologies in order to profit from these inherent properties of the nanoparticles and determine their ability to allow the design of materials that possess new characteristics, such as electrical conductivity or temperature-responsiveness.

Furthermore, the application of these NC materials with patterning techniques, such as MIMIC (micro-molding in capillaries) or FIMIC (fill-molding in capillaries) would allow further understanding of their impact on cell adhesion, by creating different physical and chemical patterns on materials and analyze their effect on cell motility and adhesion.
References


[49] Colin K. Choi, Miguel Vicente-Manzanares, Jessica Zareno, Leanna A. Whitmore, Alex Mogilner, and Alan Rick Horwitz. Actin and -actinin orchestrate the assembly and maturation of nascent


