Porous Hydrogels Doped with Nanoparticles of the Coinage Metals

Praveen (B.Sc., M.Sc.)

This thesis is presented for the degree of Doctor of Philosophy to The University of Western Australia School of Molecular Sciences October 2020

The work presented in this thesis was carried out at the University of Western Australia under the supervision of Professor Murray Baker and co-supervision of Dr. Matt Myers and Professor Reto Dorta

Praveen
Declaration

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According to The University of Western Australia’s regulations regarding Research Higher Degrees, this thesis is presented as a series of published and prepared for publication. The contributions of the candidate and co-author(s) for the papers/manuscripts comprising Chapter 2, 3, and 4 are summarized below.

**Chapter 2:**

The paper presented in Chapter 2 is first-authored by the candidate and co-authored by Dr Shuko Suzuki, Dr Christine F. Carson, Dr Matthew Myers, A/Prof. Peta L. Clode, Prof. Traian V. Chirila, Prof. Murray V. Baker and Prof. Martin Saunders, and is published as:


The candidate planned and carried out the entire experimental programme in this paper, except for the cytotoxicity assays and mechanical strength measurements, under the supervision of Dr Matthew Myers and Professor Murray Baker. The candidate analysed all the experimental results and wrote the paper based on the work. The co-authors offered comments and advice during the writing of the paper.
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Abstract

This thesis focuses on the synthesis of poly(2-hydroxyethyl methacrylate) sponges doped with silver, copper, and gold nanoparticles.

(1) Poly(2-hydroxyethyl methacrylate) sponges doped with silver nanoparticles (Ag NP-PHEMA) were prepared by UV irradiation of aqueous solutions containing 2-hydroxyethyl methacrylate (HEMA) and silver nitrate, in the presence of a crosslinking agent and a photoinitiator. The Ag NP-PHEMA sponges had polymer droplet morphology with interconnected pores, characteristic of PHEMA sponges prepared by polymerization-induced phase separation.

PHEMA hydrogel doped with Ag NPs.

For formation of Ag NP-PHEMA, the size of the polymer droplets was larger, and the rate of polymerization was increased compared to PHEMA sponges formed under similar conditions but in the absence of silver nitrate. Mechanical strength, as indicated by compression testing, was similar for Ag NP-PHEMA and PHEMA
sponges. Nanoparticles slowly leached out of Ag NP-PHEMA sponges, exerting antibacterial activity against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacteria. Notably, Ag NPs that leached were non-toxic to human corneal epithelial (HCE-T) cells.

(2) Poly(2-hydroxyethyl methacrylate) hydrogels doped with gold nanoparticles (Au-PHEMA) were prepared via photochemically- and thermally-induced phase separation polymerization of aqueous formulations containing 2-hydroxyethyl methacrylate (HEMA), a crosslinker, an initiator, and either KAuCl₄ or (CH₃)₂SAuCl. In photopolymerizations, 2,2-dimethoxy-2-phenylacetophenone (DPAP) served as photoinitiator of polymerization of HEMA but also appeared to play a role in the reduction of Au(III) to Au(0).
Abstract

for reduction of Au(III) to Au(0). The Au-PHEMA hydrogels exhibited the morphology based on a network of polymer droplets and interconnected pores characteristic of PHEMA formed via polymerization induced phase separation, with only minor differences in size of polymer droplets and mechanical properties.

(3) Poly(2-hydroxyethyl methacrylate) hydrogels incorporating copper nanoparticles (Cu-PHEMA) were prepared by a two-step process. In the first step, porous PHEMA hydrogels containing aqueous Cu(II) were formed by photo-induced phase separation polymerization in aqueous formulations containing 2-hydroxyethyl methacrylate (HEMA), a photoinitator, a crosslinker, and CuSO₄. In the second step, the Cu(II) ions were reduced to Cu(0) (Cu NPs) by hydrazine.

The Cu-PHEMA hydrogels were red-brown due to the presence of the Cu NPs, and X-ray diffraction studies confirmed the NPs were crystalline Cu and not Cu₂O. The porous
PHEMA and Cu-PHEMA hydrogels exhibited the polymer droplet morphology characteristic of sponges formed by induced phase separation polymerization of HEMA in water. The Cu NPs were stable inside the hydrogel and leaching of Cu NPs into water was very slow. Leached Cu NPs moderately inhibited the growth of *E. coli* or *S. aureus* bacteria in the surrounding medium. Incorporation of Cu NPs inside PHEMA hydrogels did not change compression strength of hydrogels.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Ag NP-PHEMA</td>
<td>Poly(2-hydroxyethyl methacrylate) doped with silver nanoparticles</td>
</tr>
<tr>
<td>Ag NPs</td>
<td>Silver nanoparticles</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerization</td>
</tr>
<tr>
<td>Au NPs</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>Au-PHEMA</td>
<td>Poly(2-hydroxyethyl methacrylate) doped with gold nanoparticles</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerization</td>
</tr>
<tr>
<td>Cu-PHEMA</td>
<td>Poly(2-hydroxyethyl methacrylate) doped with copper nanoparticles</td>
</tr>
<tr>
<td>Cu NPs</td>
<td>Copper nanoparticles</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco-modified Eagle’s medium</td>
</tr>
<tr>
<td>DPAP</td>
<td>2, 2-dimethoxy-2-phenylacetophenone</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy-dispersive X-ray microanalysis</td>
</tr>
<tr>
<td>EGDMA</td>
<td>Ethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fcc</td>
<td>Face centered cubic</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>HAADF</td>
<td>High angle annular dark-field</td>
</tr>
<tr>
<td>HCE-T</td>
<td>Immortalized human corneal epithelial</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-Hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma optical emission spectrometry</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDDA</td>
<td>Poly(diallyl dimethylammonium) chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PHEMA</td>
<td>Poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly-DL-lactic-co-glycolic acid</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly-L-lactic acid</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PVP</td>
<td>Poly(1-vinyl-2-pyrrolidinone)</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation chain transfer</td>
</tr>
<tr>
<td>RBITC</td>
<td>Rhodamine B isothiocyanate</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SEPPi</td>
<td>Solidification of emulsified polymer drops via phase inversion</td>
</tr>
<tr>
<td>STEM</td>
<td>Scanning transmission electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TSB</td>
<td>Trypticase soya broth</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XRD</td>
<td>X-Ray diffraction</td>
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<tr>
<td>3D</td>
<td>Three-Dimensional</td>
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Chapter 1.

Scaffolds in tissue engineering: A literature survey

1. BACKGROUND

Tissue engineering aims to create new tissue that either heals a damaged organ or replaces it entirely.\textsuperscript{1} To date, only a few methods are available to provide fully functional reinstallation of damaged tissue.\textsuperscript{1-5} Cell and organ transplantation to the patient’s body is generally used for the treatment of failed organs and typically requires rigorous surgeries and immunotherapies to prevent organ rejection.\textsuperscript{6} Furthermore, there are other serious limitations for transplantation of cells and organs such as donor shortage, costly therapies and an increasing number of patients requiring transplantation.\textsuperscript{1-2, 4} Abnormal cell growth development can also lead to poor tissue restoration.\textsuperscript{1-2, 4}

A new alternative approach to complicated tissue transplantation is tissue engineering. This technique will allow organs to be grown from implanted cells supported on a suitable scaffold material.\textsuperscript{1-2, 4, 7} Cells from different sources such as autologous (cells obtained from the same individuals), allogeneic (cells come from another person), xenogenic (cells belonging to individuals of different species), syngeneic (cells of identical individuals), and stem cells are cultured for the regeneration of new tissue into a scaffold.\textsuperscript{8} Scaffolds are indispensable to encourage correct development of cells into functional tissue.\textsuperscript{9} Scaffolds are essential as cell support devices upon which cells are seeded \textit{in vitro} and then encouraged to generate the foundation of a new tissue. Moreover, these scaffolds can also serve as a required growth factor or drug delivery devices.\textsuperscript{10}
Tissue engineering requires the use of polymer scaffold biomaterials made from either natural or synthetic polymers, but these materials should possess certain key characteristics:

(a) the scaffold material must be biocompatible to avoid any deleterious immune responses.\(^4\) If a scaffold is not biocompatible, immunosuppressant therapies may be vital to inhibit scaffold refusal otherwise the host may reject the implant.\(^{11-12}\)

(b) the scaffold must have three-dimensional open-pore morphology, in which the pores should have the appropriate dimensions, be interconnected, and homogeneous through the biomaterial.\(^4,7\) for cell growth and flow transport of nutrients and metabolic waste.\(^4\)

(c) the scaffold must be biodegradable to minimize the need for surgical removal after tissue regeneration has completed.\(^1,3,5\) The by-products of degradation should also be non-hazardous and able to leave the body without intervention by other organs. If the polymer is not biodegradable, it can cause problems such as irritation, inflammation and other negative side effects as well.\(^13\)
(d) the scaffold must have sufficient mechanical integrity to function from the time of implantation until the completion of the remodelling process and it should be robust enough to allow handling during surgical implantation.\textsuperscript{14}

(e) the scaffold should have properties that inhibit attachment of bacteria and associated colony formation on the surface.\textsuperscript{15}

Several natural, metallic, and synthetic scaffolds have been used in applications in tissue engineering. Natural biomaterials that have been used in tissue engineering applications include:

\begin{enumerate}
\item Silk, a naturally occurring protein produced by some insects larvae such as domesticated silkworms. Polymer scaffolds of silk are used for cartilage replacement, soft tissue augmentation and tendon reconstruction.\textsuperscript{16-18}
\item Collagen, the main structural protein in the extracellular matrix. Collagen has been used as a scaffold material for cartilage and skeletal regeneration.\textsuperscript{19-20}
\item Hyaluronic acid, a component of connective, epithelial, and neural tissues and the main component of the extracellular matrix. Scaffolds of hyaluronic acid are used for burn care and wound healing.\textsuperscript{21-22}
\item Chitosan, a polysaccharide obtained by partial deacetylation of chitin via chemical hydrolysis. It is a promising material in tissue engineering because it promotes cell adhesion without further functionalization and it can be used as a biodegradable crosslinker for other polymers for different applications in tissue engineering.\textsuperscript{23-25}
\end{enumerate}
Some metallic scaffolds are used in tissue engineering to replace damaged hard tissue. Metallic scaffolds are promising for the replacement of bone defects due the potential to use porous metal materials as a permanent structural framework. However, metallic scaffolds have a few disadvantages such as lack of biological recognition on the metallic surface and release of toxic metal ions which can lead to inflammatory response and allergic reactions.\textsuperscript{26-27}

Several synthetic polymers (e.g. poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA) and poly(DL-lactic-co-glycolic acid) (PLGA)) have been used to create scaffolds that have appropriate properties for tissue engineering applications. These polymers are considered as useful scaffold materials for tissue engineering because they allow cell growth and the polymers are fully biodegradable, but these polymers are not biocompatible.\textsuperscript{28-30} Degradation products of PLLA and PGA reduces local pH, which induces an inflammatory reaction and may lead to the death of tissue and organs.\textsuperscript{31} There are a few other polymers which are biocompatible but not biodegradable (e.g. poly(1-vinyl-2-pyrrolidinone) (PVP),\textsuperscript{32} poly(2-hydroxyethyl methacrylate)\textsuperscript{33} (PHEMA) and poly(ethylene glycol) (PEG)).\textsuperscript{34} The polymers are also relatively easy to synthesize and are cost-effective as well.

In particular, 2-hydroxyethyl methacrylate (HEMA) can be polymerized and copolymerized in diverse ways to give poly(2-hydroxyethyl methacrylate) (PHEMA) polymers (Figure 2). Depending on their formulations, PHEMA materials can have different properties that suit specific applications in the biomedical field such as internal bone fixation,\textsuperscript{35} artificial corneas,\textsuperscript{36} artificial skin,\textsuperscript{37} soft contacts,\textsuperscript{38} rhinoplasty surgery,\textsuperscript{38} surgical sutures,\textsuperscript{39} breast augmentation,\textsuperscript{40} drug delivery systems,\textsuperscript{41-43} neural regeneration\textsuperscript{44-47} and, most importantly, for tissue engineering.\textsuperscript{48-49} In most of its applications, PHEMA is used as a hydrogel—a three-dimensional interconnected network of crosslinked polymer chains that can retain a large amount of water in a swollen state.
Biocompatibility is also an important factor in tissue engineering and PHEMA is well known for its biocompatibility and very low toxicity toward cells. Porosity is important as it will give cells room for growth within the polymer matrix. Porous polymers can be prepared by different methods such as phase inversion, polymerization of high-internal-phase emulsion, the SEPPi method (solidification of emulsified polymer drops via phase inversion), electrospinning, particulate (particle) leaching, three-dimensional (3D) printing, gas foaming, and freeze-drying (also known as thermally induced phase separation). \(^{50}\)

PHEMA hydrogels with interconnected pores can be formed simply, without the need for any toxic additives, by polymerization-induced phase separation. \(^{47}\) This process involves free-radical polymerization of HEMA in water (> 60% v/v), and can be achieved photochemically or thermally. \(^{51-52}\) In this process, HEMA and water initially form a solution, but as polymerization proceeds and PHEMA chains grow, the PHEMA becomes insoluble and separates from the reaction mixture as droplets (Figure 3). These droplets eventually form a polymer sponge that contains network of interconnected pores of dimensions on the micron
Size of pores depends on the ratio of HEMA in water. On the other hand, HEMA polymerized in bulk or in the presence of only a relatively small amount of water produces, homogeneous PHEMA hydrogels with pore size <100 nm.

Figure 3. Schematic representation of formation of PHEMA sponges by polymerization-induced phase separation in water. The photo shows a PHEMA sponge formed as a cylinder of ~ 1 cm diameter and ~ 1 cm tall.

Recently, antibacterial polymers have emerged as an approach for preventing attachment or growth of bacteria on the surface of polymer scaffolds. Such polymers can be synthesized from monomers that contain functional groups possessing antibacterial activity, or by functionalising polymers with antibacterial functional groups. Polystyrene-\textit{block}-poly(4-vinylpyridine)\textsuperscript{54} copolymers and polypropylene\textsuperscript{55} polymers functionalized with phosphonium salts showed antibacterial activity against \textit{S. aureus} and \textit{E. coli}. Polymers having biguanide\textsuperscript{56}...
groups and phenol derivatives\textsuperscript{57} attached to the polymer backbone also showed antibacterial activity against \textit{S. aureus} and \textit{E. coli} bacteria.

An alternative route to antibacterial polymer materials involves the incorporation of metal nanoparticles inside the polymer matrix. Metal nanoparticles can be highly toxic to bacteria at low concentrations. The mechanism of antibacterial activity of metal nanoparticles is still not entirely known. Some researchers support the idea that antibacterial activity is directly related to interaction between bacterial cells and nanoparticles, while others support the metal ions released from metal nanoparticles showed antibacterial activity.\textsuperscript{58-60}

Metal nanoparticles are not stable and have a tendency for aggregation, which limits their applications in various fields. However, metal nanoparticles have been shown to be stabilized by a wide range of polymers, including have (polyethylene glycol),\textsuperscript{61-62} poly(vinyl alcohol),\textsuperscript{63} polystyrene,\textsuperscript{64} polyester,\textsuperscript{65} poly(diallyl dimethylammonium) chloride (PDDA),\textsuperscript{66} and poly(1-vinyl-2-pyrrolidinone) (PVP).\textsuperscript{67}

A range of techniques has been used to incorporate metal nanoparticles inside the polymer matrix, as discussed below.

\textbf{a) Chemical reduction}

Chemical reduction is one of the most common methods to synthesize metal nanoparticles. In this method, a precursor metal salt is mixed with a polymer (to act as a stabilizing agent) and reduced by a common reducing agent such as hydrazine,\textsuperscript{68} ascorbic acid,\textsuperscript{69} citrate,\textsuperscript{68,70} or sodium borohydride.\textsuperscript{69} Some polymers can also act simultaneously as reducing agent and stabilizing agent.\textsuperscript{66,71-72}

\textbf{b) Photoreduction}

In this one step method, a metal salt is pre-blended with a polymer in an appropriate
solvent. Upon irradiation, the metal ions undergo photoreduction to form metal nanoparticles, which are stabilized by the polymer.\textsuperscript{53}

c) Microwave irradiation

In this one-step procedure, a solution containing a metal salt, a stabilizing agent and a reducing agent is heated by microwave irradiation. The metal ions are reduced by the reducing agent to form metal nanoparticles, which are in turn stabilized by the stabilizing agent.\textsuperscript{73}

d) Electrospinning

Electrospinning includes three different steps to prepare metal-polymer nanocomposites. A metal salt is pre-blended with a polymer in a suitable solvent to make a uniform precursor solution, and then the solution is electrospun to generate polymer fibers containing metal ions. In the post-treatment step, metal ions in the polymer fibres are reduced by chemical, photochemical, and thermal methods.\textsuperscript{74-75}

e) Controlled/living radical polymerization

In this procedure, pre-formed metal nanoparticles are functionalized with initiators and these functionalized nanoparticles mediate a controlled/living polymerization on the surface of the metal nanoparticles. The metal nanoparticles become covered by an outer layer of polymer chains formed via reversible addition-fragmentation chain transfer (RAFT) or atom transfer radical polymerization (ATRP).\textsuperscript{76}

A focus of this thesis is the incorporation of silver nanoparticles inside PHEMA hydrogels to render hydrogels antibacterial. Silver nanoparticles (Ag NPs) are well known to
inhibit growth of bacteria. Silver nanoparticles have been incorporated into range of polymers and polymer fibres for antibacterial applications.\textsuperscript{77-78}

In many ways, the chemistry of Ag is similar to the chemistries of Cu and Au, so a logical extension of a study of Ag nanoparticles in PHEMA would explore related systems involving Cu and Au. Copper nanoparticles (Cu NPs) are considered as a cost-effective substitute for Ag NPs for antimicrobial applications. Copper nanoparticles have been incorporated into a range of polymers such as poly(ethylene glycol) (diacrylate),\textsuperscript{79} starch,\textsuperscript{80} cellulose,\textsuperscript{81} epoxy resin,\textsuperscript{82} polyethylene,\textsuperscript{83} nylon,\textsuperscript{84} polyamine,\textsuperscript{85} poly(lactic acid),\textsuperscript{86} polypropylene,\textsuperscript{55} and polystyrene\textsuperscript{87} for antimicrobial applications.

Antibacterial activity of gold nanoparticles (Au NPs) incorporated into polymers is insufficiently explored compared to that of silver and copper nanoparticles. Only a few polymers, such as poly(ɛ-caprolactone)/gelatin,\textsuperscript{88} have shown antimicrobial activity when doped with Au NPs. However, poly(N-isopropylacrylamide),\textsuperscript{89-90} graphene,\textsuperscript{91} cellulose,\textsuperscript{92} β-cyclodextrin/poly(1-vinyl-2-pyrrolidone),\textsuperscript{93} poly(vinyl alcohol),\textsuperscript{94} and wool\textsuperscript{95} doped with Au NPs have potential applications in electrosensing,\textsuperscript{96} biosensing,\textsuperscript{97} and catalysis.\textsuperscript{91}

Redox chemistry of Ag(I), Cu(II), Au(III), and Au(I) is well studied. Silver and gold salts can undergo direct photoreduction to generate Ag(0) and Au(0) respectively in alcoholic and aqueous solutions. Aqueous Cu\textsuperscript{2+} salts cannot undergo direct photoreduction to Cu NPs. Instead, Cu\textsuperscript{2+} can be reduced to Cu NPs by chemical reduction. While copper chelate complexes can generate Cu(0) by direct photoreduction via legend to metal charge transfer.\textsuperscript{98}

2. AIMS OF THIS STUDY

Incorporation of metal nanoparticles inside hydrogel matrixes can provide new nanocomposite materials that can benefit from physical (optical,\textsuperscript{99} catalytic,\textsuperscript{100} and conductive\textsuperscript{99}) and biomedical (antibacterial\textsuperscript{53}) properties of the nanoparticles. The primary aim of this project is
to prepare porous antibacterial PHEMA hydrogels doped with metal nanoparticles for tissue engineering applications. Nanoparticles were formed by chemical reduction and photoreduction. PHEMA hydrogels were formed by thermally- or photochemically-induced phase separation polymerization. Depending on the metal, the PHEMA hydrogel doped with metal nanoparticles could be synthesized in a one-step process or a two-step process.

In the one-step process, polymerization of HEMA and reduction of metal ions to metal nanoparticles occur concurrently (Figure 4). In the two-step process, the formation of PHEMA and incorporation of metal ions into the growing hydrogel occurs in the first step, and the metal ions are reduced to metal nanoparticles by chemical reduction methods in the second step (Figure 4).

Figure 4. Preparation of PHEMA hydrogels doped with metal nanoparticles.
The intent of this thesis is to explore the hypothesis "that useful, porous PHEMA materials doped with Cu, Ag, or Au nanoparticles in a single step, by polymerisation of HEMA and a crosslinker in aqueous solution containing a Cu, Ag, or Au salt." To test this hypothesis, this project had the following experimental goals:

- **Synthesis of PHEMA hydrogels doped with Cu, Ag, or Au NPs.**
  PHEMA hydrogels will be synthesized by phase separation polymerization of HEMA with a suitable crosslinker, in water and in the presence of a salt of silver, copper, or gold. Ideally, PHEMA hydrogels doped with metal nanoparticles will be obtained in one step, but the two-step process will be explored if the one-step process is not successful.

- **Characterization of morphology of the PHEMA hydrogels.**
  Morphology of the PHEMA hydrogels doped with metal nanoparticles will be characterised by SEM and confocal microscopies. Of interest here is confirmation that the porosity characteristic of PHEMA hydrogels is maintained when the hydrogel contains metal nanoparticles. Porosity and size of polymer droplets in metal-doped hydrogels will be compared with those in control (metal-free) PHEMA hydrogels.

- **Characterization of metal nanoparticles.**
  Size and distribution of the metal nanoparticles will be characterised by various electron microscopies, such as transmission electron microscopy (TEM), high angle annular dark-field scanning transmission electron microscopy (HAADF-STEM), X-ray diffraction (XRD), and energy-dispersive X-ray spectroscopy (EDS). One aspect of interest here will be the effect of concentration of the metal salt precursor of metal NPs on the size of the NPs and their distribution within the hydrogel.
• **Investigation of mechanism of formation of metal nanoparticles.**
  Preliminary experiments will be conducted to elucidate likely mechanisms for formation of metal nanoparticles. These experiments will be particularly informative when the PHEMA materials doped with metal nanoparticles are formed in a single step, and where HEMA monomer, PHEMA chains, initiators and other species may be involved in the production of nanoparticles as well as in formation of the polymer network.

• **Investigation of leaching of metal nanoparticles.**
  Nanoparticles can slowly leach from hydrogels to confer useful activity, such as antibacterial activity, and so the leaching behaviour of nanoparticles will be evaluated.

• **Biological activity of PHEMA hydrogels doped with metal nanoparticles.**
  Cytotoxicity of leached nanoparticles will be assessed *in vitro* against human corneal epithelial cells, and antibacterial activity of silver and copper nanoparticles evaluated *in vitro* against *E. coli* and *S. aureus* bacteria. Low toxicity against human cells combined with toxicity toward bacteria would be beneficial for potential biomedical applications of PHEMA hydrogels doped with the metal nanoparticles.

• **Investigation of mechanical properties of PHEMA hydrogels doped with metal nanoparticles.**
  Inclusion of metal salts in polymerization formulations causes changes in the chemistry leading to the PHEMA hydrogel (for example, degree of crosslinking), the mechanical properties of PHEMA materials doped with metal nanoparticles may differ from those of
undoped PHEMA. These possibilities will be evaluated by compression testing studies of the hydrogels doped with metal nanoparticles, and the results will be compared to those for PHEMA hydrogels without nanoparticles.

The results for Cu-PHEMA, Ag-PHEMA, and Au-PHEMA are reported in the following chapters, one chapter devoted to each metal.
3. REFERENCES


(48) Casadio, Y. S.; Brown, D. H.; Chirila, T. V.; Kraatz, H. B.; Baker, M. V., Biodegradation of poly(2-hydroxyethyl methacrylate) (PHEMA) and poly{(2-hydroxyethyl methacrylate)-co-


Chapter 2.

Poly(2-Hydroxyethyl Methacrylate) Sponges Doped with Ag Nanoparticles as Antibacterial Agents

1. BACKGROUND

A polymer scaffold for tissue engineering applications must have certain properties, such as biocompatibility,\(^1\) high porosity,\(^1\) biodegradibility,\(^2-4\) and good mechanical integrity.\(^5\) In addition, properties that inhibit attachment of bacteria and colony formation on the surface of scaffolds increase the interest in polymeric materials for biomedical applications.

Incorporation of silver nanoparticles (Ag NPs) in various materials can be appropriately engineered to suit different applications such as water filtration,\(^6\) biosensing,\(^7\) food packaging,\(^8\) food preservation\(^8,9\) and antimicrobial paints.\(^10\) The antibacterial activity of Ag NPs\(^11\) has been exploited in medical applications such as wound healing,\(^12\) burn treatment\(^13\) and arthroplasty.\(^14\) Silver nanoparticles can also prevent bacterial adhesion on textile fabrics,\(^15\) medical devices,\(^16\) dental materials,\(^17\) titanium implants,\(^18\) vascular grafts,\(^19\) and polymer surfaces.\(^20,21\) Hydrogels based on polyacrylamide,\(^22\) poly(N-isopropylacrylamide),\(^23\) and poly(acrylamide-co-vinyl alcohol),\(^24\) and cellulose fibres\(^25\) have been functionalised with Ag NPs by exposure to solutions containing the nanoparticles. Fibres of poly(L-lactide),\(^26\) polyacrylonitrile,\(^27\) silk fibroin,\(^28\) poly(3-hydroxybutyrate-co-3-hydroxyvalerate)\(^29\) containing Ag NPs have been formed by electrospinning from solutions containing Ag NPs and the dissolved polymer. In an interesting study, Henríquez et al. formed poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels incorporating AgNPs in a single step, by photochemical polymerization of 2-hydroxyethyl methacrylate (HEMA), with concomitant formation of Ag NPs by photoreduction of Ag\(^+\) ions present in the polymerization mixture.\(^30\)
2-Hydroxyethyl methacrylate (HEMA) can be polymerized and copolymerized by either thermal\textsuperscript{31} or photochemical\textsuperscript{32} free radical polymerization. When HEMA is polymerized in bulk or in the presence of relatively small amounts of water, homogeneous PHEMA hydrogels are formed. However, from aqueous mixtures (>60\% H\textsubscript{2}O by volume), HEMA undergoes a polymerization-induced phase separation, because PHEMA becomes insoluble in water when chains reach about 20 repeat units in length.\textsuperscript{33} As the growing polymer chains separate from the aqueous solution they form polymer droplets.\textsuperscript{34,35} These droplets aggregate to form a hydrogel sponge containing a network of interconnected pores of dimensions on the micron scale.\textsuperscript{34} Importantly, porosity is suitable to accommodate ingress by cells. Depending on the polymerization formulation, porous PHEMA materials can have properties tailored to suit specific biomedical applications, such as use in artificial corneas,\textsuperscript{36} artificial skin,\textsuperscript{37} rhinoplasty surgery,\textsuperscript{38} surgical sutures,\textsuperscript{38} breast prosthesis,\textsuperscript{39} drug delivery systems,\textsuperscript{31,40,41} neural regeneration\textsuperscript{42-45} and most importantly for tissue engineering.\textsuperscript{46}

Henríquez et al. incorporated Ag nanoparticles into PHEMA during a photochemical polymerization under conditions that led to homogeneous hydrogels.\textsuperscript{30} Their PHEMA materials contained up to about 12\% Ag by weight (estimated by thermogravimetric analysis) and the presence of Ag NPs had no significant effect on infrared spectra or DSC properties of the materials.\textsuperscript{30} In the present study, we have formed Ag NP-PHEMA sponges by a photo-induced phase-separation polymerization from solutions containing ~ 80\% H\textsubscript{2}O by volume (Scheme 1). Thus, unlike the previous work of Henríquez et al., our study deals with porous PHEMA materials. Since the pores have dimensions in the micron range, these PHEMA sponges are of special interest for their potential applications in tissue engineering, where the porosity of a polymeric scaffold material is important to facilitate favorable interactions between the polymer scaffold and tissue.\textsuperscript{36,47}
Scheme 1. Schematic of structure of PHEMA sponges doped with Ag nanoparticles (Ag NP-PHEMA), and their formation in one step by photopolymerization in water.

The heterogeneous nature of porous Ag NP-PHEMA sponges makes these materials inherently more complex than analogous homogeneous Ag NP-PHEMA hydrogels, and prompts many questions. For example, during formation of porous Ag NP-PHEMA sponges, there is the possibility that Ag NPs can form under different conditions, from an aqueous phase during the early stages of polymerization, and from either an aqueous phase, an organic-rich phase, or an inter-phase region during later stages of polymerization. In the resulting Ag NP-PHEMA materials, how are Ag NPs partitioned between the two phases? Do they reside within the polymer droplets, on the surface of polymer droplets, or in the pores filled with residual aqueous phase? Furthermore, since formation of Ag nanoparticles and the polymerization of HEMA both involve radicals, does formation of Ag NPs have some effect on the morphology (e.g., size of polymer droplets and porosity) or the mechanical strength of the Ag NP-PHEMA sponges? We address these questions in this paper.
Henríquez et al. did not report any study of leaching of Ag NPs from homogeneous Ag NP-PHEMA, but found that the hydrogels showed activity against *E. coli* bacteria. Because Ag NP-PHEMA sponges are microporous, however, there is the potential for Ag-NPs to be leached from these materials very quickly, which would impact upon any antibacterial activity. Thus, our interest in potential applications in tissue engineering prompted us to investigate the leaching behaviour of Ag NP-PHEMA, to assess antibacterial activity against both Gram-positive (*E. coli*) and Gram-negative (*S. aureus*) bacteria, and to test for cytotoxicity against human cells. Due to our interest in ocular applications, we chose human corneal epithelial (HCE-T) cells for the cytotoxicity study, as they have been widely and successfully used to assess the safety of various substances for ocular surface applications. Chirila et al. developed an artificial cornea comprised of a central disk of solid PHEMA (which is cut into a lens shape to facilitate clear vision), surrounded by a skirt of porous PHEMA (with pores of a size suitable for penetration by corneal cells from the surrounding tissue, to help keep the artificial cornea in place). This artificial cornea has been commercialised as the AlphaCor™ keratoprosthesis, and implanted in thousands of patients for whom a donor tissue graft would not succeed. Further research into materials for this keratoprosthesis is desirable, not only because the device can be improved by conferring antibacterial activity, but also for extending the scope of using PHEMA sponges as a biomaterial in ophthalmology.
2. EXPERIMENTAL SECTION

2.1 Materials

2-Hydroxyethyl methacrylate (HEMA; Sigma-Aldrich), 2, 2-dimethoxy-2-phenylacetophenone (DPAP; Sigma-Aldrich), ethylene glycol dimethacrylate (EGDMA; Sigma-Aldrich), and silver nitrate (Lab Supply, Sydney, Australia) were used as received without further purification. Ultrapure water was obtained from a Milli-Q system and used for sample preparation, storage and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) studies.

2.2 Preparation of PHEMA sponges doped with Ag nanoparticles (Ag NP-PHEMA)

Crosslinked hydrogels were prepared via the photopolymerization of HEMA in aqueous solution. A cylindrical quartz vial (40 mm in height, 10 mm in inside diameter) was charged with HEMA (200 µL, 214 mg, 1.64 mmol), crosslinking agent EGDMA (8.4 mg, 42 µmol), silver nitrate (16 mg, 94 µmol) then ultrapure water (0.80 mL) was added dropwise with continuous stirring to form a clear homogeneous reaction mixture. A rubber septum was fitted to the vial, nitrogen was bubbled through the solution for 10 min via a syringe, and a solution of DPAP (7.0 µL of a 64 mg/mL in ethanol solution, 1.74 µmol) was added. The vial, still sealed with the rubber septum, was positioned 12 cm from a UV lamp (100 W, 350 nm, Blak-Ray B-100 AP high Intensity UV lamp) and irradiated for 20 min. A thermometer positioned adjacent to the samples during irradiation showed that the temperature remained within 1 °C of the ambient laboratory temperature (~ 25 °C). A control hydrogel (PHEMA) without nanoparticles was synthesized in a similar manner in the absence of silver nitrate; however, due to slower polymerization rate, the reaction time had to be increased to 30 min. Polymer hydrogels were removed from quartz vials and soaked in water, and the water was changed.
twice a day for a week to remove unreacted HEMA monomer. Samples were then stored in Milli-Q water until required for other experiments. The same procedure was used, but with smaller or larger amounts of silver nitrate, to prepare Ag NP-PHEMA samples with lower or higher levels of doping with Ag NPs.

2.3 Characterization of nanoparticle-doped hydrogels

2.3.1 Scanning electron microscopy (SEM)

Hydrated polymer samples for SEM were cut into 300 µm thick sections using a Vibratome sectioning system (Vibratome 3000, Ted Pella, Inc., USA) and stored in ultrapure water. These sections were dehydrated using increasing concentrations of ethanol in a Biowave microwave processing system (250 W) fitted with a Pelco cold spot (Pelco BioWave, Ted Pella, Inc., USA). Dehydrated samples were placed into a critical point dryer (Polaron KE3000, Quorum Technologies Ltd, U.K.), and flushed ten times with liquid CO$_2$ to remove ethanol and complete the permeation of liquid CO$_2$ into the polymer samples. After 1.5 h the specimen chamber was heated slowly to the critical point of CO$_2$ and vented gently to remove CO$_2$ gas.

Dry samples were mounted on double sided copper tape on a SEM stub, and coated with platinum (~ 3 nm) using a sputter coater (Polaron SC7640, Quorum Technologies Ltd, U.K.). Samples were imaged using a FEI Verios XHR SEM (ThermoFisher, USA) at 5 kV and a beam current of 0.10 nA.

2.3.2 Transmission electron microscopy (TEM)

Hydrated polymer samples were processed for TEM in a Biowave microwave processing system. Samples were dehydrated using increasing concentrations of ethanol in aqueous solution, followed by anhydrous acetone (250 W). These dehydrated samples were then
infiltrated with increasing Procure-Araldite epoxy resin:acetone mixtures (250 W, under vacuum), embedded in 100% resin for 1 h, and polymerized at 70 °C for 24 h. Resin-embedded polymer blocks were cut into 120 nm thick sections using an ultramicrotome equipped with a diamond knife (Leica EM UC6 Ultramicrotome, Leica Microsystems, Germany), placed on copper grids and imaged at 120 kV using a JEOL 2100 TEM (JEOL Ltd, Japan) equipped with a Gatan Orius SC1000 11M pixel digital camera. Size of nanoparticles was measured using Gatan Microscopy Suit Software. Ten TEM images were collected from different areas of polymer samples and were analyzed by Gatan software to calculate the size of nanoparticles.

2.3.3 High angle annular dark field scanning transmission electron microscopy (HAADF-STEM)

The Ag NP-PHEMA samples of 120 nm thickness that had been used for routine TEM imaging were further coated with carbon (~ 20 nm) for element analysis. High angle annular dark field (HAADF) imaging and element mapping were carried out using a FEI Titan G2 80-200 TEM/STEM (ThermoFisher, USA) with ChemiSTEM Technology operating at 200 kV. The element maps were obtained by energy dispersive X-ray spectroscopy (EDS) using the Super-X detector on the FEI Titan with a probe size ~ 1 nm and a probe current of ~ 0.9 nA.

2.4 Inductively coupled plasma optical emission spectrometry (ICP-OES)

Leaching of Ag NPs from Ag NP-PHEMA was studied using an ICP-OES instrument (Agilent Technologies 5100, Agilent, USA). Standard solutions of different concentrations were prepared from an Ag standard solution (1000±3 µg/mL) in 2% HNO₃ (from High-Purity Standards) by dilution in ultrapure water. These solutions were used to generate a linear calibration curve (signal intensity as a function of concentration).
Polymer scaffolds (15 mm thickness and 10 mm diameter) immediately after synthesis were transferred to Falcon tubes containing 10 mL ultrapure water. Water was drawn off using a pipette, transferred to separate flasks, and replaced with a fresh 10 mL of ultrapure water for twice a day, for a week. After water samples had been collected for a week, 5 mL HNO₃ (70 %) was added to each sample, followed by sufficient ultrapure water to obtain a final volume of 200 mL.

Leaching of Ag⁺ from Ag NP-PHEMA (5.73 mol%) was also studied for approximately 3 months. Polymer scaffold was transferred to a Falcon tube containing 5 mL of ultrapure water. At different time intervals, the water was drawn off using a pipette, transferred to a separate Falcon tube, and replaced with a fresh 5 mL of ultrapure water. After water samples had been collected for three months, 1 mL HNO₃ (70 %) was added to each sample, followed by sufficient ultrapure water to obtain a final volume of 8 mL. All the sample solutions were filtered using non-pyrogenic 0.20 µm filters before injection into the ICP analysis system and analysis for Ag.

2.5 Mechanical testing

Compression testing of cylindrical hydrogel specimens was carried out using an Instron Materials Testing System, Model 5943 (Instron, Norwood, MA, Figure S4) equipped with a 50 N load cell, at a crosshead speed of 60% of height/min. Hydrated specimens were compressed to 75% of the initial height at 25 °C, and the stress–strain plots were recorded. After the initial compression, specimens were soaked in water, and testing was repeated two more times. The compressive moduli were computed from the slopes of the linear regions of the plots. The mean values were calculated by averaging of two or three measurements.
2.6 Antibacterial studies

2.6.1 Agar diffusion method

Antibacterial activity was investigated against *Escherichia coli* ATCC 25922 (Gram negative) and *Staphylococcus aureus* ATCC 25923 (Gram positive) bacteria. Two or three colonies of an overnight culture of the organisms on blood agar were inoculated into trypticase soya broth (TSB) and incubated at 37 °C overnight. The overnight bacterial suspensions were diluted in saline to 0.5 McFarland turbidity standards using a nephelometer, corresponding to approximately $1.5 \times 10^8$ CFU/mL. Bacterial suspensions were evenly spread on the surface of agar plates using sterile cotton swabs. Hydrated polymer samples (10 mm diameter) were cut into 2 mm thick slices using a vibratome. Polymer slices with and without silver nanoparticles were placed onto the inoculated agar surface and incubated at 37 °C for 24 h. After incubation, the diameter of the inhibition zone around the polymer scaffold was measured with a ruler. The experiment was repeated three times.

2.6.2 Preliminary assessment of leachable Ag NPs antibacterial activity in broth culture system

A preliminary assessment of the antibacterial activity of the diffusible Ag NPs from the polymer scaffolds into liquid medium was carried out by placing polymer discs (10 mm diameter $\times$ 2 mm thick) in 2 ml TSB and inoculating with *E. coli* or *S. aureus*. A volume of 10 or 100 µL of bacterial suspensions (adjusted to ~ $1.5 \times 10^6$ CFU/mL) was added to 2 mL of TSB giving a final bacterial concentrations of $7.5 \times 10^3$ and $7.1 \times 10^4$ CFU/mL, respectively. Polymer disks of 10 mm diameter and 2 mm thickness incorporating Ag NPs were placed in the broth and the mixtures incubated at 37 °C for 24 h and 48 h. Tubes containing polymer discs and bacterial suspension were examined for turbidity after 24 h and 48 h incubation. At
24 h, a loopful of liquid medium was sampled from each tube and inoculated onto a blood agar plate, incubated at 37 °C and examined for growth at 24 h and 48 h. Control experiments under the same conditions were conducted using undoped PHEMA samples. The experiment was repeated three times.

2.7 Cytotoxicity

Hydrated polymer scaffolds (15 mm thickness and 10 mm diameter) were cut into 3-4 small pieces and dried at 60 °C to constant weight. Samples were sterilised with 70% ethanol by soaking for 1 h, and then rinsed two times with PBS followed by one rinse with the cell culture medium. Samples were placed in Falcon tubes with the medium (0.2 g of dry sponge/mL) and extracted for 48 h at 37 °C. After being centrifuged at 300 rcf for 5 min (to remove small pieces of scaffolds), the supernatant extract was used in cytotoxicity studies.

Immortalized human corneal epithelial (HCE-T) cells were initially established in T75 tissue culture flasks in Dulbecco-modified Eagle’s medium (DMEM) supplemented with GlutaMax™, 10% FBS and 1% Penicillin-Streptomycin. After harvesting, cells were seeded into 96 well plates, at a density of 8,000 cells per well, and incubated at 37 °C under 5% CO₂ for 24 h. The medium was then carefully removed and replaced with 100 µL of the supernatant extract and incubated for 24 h. Normal medium was utilized as a control. Cell viability was assessed using an MTT assay. The supernatant extracts (or normal medium in the case of the control) were carefully removed and replaced in 0.5 mg/mL MTT solution in PBS (100 µL/well), and further incubated for 3 h. Then, the MTT solution was removed and 0.04 M HCl/isopropanol (100 µL/well) was added. After 5-min shaking, 80 µL of solution was transferred into a new plate, and absorbance was measured at 570 nm. The assay was performed in octuplicate for two series of experiments, and the results were presented as a mean ± standard
deviation. Influence of polymer extracts on cell morphology was also analysed and photographed by a Nikon Eclipse® TS100 microscope (Tokyo, Japan) equipped with a Nikon DS-Fi1 camera using NIS Elements® software.

2.8. Statistical analyses

The results for compression testing of cylindrical hydrogel specimens were analysed for statistical significance, using the Kruskal-Wallis test (GraphPad Prism, v. 7). For cytotoxicity testing, one-way analysis of variance (ANOVA) in conjunction with Tukey-Kramer comparisons was used to assess statistical significance (GraphPad Prism, v. 7).

3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization

3.1.1 Formation of PHEMA sponges, and polymer morphology

We have shown previously that PHEMA sponges can be produced via polymerization-induced phase separation, by the irradiation of aqueous solutions containing HEMA and EGDMA (crosslinker), with initiator radicals being formed from the photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP).48, 51 In the present work, following our previous methods, PHEMA sponges were formed in about 30 min, as indicated by conversion of the initially transparent reaction mixture (solution) into an opaque white solid. When aqueous solutions containing HEMA, DPAP, EGDMA and silver nitrate were irradiated, Ag NP-PHEMA sponges were formed more rapidly than simple PHEMA sponges were formed under similar conditions without silver nitrate. PHEMA sponges were white, while Ag NP-PHEMA sponges were yellow-brown (Figure 1).
The rate of polymerization increased with increasing proportion of AgNO₃ in the reaction mixture; sponges were formed within 10 min and 20 min when concentration of AgNO₃ was 14.0 mol% and 5.73 mol% respectively, relative to HEMA. This result suggests that during the formation of Ag NP-PHEMA, radical species generated by photoreduction of silver ions supplement radicals generated by photolysis of DPAP to provide additional radicals for initiation of polymerization of HEMA. Photolysis of DPAP results in formation of benzoyl and methyl radicals, with benzoyl radicals being the predominant initiator species in polymerization of acrylates. It has been reported that in aqueous or ethanolic solution, photoreduction of silver ions involves electron transfer from a molecule of water or ethanol to an excited state silver ion, generating Ag NPs and radical species (HO• or HOĊH₂CH₃). Similar processes presumably occur in our photopolymerizations, to produce HO• (from H₂O) or species containing -OĊH₂CH₂O- moieties (from HOCH₂CH₂O groups of HEMA or PHEMA, analogous with HOĊH₂CH₃ species formed in ethanolic solutions). In principle, HO• radicals could initiate growth of new PHEMA chains, while -OĊH₂CH₂O- moieties in PHEMA chains could initiate branching. Nevertheless, the initiation processes arising from photolysis of DPAP must be the dominant initiation processes, since in the absence of DPAP; formation of Ag NP-PHEMA sponges was not complete even after 3-4 h irradiation.

**Figure 1.** Photograph of PHEMA and Ag NP-PHEMA samples.
SEM images confirmed that the PHEMA and Ag NP-PHEMA sponges had morphologies based on polymer droplets and a network of interconnected pores (Figure 2). For PHEMA, the diameter of the polymer droplets and the size of interconnected pores in PHEMA scaffolds were approximately 2 µm and 10-20 µm respectively. In contrast, for Ag NP-PHEMA, polymer droplets packed closer to each other and size of polymer droplets increased to 2.5 µm and 3.0 µm as concentration of silver nitrate increased from 2.87 mol % (Figure 2B) to 5.73 mol% (Figure 2C) respectively, while polymer droplet size decreased to 2.0 µm when concentration of silver nitrate increased to 14.0 mol% in reaction mixture.

The size of the polymer droplets that comprised the Ag NP-PHEMA sponges initially increased (Figure 2A, 2B) as the concentration of AgNO₃ in the polymerization mixture increased. We attribute this result to the generation of a additional radicals in the polymerization mixture by photoreduction of silver ions. Previous studies of polymerization induced phase-separation of PHEMA have shown that increasing the number of initiator radicals results in an increase the size of polymer droplets in the PHEMA sponge. When the concentration of AgNO₃ in the polymerization mixture was increased further, the polymer droplet size was reduced (Figure 2D). We attribute this result to the increase in ionic strength of the polymerization medium, causing the growing polymer chains to be “salted out” earlier in the polymerization-induced phase separation process.
3.1.2 Size and distribution of Ag nanoparticles

The size and distribution of the Ag nanoparticles were examined using TEM. We were especially interested to determine whether nanoparticles were dispersed throughout the PHEMA droplets or whether they were concentrated on the surfaces of PHEMA droplets (i.e. at the polymer/solution interface). In bright field TEM image recorded at the lowest resolution (Figure 3A) the droplet morphology of the PHEMA sponge seen in the SEM images (Figure 2) is again apparent. During sample preparation for TEM analysis, Araldite epoxy resin
infiltrated into the hydrogel and occupied the pores between the polymer droplets, providing structural integrity for sectioning. Thus, in the TEM images, the polymer droplets appear as higher intensity (lighter) regions, while darker areas represent pores filled with resin (see Fig 3A). Lighter and darker regions in the higher resolution images (Figure 3B-D) similarly represent polymer droplets and pores respectively, although the images show areas that are too small to contain whole polymer droplets.

The nanoparticles ranged from 2 nm to 23 nm in diameter for Ag NP-PHEMA (2.87 mol %), and as can be seen in Figure 3A and Figure 3B, were mostly located on or near the surface of the polymer droplets. When the feed ratio of AgNO$_3$ was increased to 5.73 mol % and 14.0 mol%, the diameter of the nanoparticles decreased to 2 nm to 12 nm (Figure 3C) and 2 nm to 9 nm (Figure 3D) respectively, and the nanoparticles appeared to become distributed throughout the polymer droplet rather than concentrated at the surface (Figure 3 C,D).

A decrease in the size of Ag NPs as the concentration of AgNO$_3$ is increased has been observed previously,$^{56,57}$ and has been attributed to a reduction of silver ions at higher concentration resulting in a larger number of nuclei generated, which in turn leads to faster nucleation of silver nanoparticles and smaller nanoparticles being formed.
Figure 3. Bright field transmission electron micrographs of 120 nm thick slices of Ag NP-PHEMA impregnated with Araldite epoxy resin. (A) Low resolution TEM image of an Ag NP PHEMA sample prepared from a mixture containing 2.87 mol% AgNO₃ relative to HEMA. The bright regions (indicative of light elements) correspond to PHEMA droplets (contain predominantly C, H, O). The dark regions (indicative of heavier elements) correspond to the resin infused into the sample to make it sufficiently robust for sectioning prior to TEM studies. The resin contains Cl, S, and Si, in addition to C, H, and O. (B) - (D) High resolution TEM images of Ag NP PHEMA samples prepared from polymerization mixtures containing 2.87 mol%, 5.73 mol%, and 14.0 mol% AgNO₃ relative to HEMA. The black spots correspond to Ag NPs.
High angle annular dark field (HAADF) STEM images and energy-dispersive X-ray spectroscopy (EDS) studies of Ag NP-PHEMA samples are shown in Figure 4. In the normal HAADF image of Ag NP-PHEMA (5.73 mol%) Figure 4A, the dark area represents the

Figure 4. STEM analysis of Ag NP-PHEMA (5.73 mol% AgNO3 relative to HEMA). (A) High angle annular dark field (HAADF) image of the sample. (B) Ag element analysis map. (C) EDS spectrum of area 1 from the image in (A). (D) EDS spectrum of area 2 from the image in (A). (Note: Cu peaks arise from copper grid used as the sample support. Cl, Si, S peaks arise from the resin used for sample preparation.)
HAADF image of Ag NP-PHEMA (5.73 mol%) Figure 4A, the dark area represents the polymer matrix while the brighter spots indicate heavy metals. STEM-EDS element mapping Figure 4B confirmed that the bright spots are related to silver metal. The results of EDS studies further confirm the conclusions drawn from the HAADF images. EDS of area 1 in a dark region of image (A) showed no Ag present, while EDS of area 2 of image (A) containing some bright spots showed the presence of Ag.

### 3.2 Leaching experiments

To remove any unreacted monomers prior to subsequent studies, PHEMA and Ag NP-PHEMA samples were soaked in water and the water was changed two times a day for a week. Some silver was leached from the Ag NP-PHEMA during this washing process and the amount of Ag present in the washings was measured by ICP-OES. We found that approximately 52-55% of silver initially present in the Ag NP-PHEMA formulation was removed during the washing step, and we therefore estimate that 45-48% of silver remained in polymer scaffolds. We tentatively suggest that during this initial washing process, any unreduced silver ions and silver nanoparticles suspended in the aqueous medium within the pores of the Ag NP-PHEMA sponge, while the remaining silver nanoparticles, being trapped between polymer chains within polymer droplets, was removed more slowly. When rinsing of an Ag NP-PHEMA (5.73 mol%) sample that had been washed with water over 9 days was subjected to extraction over an additional 81 days, only ~ 1% of silver present in the initial formulation ended was removed during the additional extraction period.
Figure 5. Concentration of Ag⁺ leached from Ag NP-PHEMA (5.73 mol%). The points joined by the red curve (referenced to the concentration axis on the left) shows a period of rapid leaching during the first 10 days, with the rate of leaching declining rapidly to near zero by day 12. Data for day 16 onwards (points with no line, plotted on a magnified scale, concentration axis on the right) shows that leaching actually continues at a low rate for many weeks.

3.3 Mechanical testing

Mechanical properties of PHEMA and Ag NP-PHEMA (5.73 mol%) scaffolds were assessed using an Instron material testing system. Compressive modulus was determined from the initial slope of the stress vs strain plot (i.e. 0-20% strain) and strength was measured at 70% strain. Compressibility for both types of scaffolds proved to be essentially reversible over three compression cycles with re-equilibration in water between each run. Figures 6A and 6B show stress-strain curves of PHEMA and Ag NP-PHEMA (5.73 mol%) scaffolds. The PHEMA sponge displayed the higher initial strength, but its compressive modulus and strength diminished appreciably with repeated compression cycles (i.e. two more times, Figure 6C and 6D). Interestingly, the compressive strength of the Ag NP-PHEMA matrix did not change
appreciably after repeated compression cycles, although its compressive modulus decreased after the first run (Figure 6C and 6D). The scaffold structure of PHEMA was altered by the presence of Ag NPs, as the polymer droplet size increased from 2 μm to 3 μm, and this may have affected the observed mechanical property. PHEMA and Ag NP-PHEMA sponges were not broken after three repeat compression tests indicating good mechanical strength of the sponges.

**Figure 6.** Compressive characteristics of hydrated PHEMA scaffolds. The first, second and third repeated compression stress-strain curves of (A) PHEMA and (B) Ag NP-PHEMA. Compressive (C) modulus and (D) strength. The bar represents mean ± standard deviation from two (Ag NP-PHEMA) or three (PHEMA) separate measurements. A Kruskal-Wallis test was used to assess statistical significance in each sample. * p < 0.05.
3.4 Cytotoxicity

PHEMA is well known for its biocompatibility, in particular its low toxicity toward cells. In biomaterials applications, Ag NP-PHEMA materials would be in contact with cells, so it is necessary to assess the toxic effects of nanoparticles leached from Ag NP-PHEMA. Samples of PHEMA and Ag NP-PHEMA were extracted in cell culture medium, and the extracts were then added to HCE-T cells cultured in 96-well plates and the plates were incubated for 24 h. An MTT assay showed that there was no significant cytotoxicity of Ag NPs leaching from Ag NP-PHEMA — the percentages of viable cells (% of control, no extract added) were 96±13% and 101±14% for HCE-T cells treated with PHEMA and Ag NP-PHEMA extracts, respectively. Furthermore, the morphology of HCE-T cells was not affected by exposure to the extract from PHEMA or Ag NP-PHEMA scaffolds for two days (Figure 7). Cells grown in the presence of PHEMA (Figure 7B) and Ag NP-PHEMA (Figure 7C) extracts showed typical corneal epithelial cell morphology (i.e. were nearly identical to the control, Figure 7A), suggesting good cytocompatibility of these polymer scaffolds.

![Figure 7](image.png)

**Figure 7.** Morphology of cells grown in: (A) control medium; (B) PHEMA extract; and (C) Ag NP-PHEMA extract.
3.5 Antibacterial properties

The antibacterial activity of PHEMA and Ag NP-PHEMA scaffolds was evaluated against *E. coli* and *S. aureus*, by measuring the zone of inhibition of bacterial growth around disks placed on Mueller-Hinton agar medium (Figure 8). No inhibition was seen around PHEMA disks, but Ag NP-PHEMA disks showed a prominent zone of inhibition for both *E. coli* and *S. aureus* indicating antibacterial activity.

![Image of inhibition zones](image)

**Figure 8.** Formation of inhibition zone around PHEMA and Ag NP-PHEMA discs against (A) *E. coli* and (B) *S. aureus* after 24 h incubation.

Further assessment of the antibacterial activity of Ag NP-PHEMA was carried out using a broth test. *E. coli* and *S. aureus* bacteria were inoculated into liquid medium containing PHEMA discs (Figures 9A and 9C respectively). The bacterial suspensions were turbid after 24 h of incubation, indicative of bacterial growth. In the presence of Ag NP-PHEMA discs, however, growth medium inoculated with *E. coli* or *S. aureus* (Figures B and D respectively) remained clear at 24 h and 48 h, indicating that the discs had leached sufficient antibacterial activity to prevent bacterial growth in the surrounding medium. The persistence of viable organisms was checked at 24 h by sampling a loopful of suspension from the liquid cultures.
and inoculating onto blood agar. After overnight incubation there was no growth of bacteria on agar plates inoculated from the 48 h liquid culture containing Ag NP-PHEMA discs.

Figure 9. Photograph of *E. coli* liquid culture incubated with (A) PHEMA (B) Ag NP-PHEMA and *S. aureus* liquid culture incubated with (C) PHEMA (D) Ag NP-PHEMA. Picture taken after 48 h incubation.

4. CONCLUSION

Porous PHEMA sponges doped with Ag nanoparticles can be synthesized in a simple, one step procedure from common starting materials. The presence of Ag nanoparticles does not have a significant impact on the appearance, morphology, or mechanical properties of the PHEMA sponges. The PHEMA sponges doped with Ag nanoparticles are non-toxic towards human corneal epithelial cells, but show activity against *E. coli* and *S. aureus* bacteria.
5. SUPPORTING INFORMATION

5.1 STEM-EDS analysis of Ag NP-PHEMA (5.73 mol%)

STEM-EDS analysis of PHEMA samples was undertaken according to procedures described in Section 2.3.3.

Figure S1. Scanning transmission electron microscopy (STEM) and energy dispersive X-ray spectroscopy (EDS) analysis of Ag NP-PHEMA (5.73 mol%).

(A) High angle annular dark field (HAADF) STEM image of Ag NP-PHEMA.

(B) Silver EDS map.

(C) Carbon EDS map.

(D) Oxygen EDS map.
5.2 Elemental analysis of Ag NP-PHEMA (5.73 mol\%)

Elemental analysis of PHEMA samples was undertaken according to procedures described in Section 2.3.3.

Figure S2. Elemental analysis of Ag NP-PHEMA (5.73 mol\%).
(A) HAADF-STEM image of Ag NP-PHEMA.
(B) EDS spectrum of area 1.
(C) EDS spectrum of area 2.
5.3 FT ATR-IR spectra of PHEMA and Ag NP-PHEMA

PHEMA and Ag NP-PHEMA hydrogel samples were cut into small sections and excess water was removed using kimwipes. FT ATR-IR spectra of the polymer samples were recorded at room temperature using PerkinElmer Spectrum One FT-IR spectrometer.

![FT ATR-IR spectra of (A) PHEMA, and (B) Ag NP-PHEMA (5.73 mol%).](image)

Figure S3. FT ATR-IR spectra of (A) PHEMA, and (B) Ag NP-PHEMA (5.73 mol%).
5.4 Compression testing of PHEMA hydrogels

Compression strength testing data were recorded according to the procedure described in Section 2.5.

Figure S4. Photographs showing compression testing of cylindrical hydrogels using the Instron Materials Testing System.
6. REFERENCES


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Chapter 3.

Poly(2-Hydroxyethyl Methacrylate) Hydrogels Doped with Gold Nanoparticles

1. BACKGROUND

The unique chemical, physical, and optical properties of gold nanoparticles\(^1\) (henceforth, Au NPs) have attracted increasing interest in such diverse fields as biomedicine,\(^2,3\) drug delivery,\(^2\) antimicrobial materials,\(^4\) biosensors\(^5\) and catalysis.\(^6-10\) Gold nanoparticles have been incorporated into matrices based on poly(N-isopropylacrylamide),\(^11,12\) graphene,\(^13\) methacrylated gelatin,\(^14\) polystyrene,\(^15\) and nylon-11.\(^16\) Fibres based on polymers such as cellulose,\(^17\) β-cyclodextrin/poly(N-vinylpyrrolidone),\(^18\) poly(vinyl alcohol),\(^19\) and wool\(^20\) have been also functionalized with Au NPs. Common methods to introduce Au NPs into polymers include: (1) \textit{in situ} chemical reduction of Au salts inside polymer hydrogels;\(^11-13,17,18\) (2) electrospinning fibers from a solution containing a polymer and Au NPs;\(^19\) and (3) evaporation of Au onto polymer films.\(^16\) Polymers such as poly(N-vinyl-2-pyrrolidinone),\(^21\) poly(ethylene glycol),\(^22\) chitosan,\(^23\) poly(diallyldimethylammonium chloride),\(^24\) poly(ethylene oxide) - poly(propylene oxide) - poly(ethylene oxide) block copolymer\(^25\) can act as both \textit{in situ} reducing agents and as stabilizers of metal nanoparticles. Functionalizing these polymers with Au NPs can thus be achieved by using Au salts as nanoparticle precursors and using the polymer to both reduce the Au salts and then to stabilize/immobilize the resulting nanoparticles and thus a separate synthesis of Au nanoparticles is not required.

Poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels can be synthesized by microwave-assisted polymerization,\(^26\) \(\gamma\)-irradiation,\(^27\) UV-irradiation\(^28,29\) and thermal polymerization of 2-hydroxyethyl methacrylate (HEMA).\(^30\) When HEMA polymerizes in water, it produces a specific morphology of hydrogels largely dependent on its molecular
weight and extent of crosslinking. PHEMA chains having molecular weight >2800 Da (which is the case in this study) are not water soluble and separate from water to form a 3D network of polymer droplets with interconnected pores. PHEMA hydrogels have been used in many biomedical applications such as drug release, artificial cornea, soft contact lenses, and tissue engineering. In previous work in our laboratory, we have synthesized PHEMA hydrogels doped with silver nanoparticles (Ag NPs) in one step using simultaneous photo-induced phase separation polymerization of HEMA with concurrent photoreduction of Ag ions in aqueous solution.

In this paper, we report the synthesis of PHEMA hydrogels doped with Au NPs using either a photochemical or thermal induced phase separation polymerization of HEMA in water containing either Au(I) or Au(III) ions. For the photopolymerization, 2,2-dimethoxy-2-phenylacetophenone (DPAP) was used as a photoinitiator to form radicals that both initiated the polymerization of HEMA and reduced Au(III) ions to Au(0). HEMA monomers contain hydroxyl groups that can also reduce Au(III) ions and stabilize Au NPs. The effect of concentration of Au(I) and Au(III) ions on polymer morphology as well as on size and distribution of Au NPs inside polymer hydrogels was investigated. In addition, we report the synthesis of PHEMA hydrogels doped with Au NPs in a one-step thermal procedure, in which HEMA and/or PHEMA chains acted as a reducing agent and PHEMA chains acted as an immobilizing/stabilizing agent. It has previously been reported that some alcohols and polymers bearing hydroxyl groups can reduce Au cations and stabilize Au NPs. The morphology of these polymer hydrogels was characterized by scanning electron microscopy (SEM) and confocal microscopy. The size and distribution of Au NPs inside PHEMA hydrogels were characterized by transmission electron microscopy (TEM) and high angle annular dark-field scanning transmission electron microscopy (HAADF-STEM). The leaching of the Au NPs into aqueous media was studied by inductively coupled plasma optical
emission spectrometry (ICP-OES). The mechanical strength of the Au NPs-doped PHEMA hydrogels was evaluated in a mechanical tensile/compression testing system.

Scheme 1. Schematic representation of formation of Au-PHEMA hydrogels in one step via photochemical or thermal polymerization in water.

2. EXPERIMENTAL SECTION

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA; Esstech Inc., USA), ethylene glycol dimethacrylate (EGDMA; Sigma-Aldrich), 2,2-dimethoxy-2-phenylacetophenone (DPAP; Sigma-Aldrich), potassium persulfate (Unilab, Ajax Chemical Co Pty Ltd, Sydney), ethylene glycol (Sigma Chemicals Pty Ltd, WA), poly(vinyl alcohol) (PVA, average $M_w$ 13-23 kDa, 87-89% hydrolysed; Polysciences, Inc., Warrington, PA), and rhodamine B isothiocyanate (Aldrich) were used as received without further purification. Potassium tetrachloroaurate ($\text{KAuCl}_4$) was
synthesized by modification of literature methods. Chloro(dimethylsulfide)gold(I) was synthesized according to literature procedures. Ultrapure water was obtained from a Milli-Q system and used for sample preparation and storage of hydrogels.

2.2. Preparation of PHEMA Hydrogels Doped with Au NPs by Photo-induced Phase Separation Polymerization

The hydrogels PHEMA<sub>hν</sub> and Au-PHEMA<sub>hν</sub> #1-5 were prepared as described previously for their Ag NP counterparts by photo-induced phase separation polymerization of HEMA in water in the presence of different precursors of Au NPs, according to formulations in Table 1. The reactions were initiated by DPAP (4.0 µL of a 64 mg/mL in ethanol solution, 1.0 µmol) and under irradiation (Blak-Ray B-100 AP high-Intensity UV lamp, 100 W, 350 nm) for 30 min. The cylindrical hydrogel samples were carefully removed from the vials (with the aid of a small spatula) and soaked in ultrapure water (~ 10 mL) with the water being replaced twice a day for 5 days. When this rinsing process was completed, the water washings were combined and the Au content was measured using ICP-OES. The hydrogel samples were stored in water until needed for further studies (SEM, TEM, XRD, etc.).

2.3. Preparation of PHEMA Hydrogels Doped with Au NPs by Thermally-induced Phase Separation Polymerization

A borosilicate glass test tube (75 mm in height, 8 mm in inside diameter) was charged with HEMA (250 µL, 268 mg, 2.0 mmol), EGDMA (6 µL, 6.3 mg, 31.8 µmol), KAuCl<sub>4</sub> (1.1 mg, 3.0 µmol) and a solution of potassium persulfate (4.5 mg, 17.0 µmol) in ultrapure water (0.78 mL). The mixture was stirred for 5 min, then the test tube was sealed with a rubber septum and the solution was purged with nitrogen for 10 min via a syringe. The test tube was then placed in a water bath and maintained at ~ 70 °C temperature for 60 min.
During this time the reaction mixture changed from a yellow solution to a mauve solution, and, which became turbid after about 30 min and finished as an opaque mauve solid (Au-PHEMAΔ). The cylindrical polymer sample was carefully removed from the test tube (with the aid of a small spatula) and soaked in ultrapure water (~ 10 mL) in a glass vial, with the water being replaced twice a day for 5 days. When this rinsing process was complete, the leached Au content in the combined water washings was measured using ICP-OES. A control hydrogel (PHEMAΔ) was synthesized by the same procedure but in the absence of KAuCl₄. The polymerization mixture became turbid within 5 min of commencement of heating at ~ 70 °C and finished as an opaque white solid. Polymer hydrogels were stored in water until needed for further studies (SEM, TEM, XRD, etc.).
Table 1. Formulations of PHEMA and Au-PHEMA hydrogels

<table>
<thead>
<tr>
<th>Hydrogel $^{a}$</th>
<th>HEMA (mmol)</th>
<th>EGDMA (mmol)</th>
<th>KAuCl$_4$ (µmol, mol% of HEMA)</th>
<th>(CH$_3$)$_2$SAuCl (µmol, mol% of HEMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHEMA$_{hv}$</td>
<td>1.0</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Au-PHEMA$_{hv}$ #1</td>
<td>1.0</td>
<td>0.02</td>
<td>0.2 (0.02 mol%)</td>
<td>-</td>
</tr>
<tr>
<td>Au-PHEMA$_{hv}$ #2</td>
<td>1.0</td>
<td>0.02</td>
<td>0.8 (0.08 mol%)</td>
<td>-</td>
</tr>
<tr>
<td>Au-PHEMA$_{hv}$ #3</td>
<td>1.0</td>
<td>0.02</td>
<td>1.4 (0.14 mol%)</td>
<td>-</td>
</tr>
<tr>
<td>Au-PHEMA$_{hv}$ #4</td>
<td>1.0</td>
<td>0.02</td>
<td>-</td>
<td>1.4 (0.14 mol%)</td>
</tr>
<tr>
<td>Au-PHEMA$_{hv}$ #5</td>
<td>1.0</td>
<td>0.02</td>
<td>-</td>
<td>2.7 (0.27 mol%)</td>
</tr>
<tr>
<td>PHEMA$_{\Delta}$</td>
<td>2.0</td>
<td>0.032</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Au-PHEMA$_{\Delta}$</td>
<td>2.0</td>
<td>0.032</td>
<td>3.0 (0.15 mol%)</td>
<td>-</td>
</tr>
</tbody>
</table>

$^{a}$ Hydrogels PHEMA$_{hv}$ and Au-PHEMA$_{hv}$ were synthesized according to the procedure described in Section 2.2, in water (0.4 mL) with DPAP (1.0 µmol) as a photoinitiator. Hydrogels PHEMA$_{\Delta}$ and Au-PHEMA$_{\Delta}$ were synthesized according to the procedure described in Section 2.3, in water (0.78 mL) with potassium persulfate (17 µmol) as thermal initiator.
2.4. Characterization of Morphology and Mechanical Strength of Hydrogels

The morphology of the hydrogels was examined by scanning electron microscopy (SEM) and confocal microscopy. Before characterization by SEM, samples were vibratomed to produce thin sections (400 µm thickness), dried in a critical point dryer (Polaron KE3000, Quorum Technologies Ltd, U.K.) according to the procedure described in our previous work, and coated with 3 nm platinum using a sputter coater (Polaron SC7640, Quorum Technologies Ltd, U.K.). SEM was performed using a FEI Verios XHR SEM (ThermoFisher, USA) operating at 3 kV with a beam current of 100 pA.

To prepare samples for confocal microscopy, hydrogel sections (400 µm thickness) were functionalized in a 0.05% w/v aqueous solution of fluorescent rhodamine B isothiocyanate (RBITC) for 12 h at 37 °C in dark. To remove excess RBITC, the sections were subsequently soaked in Milli-Q water for 3 days at 6 °C, with the water being replaced every 12 h. Following a final thorough rinsing in water, hydrogel samples were stored in the dark at 6 °C to prevent the samples from photobleaching. Hydrated polymer samples were placed on glass-bottom microwell dishes (35 mm petri dish, 10 mm microwell and 0.16-0.19 mm cover glass) and imaged using a confocal microscope (Nikon Ti-E inverted motorized microscope equipped with Nikon A1Si spectral detector and NIS-AR Elements software). Images were collected with a resolution 1024 × 1024 pixel, 5.9 µs dwell time, and 20× dry objectives using a 561 nm laser for RBITC excitation.

The compression tests of hydrated, cylindrical PHEMA specimens were performed using an Instron 5943 microtester (Instron, Norwood, MA) with a 50-N load cell and recorded under a crosshead speed of 60% of height/min to 75% of the initial height at 25°C. Compressive stress versus strain plot was then generated. The initial slope of the plot (i.e. 0-20% strain) was used to determine the compressive modulus, and strength was measured at 70% strain. The
mean values were calculated from two (Au-PHEMAΔ) or three (PHEMAΔ) measurements. The method of statistical analysis used to compare the compression results of specimens was a Student’s t-test (GraphPad Prism, v. 7).

2.5. Characterization of Au NPs

The Au NPs were characterized using a combination of UV-visible spectroscopy, transmission electron microscopy (TEM), high angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and X-ray diffraction. The UV-visible absorption spectra of the nanoparticles in suspensions were measured with a Cary 60 UV-Vis spectrometer (Agilent, CA, USA; 250 to 700 nm) equipped with a xenon flash lamp and a double beam optical configuration.

For TEM studies, hydrogels were embedded in Procure-Araldite epoxy resin. After curing of the epoxy resin at 60 °C, 150-nm thick sections were cut and mounted onto copper TEM grids, and samples were prepared for TEM analysis according to a previously described procedure.\textsuperscript{36} Routine TEM imaging was performed at 120 kV using a JEOL 2100 TEM (JEOL Ltd, Japan) equipped with an 11 M pixel digital camera (Gatan Orius SC1000). For HAADF-STEM, similar polymer sections (150 nm thickness, embedded in Procure-Araldite epoxy resin) were coated with carbon (~ 20 nm) using a sputter coater and imaged using a FEI Titan G2 80-200 TEM/STEM (ThermoFisher, USA) with ChemiSTEM Technology, operating at 200 kV. Element maps were obtained by energy-dispersive X-ray spectroscopy (EDS) using the Super-X detector on the FEI Titan with a probe size of ~ 1 nm and a probe current of ~ 0.9 nA.

Prior to X-ray diffraction studies, the hydrogel samples were dried overnight in a vacuum oven at 50 °C. The dry polymer samples were ground into a fine powder and placed
on a monocrystalline silicon sample holder (Sil'tronix Silicon Technologies, Archamps, France). X-ray diffraction was performed using a Panalytical Empyrean X-ray powder diffractometer (Malvern Panalytical Ltd, Malvern, UK), using CuKα radiation collected in reflection (Bragg-Brentano) geometry using a programmable spinner stage, with a step size of 0.01° and an acquisition time of 3000 s. Qualitative analysis of the composition of the samples was conducted using the HighScore Plus diffraction software.\textsuperscript{41}

The concentration of leached Au in washings of Au-PHEMA hydrogels was determined using ICP-OES (Agilent Technologies 5100 Series ICP-OES, Agilent, USA). Standard solutions of different concentrations were prepared from an Au standard solution (1000±3 \( \mu \text{g/mL} \)) in 2\% HCl (from High-Purity Standards, North Charleston, SC, USA) by dilution with ultrapure water. These solutions were used to generate a linear calibration curve (signal intensity as a function of concentration).

3. RESULTS AND DISCUSSION

3.1. Preparation of PHEMA and Au-PHEMA Hydrogels

PHEMA (with no Au NPs) and Au-PHEMA hydrogels were prepared from aqueous solutions containing HEMA monomer, EGDMA as a crosslinker, and an initiator, using polymerization-induced phase separation (see Table 1 for details). For Au-PHEMA hydrogels, either KAuCl\(_4\) or (CH\(_3\))\(_2\)SAuCl were used as the precursor for the Au NPs. Polymerization was achieved thermally or photochemically, using K\(_2\)S\(_2\)O\(_8\) as a thermal initiator or DPAP as a photoinitiator. During phase separation, polymer chains form and evolve into droplets. These droplets slowly separate from the aqueous phase and form a three-dimensional interconnected porous white opaque hydrogel.\textsuperscript{28,42,43} PHEMA hydrogels are opaque white, while Au-PHEMA hydrogels were pink-purple with the coloration attributed to the presence of Au NPs (Figure 1). In one case (Au-PHEMA\(_{hv}\) prepared from a formulation containing 0.27 mol\% (CH\(_3\))\(_2\)SAuCl (Au-
PHEMA\textsubscript{hν} #5), Au NPs began to form as soon as the reagents were mixed, and some settled as a purple precipitate at the base of the test tube, while the Au-PHEMA\textsubscript{hν} hydrogel that was formed was uniform in appearance. In all other cases, noticeable formation of Au NPs, as indicated by onset of a purple colouration, did not commence until the polymerization mixture was heated (for Au-PHEMA\textsubscript{Δ} samples) or irradiated (for Au-PHEMA\textsubscript{hν} samples).

![Figure 1. PHEMA and Au-PHEMA hydrogels prepared photochemically.](image)

Immediately after preparation, the hydrogels were washed in water for five days to remove the unreacted monomer and any loosely bound Au salts or Au NPs. The concentrations of Au in the combined washings for each of the samples were measured using ICP-OES. The results showed that, generally, ≥ 95% of the Au in the initial polymerization mixture was retained in the hydrogel, regardless of the type of polymerization (thermal or photochemical) or the source Au compound. The only exceptional case was hydrogel Au-PHEMA\textsubscript{hν} #5, which retained only 40% of the Au present in the original formulation mixture. This low retention of Au is presumably a consequence of much of the (CH\textsubscript{3})\textsubscript{2}SAuCl in the initial formulation for Au-PHEMA\textsubscript{hν} #5 (0.27 mol%) being reduced to Au NPs that precipitated from the mixture before significant polymerization occurred.
3.2. Mechanism of Formation of Au NPs

To gain insight into the formation and stabilization of Au NPs inside the hydrogels by photo- and thermal polymerization, additional experiments were conducted according to the formulations in Figure 2 and Figure 4.

![Figure 2](image.png)

**Figure 2.** Results of irradiation of various formulations containing Au(III). Reactions were carried out in borosilicate glass test tubes (75 mm in height, 8 mm in inside diameter) at room temperature. Reaction mixtures were purged with nitrogen for 5 min and sealed with rubber septum before exposing to UV lamp. Mixtures in Tests 1-3 contained KAuCl₄ (1.8 μmol) in H₂O (0.52 mL), and, as indicated, HEMA (1 mmol) and DPAP (0.5 μmol). Mixtures in Tests 4 and 5 contained KAuCl₄ (1.8 μmol) and PVA (MW 13-23 kD, 40 mg) in H₂O (1.0 mL), and, as indicated, DPAP (0.5 μmol).

Irradiation of solutions of KAuCl₄ in water (Figure 2, Test 1) or water/ethylene glycol mixtures (Figure S4, Test 6) for 5 h did not result in formation of Au NPs. In these experiments, only an absorption band near 310 nm corresponding to AuCl₄⁻ in aqueous solution was
observed in the UV-visible absorption spectrum (Figure 3, Test 1). These results show that neither H₂O nor ethylene glycol reduce AuCl₄⁻ under the experimental conditions.

Irradiation of a solution of KAuCl₄ in water containing HEMA (Figure 2, Test 2) resulted in a pale pink coloration, indicating formation of some Au NPs and suggesting that HEMA can reduce AuCl₄⁻ to Au NPs. After 5 h of irradiation, however, most of the AuCl₄⁻ had not been reduced, as indicated by the pale coloration of the mixture and the presence of an absorption band near 310 nm due to residual Au(III) (Figure 3, Test 2). Thus, reduction of Au(III) by HEMA alone (either by the hydroxyethyl moiety or the acrylate moiety) cannot account for the formation of Au(0) in the form of Au NPs in the Au-PHEMA sponge.

In a separate experiment (not shown in Figure 2), we found that when a suspension of DPAP (0.5 μmol) in a solution of KAuCl₄ (1.8 μmol) in H₂O (0.52 mL) was stirred and irradiated for 20 min, a burgundy precipitate (presumably Au NPs) was formed but the solution remained yellow. This experiment was complicated by the insolubility of the DPAP in the reaction mixture, and by the precipitation of the Au NPs from the reaction mixture. Nevertheless, this result suggests that DPAP can reduce Au(III) to form Au NPs. DPAP is known to form ketyl radicals under photochemical conditions, and Marin et al. have already reported that ketyl radicals are powerful reducing agents that can reduce Au(III) ions to Au(0).⁴⁴

In contrast to the above results, irradiation of a solution of KAuCl₄ in water containing both HEMA and DPAP (Figure 2, Test 3) resulted in complete reduction of Au(III) within 20 min. The dark burgundy colored solution showed a strong absorption band at 540 nm in UV-visible spectrum for Au NPs and no peak near 320 nm, in the region expected for Au(III) ions (Figure 3, Test 3). This result again suggests that DPAP can reduce Au(III) to form Au NPs, but we cannot exclude the possibility that PHEMA, formed by polymerization initiated by DPAP, also serves as reducing agent. Indeed, there is insufficient DPAP used on this
experiment for DPAP alone to account for all the reduction of Au(III) to Au NPs. Au NPs have been reported to autocatalyse further reduction of Au(III),\textsuperscript{45} and in our experiments Au NPs may catalyse reduction of Au(III) by HEMA, by its C=C or hydroxyethyl moieties. Olefinic groups are known to be strong reducing agents for Au(III) under certain conditions\textsuperscript{46} but we cannot assess the ability of the C=C group in HEMA directly in our work due to olefinic polymerization of HEMA under the reaction conditions, the polymer PHEMA also being a potential reducing agent. Interestingly, in this experiment, the Au NPs remained in solution, presumably being stabilized by PHEMA that was formed during the experiment.

The poor solubility of PHEMA in water prevented us from assessing the influence of the polymer on formation of Au NPs, so we used poly(vinyl alcohol) (PVA) as a model system. PVA, like PHEMA, is rich in aliphatic alcohol moieties. Irradiation of aqueous solutions of KAuCl\textsubscript{4} containing PVA for 5 h (Figure 2, Test 4) resulted in a pale pink coloration indicative of formation of some Au NPs. When the experiment was repeated with DPAP (Figure 2, Test 5), Au(III) was reduced to Au NPs within 20 min, as indicated by formation of a dark burgundy-coloured solution having $\lambda_{\text{max}} = 570$ nm (Supporting Information Figure S5). This result is consistent with a sequence where DPAP reduces some Au(III) to Au NPs, and the Au NPs in turn catalyse reduction of more Au(III) by aliphatic alcohol moieties in PVA. Similar chemistry should occur for Au(III) in the presence of DPAP and PHEMA.

When Au(III) ions in water-ethylene glycol mixture were irradiated for 5 h without DPAP present (Figure S4, Test 6) (Supporting Information Figure S4), the colour of reaction mixture did not change. On the other hand, when Au(III) ions in water-ethylene glycol mixture were irradiated for 20 min in the presence of DPAP initiator (Figure S4, Test 7) (Supporting Information Figure S4), formation of Au NPs was indicated by the reaction mixture becoming dark burgundy in colour and showing an absorption band at 540 nm (Supporting Information
Figure S5). This result is again consistent with a model where DPAP serves to reduce some Au(III) to form Au NPs, with the Au NPs catalysing further reduction of Au(III), in this case with ethylene glycol serving as the reducing agent. In this experiment, however, in the absence of a surfactant-type polymer$^{47,48}$ (such as PVA or PHEMA), the Au NPs were not stable and precipitated out after 24 h.

Taken together, the results of these experiments suggest that aliphatic alcohol groups in HEMA, PHEMA or PVA can function as the reducing agents for Au(III) to generate Au NPs, but the reduction of Au(III) to Au NPs by initiator radicals (formed from DPAP) is faster, and is a crucial first step in the process.
Figure 3. The UV-visible absorbance spectra of aqueous KAuCl₄ solution (Test 1), solution of KAuCl₄ in aqueous-HEMA reaction mixture after 5h of irradiation (Test 2), and solution of KAuCl₄ and DPAP in aqueous-HEMA reaction mixture after 20 min of irradiation (Test 3).

While DPAP clearly plays an important role in reduction of Au(III) to form Au NPs under photochemical conditions, the same is not the case when the gold source is (CH₃)₂SAuCl. A mixture containing (CH₃)₂SAuCl (1.4 μmol), H₂O (0.52 mL), and HEMA (1 mmol) under nitrogen was initially colourless but became turbid and mauve coloured within 5 min under ambient laboratory light. This result shows that under low-light conditions, a water-HEMA mixture is sufficient to reduce Au(I) to form Au NPs. When the experiment was repeated with DPAP (0.5 μmol) added to the mixture, the outcome was the same, suggesting that DPAP has no direct role in reduction of Au(I) to Au NPs.
Figure 4. Results of heating of various formulations containing Au(III). Reactions were carried out in borosilicate glass test tubes (75 mm in height, 8 mm in inside diameter) at room temperature. Reaction mixtures were purged with nitrogen for 5 min and sealed with rubber septum before heating to ~70 °C. Mixtures in Tests 8-11 contained KAuCl₄ (3.5 μmol) in H₂O (0.80 mL), and, as indicated, HEMA (2 mmol) and K₂S₂O₈ (16 μmol). The mixture in Test 12 contained KAuCl₄ (3.5 μmol) and PVA (MW 13-23 kD, 80 mg) in H₂O (1.6 mL).

Thermal polymerizations of HEMA used potassium persulfate as initiator, which undergoes thermolysis to form sulfate radicals. Sulfate radicals initiate polymerization, but they can also react with water to produce hydroxyl radicals, which themselves can also initiate polymerization. Test experiments showed that at ~ 70 °C, water alone (Figure 4, Test 8) or K₂S₂O₈ and any species arising from its thermolysis in water (Figure 4, Test 9) do not reduce Au(III) to form Au NPs.

In water at 70 °C, HEMA demonstrates a weak ability to reduce Au(III), as indicated by the initially yellow Au(III)/HEMA/water solution turning pale pink after 5 h (Figure 4, Test 10). The UV-visible spectrum of the solution displayed a weak absorption band centred near
560 nm (Figure 5, Test 10), consistent with the formation of Au NPs. In the presence of both K$_2$S$_2$O$_8$ and HEMA, however, Au(III) is rapidly reduced to form Au NPs (Figure 4, Test 11). The UV-visible spectrum of solution displayed an absorption band at 550 nm (Figure 5, Test 11), consistent with formation of Au NPs, which were stable in (i.e., did not precipitate from) the reaction mixture. Under the condition of this experiment, HEMA would be polymerised to form PHEMA, which should stabilise Au NPs in solution and, as discussed above, should also serve as a reducing agent for AuCl$_4^-$
. PVA also served as efficient reagents to reduce Au(III) to Au NPs and stabilise the Au NPs under the reaction conditions (Figure 4, Test 12).
Figure 5. The UV-visible absorbance spectra of solution of KAuCl₄ in aqueous-HEMA reaction mixture after 5h of heating (Test 10), solution of KAuCl₄ and K₂S₂O₈ in aqueous-HEMA reaction mixture after 20 min of heating (Test 11), and solution of KAuCl₄ in aqueous-PVA reaction mixture after 30 min of heating (Test 12).

3.3. Morphology of the PHEMA Hydrogels

The PHEMAΔ and Au-PHEMAΔ hydrogels showed the expected polymer droplet morphology (Figure 6). The appearance of the polymer droplets was similar in the images recorded by confocal microscopy and SEM, indicating that the morphology is robust enough to withstand the drying process prior to the SEM studies. The average size of polymer droplets was slightly larger for Au-PHEMAΔ (~ 7 μm) than for PHEMAΔ (~ 4.5 μm). We tentatively suggest that this difference in size is due to differences in solubility of the PHEMA formed in each case. This suggestion is consistent with our observation that the onset of turbidity due to separation of the polymer phase from the aqueous phase occurred after ~ 5 min in experiments leading to PHEMAΔ, but did not occur until ~ 30 min in experiments leading to Au-PHEMAΔ. In previous studies of PHEMA/PEGMA sponges (PEGMA = poly(ethylene glycol) methacrylate) we
found that size of polymer droplets increased as the solubility of the polymer increased. In the present study, we suggest that reduction of Au(III) by PHEMA chains formed during the early stages of formation of Au-PHEMA would involve generation of radical sites on PHEMA, which would in turn initiate polymerization and lead to branched PHEMA; branched polymers are typically more soluble than their non-branched counterparts.

The PHEMA and the Au-PHEMA hydrogels prepared using 0.02 or 0.08 mol% Au(III) as the source of Au NPs also showed the expected polymer droplet morphology (Figure 7). According to the SEM images, the size of polymer droplets was ~ 2.0 µm for PHEMA but ~ 3.5 µm for the Au-PHEMA samples prepared using 0.02 and 0.08 mol% Au(III). Confocal microscopy of the Au-PHEMA hydrogel prepared using 0.12 mol% Au(III), however, showed a morphology that, while clearly porous, had only poorly formed droplets in a branch-like network. This morphology was not robust, and collapsed during drying, so that no droplet morphology was apparent when the sample was examined by SEM. The dehydration processes used in sample preparation can affect the native morphology of hydrogels.

Our findings in Section 3.2 indicate that some of the initiator (DPAP) must be consumed to reduce Au(III) ions to Au NPs. When the concentration of Au(III) increases, more DPAP will be consumed to reduce Au(III) ions to Au NPs, leaving less DPAP to initiate polymerization. As a result, polymerization and crosslinking likely occurs less efficiently as the concentration of Au(III) increases. It has been already reported by Chirila et al. that the size of PHEMA polymer droplets increased as the degree of crosslinking decreased.
Figure 6. Confocal microscopy images (upper panels) and SEM images (lower panels) of a PHEMA$_\Delta$ hydrogel and a Au-PHEMA$_\Delta$ hydrogel. The hydrogels were synthesized by thermally induced phase separation polymerization according to the formulations in Table 1. PHEMA$_\Delta$ hydrogel synthesized without KAuCl$_4$ and Au-PHEMA$_\Delta$ hydrogel was synthesized using 0.15 mol% of KAuCl$_4$ relative to HEMA. (A 3D confocal image of Au-PHEMA$_\Delta$ hydrogel was also recorded—sees Supporting Information S6.)
Figure 7. Confocal microscopy images (upper panels) and SEM images (lower panels) of a PHEMA<sub>hν</sub> hydrogel and Au-PHEMA<sub>hν</sub> #1-3 hydrogels incorporating Au from KAuCl<sub>4</sub>. The hydrogels were synthesized by photochemically induced phase separation polymerization. Au-PHEMA<sub>hν</sub> #1, Au-PHEMA<sub>hν</sub> #2, and Au-PHEMA<sub>hν</sub> #3 hydrogels were synthesized using 0.02, 0.08, and 0.14 mol% of KAuCl<sub>4</sub> relative to HEMA according to the formulations in Table 1.

For Au-PHEMA<sub>hν</sub> hydrogels prepared using a Au(I) compound, (CH<sub>3</sub>)<sub>3</sub>SAuCl, as the precursor of Au NPs, formulations containing 0.14 mol% Au(I) resulted in a stable polymer droplet morphology (Figure 8). In these Au-PHEMA<sub>hν</sub> hydrogels the average droplet size was ~3.5 μm, again slightly larger than for the corresponding Au-free PHEMA<sub>hν</sub> hydrogel. For Au-PHEMA<sub>hν</sub> hydrogels prepared using formulations containing 0.27 mol% Au(I), a polymer droplet morphology with much larger droplets (~10 μm), which collapsed during drying prior to SEM, so that SEM images showed features that appeared as collapsed bubbles (Figure 6). Interestingly, stable polymer droplet morphologies could be achieved from formulations containing higher concentrations of Au(I) than for Au(III), which may reflect greater
consumption of DPAP when Au(III) is reduced to Au NPs than when Au(I) is reduced to Au NPs.

**Figure 8.** Confocal microscopy images (upper panels) and SEM images (lower panels) of a PHEMA$_{hv}$ hydrogel and Au-PHEMA$_{hv}$ hydrogels incorporating Au from (CH$_3$)$_2$SAuCl. The hydrogels were obtained by photochemically induced phase separation polymerization. Au-PHEMA$_{hv}$ #4 and Au-PHEMA$_{hv}$ #5 were synthesized using 0.14 and 0.27 mol% (CH$_3$)$_2$SAuCl relative to HEMA, and other details of the formulations are summarised in Table 1.

### 3.4. Characterization of Au NPs within Hydrogels

The size and distribution of the Au NPs within the hydrogel matrix were characterized using TEM. In the bright-field transmission electron micrographs of the hydrogels (Figure 9), dark regions represent pores that were filled with resin during sample preparation, lighter regions represent polymer matrix, and Au NPs appear as black spherical dots of different sizes. For
Au-PHEMA$_{hv}$ #1 (0.02 mol% Au(III)), the Au NP diameters ranged from 40 to 80 nm, and the nanoparticles were distributed inside polymer droplets. These results suggest that the Au NPs form prior to or during polymerization of HEMA, which seems reasonable since DPAP (which can reduce Au(III)) is present in sufficient amount to reduce all Au(III) present in the mixture. Thus, when Au NPs are being formed, there is a high concentration of Au (all Au(III) is reduced to Au(0) by DPAP) resulting in large Au NPs, and they form before polymerization is complete, so the Au NPs are embedded within the polymer droplets.

At a much higher Au(III) concentration (0.14 mol% in Au-PHEMA$_{hv}$ #3), the nanoparticles were smaller (2-20 nm), and were all located near the edge of the polymer droplets, sometimes forming aggregates. We note that the polymerization mixture contains insufficient DPAP (1.0 µmol) to fully reduce the Au(III) to Au(0), so polymerization likely occurred before formation of Au NPs, resulting in the Au NPs being located at the surface of the polymer droplets. The smaller size of the Au NPs may be a consequence of Au NPs being formed in the presence of PHEMA chains, which can stabilize the Au NPs in solution and presumably inhibit further growth, or by Au NPs being formed under conditions where reduction of Au(III) occurs slowly, so that the steady state concentration of Au available for incorporation into Au NPs is low. For polymerizations from formulations involving intermediate concentrations of Au(III) (Au-PHEMA$_{hv}$ #2, 0.08 mol%) the Au-PHEMA$_{hv}$ hydrogel has a form that is intermediate between the other two cases—a few large Au NPs are located within the polymer droplets, while many smaller Au NPs are located near the surface of the droplets.

In Au-PHEMA$_{hv}$ samples prepared using 0.14 or 0.27 mol% (CH$_3$)$_2$SAuCl as a precursor of Au NPs, the hydrogels had Au NPs of 2-40 nm diameter, distributed throughout the polymer droplets. This result is consistent with the suggestion that Au NPs are distributed throughout the polymer droplets when the Au NPs are formed prior to or during
polymerization, and we have shown that water-HEMA mixture reduces \((\text{CH}_3)_2\text{SAuCl}\) to form Au NPs even under conditions of low light.

For the Au-PHEMA\(_\Delta\) hydrogel synthesized by thermal polymerization using Au(III) ions as Au NPs precursor, 20-60 nm size Au NPs formed and nanoparticles were distributed within the polymer droplets rather than at their surfaces. This result suggests that Au NPs form before or at the same time as polymerization of HEMA, and is consistent with our observations that Au(III) is reduced to Au NPs in the presence of both HEMA and \(\text{K}_2\text{S}_2\text{O}_8\).
Figure 9. Bright-field transmission electron micrographs of hydrogels synthesized by photochemical polymerization of HEMA from formulations containing KAuCl₄ (Au-PHEMAᵥ #1-#3) or (CH₃)₂SAuCl (Au-PHEMAᵥ #4-#5) or thermal polymerization of HEMA from a formulation containing KAuCl₄ (Au-PHEMAₐ). In the image for Au-PHEMAᵥ #3, the arrows highlight aggregates of Au NPs and the inset shows a cluster of Au NPs at higher magnification.
The Au NPs were further characterized using high angle annular dark-field scanning transmission electron microscopy (HAADF-STEM). In the dark field image of an Au-PHEMA₃₅ hydrogel (Figure 10A), dark regions represent the polymer matrix and the resin within which the sample is embedded, while bright spots represent heavy atoms. Energy dispersive X-ray spectroscopy (EDS) mapping of polymer samples confirmed that the bright spots are Au NPs (Figure 10B). Two different EDS spectra were recorded. Zone 1 represented a background spectrum where no Au NPs were present, while zone 2 contained Au NPs. No peaks associated with Au were recorded in zone 1 (Figure 10C), while the presence of Au was observed at 2.2 keV and 9.7 keV in zone 2 (Figure 10D). Carbon and oxygen EDS maps were also recorded for Au-PHEMA₃₅ hydrogels (see Supporting Information Figure S2). HAADF-STEM and EDS studies of Au NPs in Au-PHEMA₃₅ #4 polymer hydrogel yielded analogous results to those obtained for the Au-PHEMA₃₅ sample (see Supporting Information Figure S3).
Figure 10. STEM analysis of Au-PHEMAΔ (0.15 mol% Au(III) relative to HEMA). (A) High angle annular dark field (HAADF) image of the sample. (B) Au element analysis map. (C) EDS spectrum of Zone 1 from the image in (A). (D) EDS spectrum of Zone 2 from the image in (A). (Note: Cu peaks arise from copper grid used as the sample support. N, F peaks arise from the resin used for sample preparation.)
3.5. X-ray Diffraction (XRD) Studies

XRD confirmed the phase purity of Au NPs inside Au-PHEMA$_\Delta$ and Au-PHEMA$_{hv}$ #5 polymers (Figure 11). XRD patterns obtained from both samples show a broad peak related to amorphous PHEMA polymer, as well as peaks that appear at 38.20°, 44.41°, 64.54°, 77.50°, and 81.68°, related to the (111), (200), (220), (311), and (222) planes of the crystalline unit cell of elemental gold as reported in 04-0784 file of the JCPDS database. These data indicate that gold nanoparticles have a face-centered cubic (fcc) crystalline structure.$^{53}$

![Figure 11. XRD patterns obtained from powdered samples of the hydrogels Au-PHEMA$_\Delta$ and Au-PHEMA$_{hv}$ #5.](image-url)
3.6. Mechanical Strength

Mechanical strength measurements of PHEMAΔ and Au-PHEMAΔ hydrogels were carried out using an Instron 5943 microtester system. The results of compression tests are shown in Figure 12. All hydrogels displayed good mechanical strengths—the specimens did not break at the end of the compression test, and they recovered to their original dimensions after removal of the compression force. Au-PHEMAΔ hydrogels were significantly stiffer than PHEMAΔ hydrogels, with the value of compressive strength at 70% strain for the Au-PHEMAΔ samples approximately twice that for PHEMAΔ samples (p < 0.05). The Au NPs make up only a tiny fraction of the volume of the Au-PHEMAΔ sample, so the presence of Au NPs is unlikely to account for the higher compressive strength of Au-PHEMAΔ vs PHEMAΔ. We note that inclusion of Au in the polymerization formulation affected the morphology of the resulting hydrogel, increasing the polymer droplet size from 4.5 µm (in PHEMAΔ) to 7.0 µm (in Au-PHEMAΔ), and this change in morphology may have affected the mechanical strength of the hydrogels.
4. CONCLUSION

Porous PHEMA hydrogels doped with Au NPs can be prepared by a simple, one step, photochemically- or thermally-induced phase separation of HEMA in aqueous solution. In photochemical polymerizations, DPAP plays a key role as both photoinitiator of polymerization of HEMA as well as reducing agent to convert Au(I) or Au(III) to Au NPs. In thermal polymerizations, neither the initiator K2S2O8 nor HEMA monomer were able to reduce Au(III) to Au NPs, but did so in combination, suggesting that in these experiments the polymer PHEMA played an important role as a reducing agent in the formation of Au NPs. PHEMA also appeared to stabilize the Au NPs. In most cases, approximately 95% of the Au in the initial polymerization mixture was incorporated into the Au-PHEMA hydrogel; the Au NPs were not aggregated, and were distributed throughout the polymer droplets in the hydrogels.
5. SUPPORTING INFORMATION

5.1 Infrared Spectra

Samples of PHEMAΔ and Au-PHEMAΔ hydrogels were dried in vacuum oven overnight and ground into fine powders. FT ATR-IR spectra of the powdered PHEMAΔ and Au-PHEMAΔ hydrogels (Figure S1) were recorded at room temperature using PerkinElmer Spectrum One FT-IR spectrometer.

The IR spectra of both PHEMAΔ and Au-PHEMAΔ samples were essentially identical and showed signals expected for PHEMA hydrogels. Key signals include: 3367 cm⁻¹ (broad, OH stretch), 2945 and 2951 cm⁻¹ (CH stretches of CH₃ and CH₂ groups), 1708 cm⁻¹ (C=O stretch), 1163 cm⁻¹ (C-O-C), 1075 cm⁻¹ (C–OH stretch). No peak was observed for unreacted HEMA (1650 cm⁻¹ C=C).
Figure S1. FT ATR-IR spectra of (A) PHEMAΔ and (B) Au-PHEMAΔ.
5.2 STEM-EDS analysis of Au-PHEMA₆ sample

STEM-EDS analysis was undertaken according to procedures described in Section 2.5.

**Figure S2.** Scanning transmission electron microscopy (STEM) and energy dispersive X-ray spectroscopy (EDS) analysis of Au-PHEMA₆ (A) High angle annular dark field (HAADF) STEM image of Au-PHEMA₆. (B) Au EDS map. (C) Carbon EDS map. (D) Oxygen EDS map. (E) EDS spectrum of Zone 1. (F) EDS spectrum of Zone 2.
5.3 STEM-EDS analysis of Au-PHEMA\textsubscript{hν} #4 sample

Figure S3. Scanning transmission electron microscopy (STEM) and energy dispersive X-ray spectroscopy (EDS) analysis of Au-PHEMA\textsubscript{hν} #4. (A) High angle annular dark field (HAADF) STEM image of Au-PHEMA\textsubscript{hν} #4. (B) Au EDS map. (C) Carbon EDS map. (D) Oxygen EDS map. (E) EDS spectrum of Zone 1. (F) EDS spectrum of Zone 2.
5.4 Formation of Au NPs in water-ethylene glycol mixtures by under photochemical conditions.

**Figure S4.** Results of irradiation of two mixtures (Test 6 and Test 7) containing KAuCl$_4$ (3.5 µmol), H$_2$O (0.9 mL), ethylene glycol (7 mmol), and, as indicated DPAP (1.0 µmol). Reactions were carried out in borosilicate glass test tubes (75 mm in height, 8 mm in inside diameter) at room temperature. Reaction mixtures were purged with nitrogen for 5 min and sealed with rubber septum before exposure to UV lamp.
5.5 UV-Visible absorbance spectra of solutions prepared in Test 5 and Test 7 (irradiation of aqueous Au(III) in the presence of DPAP and PVA or DPAP and ethylene glycol)

**Figure S5.** The UV-Visible absorbance spectra of (a) a sample prepared by dissolution of KAuCl₄ (1.8 µmol), DPAP (0.5 µmol) and poly(vinyl alcohol) (40 mg) in water (1.0 mL), after 20 min of irradiation [Test 5], and (b) a sample prepared by dissolution of KAuCl₄ (3.5 µmol), ethylene glycol (7 mmol), and DPAP (1.0 µmol) in water (0.9 mL), after 20 min of irradiation [Test 7]. Samples were irradiated in borosilicate glass test tubes using a Blak-Ray B-100 AP high-Intensity UV lamp (100 W, 350 nm) and transferred to quartz cuvettes for recording of the UV-Vis spectra.
5.6 Three dimensional (3D) confocal images of Au-PHEMA$_\Delta$ sample

Sample preparation for 3D confocal imaging was similar as described in Section 2.4 of the main manuscript. Images were collected with a resolution 1024 x 1024 pixel, 2.4 pixel dwell, and 20x dry objectives using 561 nm laser for excitation. Images were collected to 13 $\mu$m depth of polymer hydrogel.

Figure S6. 3D confocal images of Au-PHEMA$_\Delta$ hydrogel.
6. REFERENCES


(28) Paterson, S. M.; Shadforth, A. M. A.; Brown, D. H.; Madden, P. W.; Chirila, T. V.; Baker, M. V. The synthesis and degradation of collagenase-degradable poly(2-hydroxyethyl


Chapter 4.

Poly(2-Hydroxyethyl Methacrylate) Hydrogels Doped with Copper Nanoparticles

1. BACKGROUND

Copper nanoparticles (Cu NPs) have been used in applications as diverse as water purification [1], sensors [2], catalysts [3], conductive ink [4], inkjet-printing [5], and antimicrobial agents [6]. Incorporation of Cu NPs into polymer matrices has attracted increasing interest because of the unique chemical, physical, and biological properties that are imparted to the polymer materials [7,8,3,9,10]. Previously, Cu NPs have been incorporated into a range of different polymers, including poly(2-phenoxyethyl acrylate) [11], poly(o-toluidine) [7], polypropylene [12], cellulose acetate [13], poly(vinylmethyl ketone) [9], polyvinylidenefluoride [9], polyaniline [14], polyurethane [15], poly(acrylic acid) [16], and polyethylene [17]. The nanoparticles have been incorporated into polymer matrix using several different methods; including (a) blending of preformed nanoparticles with monomers or polymers [18-20] (b) in situ chemical reduction and photoreduction of metal ions inside preformed polymer or polymerization mixture containing monomers [21-23] (c) deposition of nanoparticles on polymer surfaces by chemical vapour deposition, spin coating, pulsed laser deposition [24-26].

Hydrogels are materials that are flexible and can retain high water content without dissolution and which can therefore mimic the body’s soft tissues. Hydrogels may also possess a 3-D network and biodegradable bonds. Such properties have promoted interest in using hydrogels for tissue engineering applications [27]. Importantly, the incorporation of metallic nanoparticles into hydrogels can inhibit the attachment of bacteria onto the hydrogel’s surface, while the polymer chains within the hydrogel network can stabilize the nanoparticles [23].
In this study, we explore incorporation of Cu NPs into poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels. PHEMA is well known for its excellent biocompatibility. Via a simple polymerization-induced phase separation process from water, PHEMA can easily be fabricated in a porous form suitable for applications in tissue engineering [28-30]. We were interested to see whether Cu NPs would confer useful antibacterial properties to the PHEMA hydrogels. Crosslinked PHEMA hydrogels were prepared by photo-induced phase separation polymerization of 2-hydroxyethyl methacrylate (HEMA) in water containing different concentrations of Cu(II) ions. Subsequently, the Cu(II) ions inside the PHEMA hydrogels were reduced to Cu(0) using hydrazine hydrate as a reducing agent. The morphology of hydrogels was studied by scanning electron microscopy (SEM). Nanoparticles were characterized by transmission electron microscopy (TEM) and high angle annular dark-field scanning transmission electron microscopy (HAADF-STEM). Leaching behaviour of Cu NPs from PHEMA hydrogel was studied by inductively coupled plasma optical emission spectrometry (ICP-OES). The mechanical strength of the Cu-PHEMA hydrogels was measured by compression strength testing.

2. EXPERIMENTAL SECTION

2.1 Materials

2-Hydroxyethyl methacrylate (HEMA), 2, 2-dimethoxy-2-phenylacetophenone (DPAP), and ethylene glycol dimethacrylate (EGDMA) were all supplied by Sigma-Aldrich (USA); copper(II) sulfate pentahydrate and hydrazine hydrate were supplied by Fluka (Switzerland). All reagents were used as received without further purification. Ultrapure water was obtained from a Milli-Q system and used for sample preparation, storage and ICP studies.
2.2 Preparation of PHEMA hydrogels doped with Cu NPs

Seven samples of Cu-PHEMA nanocomposite hydrogel H1 (Table 1) were prepared via the photopolymerization of HEMA in water. A round bottom flask was charged with HEMA (1.5 mL, 1.6 g, 12.33 mmol), crosslinking agent EGDMA (30 µL, 31.53 mg, 0.16 mmol), CuSO$_4$·5H$_2$O (150 mg, 0.6 mmol) and then Milli-Q water (4.5 mL) was added dropwise with continuous stirring to form a clear homogeneous reaction mixture. A solution of DPAP (35 µL of a 64 mg/mL in ethanol solution, 8.74 µmol) was added and the mixture was stirred for 2 min, and then transferred to 7 quartz vials (30 mm in height, 8 mm inside diameter). Nitrogen was bubbled through the contents of each vial for 5 min, and then the vials were sealed with a rubber septum and irradiated (100 W, 350 nm, Blak-Ray B-100 AP high Intensity UV lamp, and positioned 12 cm from the vials) for 30 min. Seven control hydrogels (PHEMA) without nanoparticles were synthesized in a similar manner but in the absence of CuSO$_4$·5H$_2$O. After irradiation the copper-containing reaction mixtures had formed opaque pale blue hydrogels, while the controls had formed opaque white hydrogels. The hydrogels were removed from the quartz vials and transferred to Milli-Q water (~50 mL) for 2 h, to remove loosely bound copper ions and unreacted monomer, and were then transferred to Milli-Q water (350 mL) containing N$_2$H$_4$·H$_2$O (20.0 mg/mL) at 80 °C for 5 h. The same procedure was used to prepare Cu-PHEMA hydrogels H2-H7, according to formulations and temperatures listed in Table 1.

Hydrogel Cu-PHEMA H4 was used immediately for studies of leaching of Cu NPs (see below: "Analysis by ICP-OES"). The remaining hydrogels were soaked in Milli-Q water for 5 days, with water changes twice daily (to remove any unreacted monomers or loosely bound Cu NPs), and were then stored in water until used for further studies.
Table 1 Experimental details for the preparation of Cu-PHEMA hydrogels

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<th>Hydrogels&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CuSO$_4$·5H$_2$O (mol%, relative to HEMA)</th>
<th>N$_2$H$_4$·H$_2$O (mg/mL)</th>
<th>Reaction temperature (°C)</th>
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<sup>a</sup> All hydrogels except Cu-PHEMA H7 were prepared by photochemical polymerization of mixtures of HEMA (1.5 mL), EGDMA (a crosslinker, 30 µL), DPAP (initiator, 2.24 mg), and the indicated amount of CuSO$_4$·5H$_2$O, in ultrapure water (4.5 mL). Once formed, hydrogels were rinsed twice in water then transferred to solutions containing the indicated concentration of N$_2$H$_4$·H$_2$O in water (350 mL) and heated at the indicated temperature for 5 h.<sup>b</sup> Hydrogels H7 were synthesized as outlined above but without any DPAP initiator. <sup>c</sup> Hydrogels H8 were synthesized as outlined above but without any CuSO$_4$ in polymerization mixture and were then freeze-dried for 24 h. The dry H8 hydrogels were rehydrated in CuSO$_4$ solution (9.7 mol% CuSO$_4$ relative to HEMA, in 4.5 mL water) for 2 h, then washed with water twice before being transferred to solutions containing 20 mg/mL N$_2$H$_4$·H$_2$O in water (350 mL) and heated at 80 °C for 5 h.
2.3 Characterization of Cu NPs inside PHEMA hydrogels

The morphology of hydrogels was characterized by SEM. Hydrogel samples were dried in a critical point dryer (Polaron KE3000, Quorum Technologies Ltd, U.K.) and coated with platinum (~ 3 nm; Polaron SC7640, Quorum Technologies Ltd, U.K.) before collecting the images by SEM. The samples were dried and coated for SEM analysis according to a procedure described in our previous work [23]. The Cu NPs were characterized using TEM and HAADF-STEM as previously described [23].

X-Ray diffraction (XRD) patterns of Cu NPs inside Cu-PHEMA hydrogel H2 were recorded for three consecutive weeks. After preparation, the hydrogel was stored in water for 21 days at 6 °C, with water changed twice a day for the first 4 days. On days 7, 14, and 21, small pieces were cut from the sample, dried in vacuum oven for 48 h, and ground into fine powders. Dry fine powder was placed on a monocrystalline silicon sample holder and the XRD pattern was recorded. Data were collected on a Panalytical Empyrean X-ray powder diffractometer using CuKα radiation collected in reflection (Bragg-Brentano) geometry using a programmable spinner stage. Data were collected on a continuous scan mode from 20 to 80 degrees at 20 using a step size and count time of 0.01° and 3000s respectively. Qualitative analysis of the composition of the samples was conducted using the HighScore Plus diffraction software [31].

2.4 Analysis by ICP-OES

The Cu-PHEMA H4 hydrogels were transferred from the hydrazine solution (see above: "Preparation of PHEMA hydrogels doped with Cu NPs") into 50 mL water in a stoppered flask and stored in an oven at 37 °C. At 3-day intervals for 3 months, the water was drawn off using a pipette, transferred to a separate flask, and replaced with fresh 50 mL water. After all the samples (extracts of NPs from hydrogels) had been collected, HNO₃ (70%, 5 mL) was added
to each sample, followed by sufficient ultrapure water to obtain a final volume of 80 mL. The 80 mL samples were analysed by ICP-OES (Agilent Technologies ICP-OES 5100). A standard solution of copper (1000 ± 3 µg/mL) in 2% HNO₃ (from High-Purity Standards, North Charleston, South Carolina) was diluted in ultrapure water to prepare different concentrations of copper solutions and generate a linear calibration curve (signal intensity as a function of concentration). Leaching of copper from the Cu-PHEMA H2 hydrogels was also studied for 10 days according to procedure described above.

2.5 Cytotoxicity and antibacterial studies

PHEMA and Cu-PHEMA H2 hydrogels were soaked in distilled water for one week with water changes twice a day. The cylindrical hydrogels were sliced into discs 1-2 mm thick, and further cut into small pieces. The pieces were dried overnight in an oven at 60 °C and weighed. Dried samples were sterilized for 1 h in 70% ethanol, washed two times with PBS (10 min each) and rinsed with cell culture medium. In a sterile environment, sponges were transferred into 50 mL Falcon tubes containing culture medium (1 mL of culture medium per 0.2 g of dry hydrogel) and incubated for 48 h at 37 °C in a bead bath. The mixtures were transferred to 15 mL Falcon tubes and centrifuged at 300 rcf for 5 min to separate small pieces of the hydrogel, and the supernatant extracts were used in cytotoxicity studies.

Immortalized human corneal epithelial (HCE-T) cells were used to for the cytotoxicity study. HCE-T cells were grown on T75 flask in Dulbecco-modified Eagle’s medium (DMEM) supplemented with GlutaMAX™, 10% FBS and 1% penicillin-streptomycin. Cells were passaged using Versene and TrypLE®, seeded into 96 well plates, at a density of 8000 cells per well, and incubated for 24 h at 37 °C under 5% CO₂. The medium was removed and replaced by 100 µL of the supernatant extracts from the hydrogels, and the plates were incubated for an additional 24 h. For controls, normal medium was used in place of supernatant extract. Media
with/without extracts were removed and replaced by 100 µL/well of 0.5 mg/mL MTT solution in PBS and the plates were incubated for 3 h. The MTT solutions were then replaced by 100 µL/well of 0.04 M HCl/isopropanol to dissolve the purple/blue crystals formed inside the cells. After 5 min of shaking, 80 µL aliquots of solutions were transferred to a new plate and absorbance was measured at 570 nm. Two series of experiments, each in octuplicate, were performed and the relative cell viability (%) was expressed as a percentage relative to the control wells and presented as a mean ± standard deviation. Effect of extracts on cell morphology was also studied and photographs were taken by Nikon Eclipse TS 100 equipped with a Nikon DS-Fi1 camera using NIS Elements software.

Antibacterial activity of leachable Cu NPs from PHEMA hydrogels was carried out by the Broth culture test [23]. Both *E. coli* and *S. aureus* bacterial suspensions were prepared by adding 10 µL of bacterial suspensions (adjusted to $1.5 \times 10^6$ CFU/mL) into 2 mL TSB (trypticase soya broth) to get a final concentration of $7.5 \times 10^3$ CFU/mL in four different tubes. PHEMA and Cu-PHEMA H2 hydrogel discs (8 mm in diameter and 2 mm in thickness) were placed inside *E. coli* and *S. aureus* bacterial suspensions in 2 mL of TSB and incubated at 37 °C for 24 h. The turbidity of bacterial suspension was recorded after 24 h of incubation. The experiments were repeated three times.

### 2.6 Mechanical testing

Compression testing of cylindrical hydrogel specimens was carried out as previously described [23]. Mechanical properties of PHEMA and Cu-PHEMA H2 hydrogels were evaluated in terms of compressive strength. Compressive strength was measured at 70% strain and compressive modulus was calculated from the initial slope of the stress versus strain plot.
3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization of hydrogels

The Cu-PHEMA hydrogels were synthesized by phase separation polymerization of HEMA (26 % w/w) in water containing crosslinker (EGDMA), photoinitiator (DPAP) and various concentrations of Cu(II) ions (from CuSO$_4$), as summarised in Table 1. Upon irradiation with UV light, the DPAP generated radicals that initiated radical polymerization of HEMA monomer. After 30 min of irradiation, the initially transparent blue reaction solutions had turned to opaque blue hydrogels. The hydrogels remained blue after rinsing in ultrapure water, suggesting the presence of Cu(II) ions within the hydrogel sponge. PHEMA hydrogel (reference hydrogel) synthesised in the absence of Cu(II) ions was opaque white. Cu-PHEMA hydrogels containing Cu(II) ions were heated at various temperatures in solutions containing various concentrations hydrazine to reduce the Cu(II) ions to Cu nanoparticles, and resulting in a change of color to red-brown (Fig. 1).

Four hydrogels were synthesized as controls. PHEMA H1 and PHEMA H2 were synthesized as copper-free controls. For PHEMA H1 there was no CuSO$_4$ in the polymerization mixture and the hydrogel was not heated in a hydrazine solution. PHEMA H2 was formed in a similar way to PHEMA H1 but was subsequently heated in a hydrazine solution at 80 °C. Cu PHEMA H7 was synthesized without any DPAP initiator in the initial polymerization mixture, to test whether CuSO$_4$ could initiate polymerization. For Cu PHEMA H8, instead of introducing Cu(II) as CuSO$_4$ in the initial polymerization mixture, a Cu-free PHEMA sample was formed and dried, and a solution of CuSO$_4$ was introduced to the dried PHEMA hydrogel, prior reduction with hydrazine solution.
The morphology of the PHEMA hydrogels was examined by SEM. The micrographs (Fig. 2) confirmed the polymer droplet morphology and the presence of a 3D network of interconnected pores in hydrogels formed by polymerization of HEMA initiated by DPAP. The size of polymer droplets increased as the concentration of Cu(II) ions increased in the polymerization mixture. For the PHEMA H1 and PHEMA H2 control samples without Cu NPs, the size of polymer droplets was 2-3 µm (Fig. 2a and 2b). The size of the polymer droplets increased to ~5.0 µm (Cu PHEMA H1, Fig. 2c) and ~7.0 µm (Cu PHEMA H4, Fig. 2d), as the concentration of Cu(II) ions relative to HEMA was increased from 4.8 mol% to 9.7 mol%, respectively, suggesting that the size change of the polymer droplets is related to the concentration of Cu(II) ions. It has been shown [32,33] that irradiation of Cu(II) ions in aqueous media produces Cu(I) ions that can initiate the polymerization of acrylate monomers. It has also been shown [34] that in polymerization of HEMA, when the initiator concentration increases, the size of the resultant PHEMA polymer droplets also increases. When a solution of HEMA and EGDMA in water containing CuSO₄ (9.7 mol%) in the absence of DPAP initiator was irradiated for 1 h, a pale blue hydrogel formed (Cu-PHEMA H7). This result suggests that Cu(I) ions generated photochemically from Cu(II) may be initiating polymerization. According to Fig. 2e and 2f, however, Cu-PHEMA H7 hydrogels had different
morphologies to the hydrogels formed in the presence of the initiator DPAP. Rather than showing a morphology based on polymer droplets (Fig. 2a-d), Cu PHEMA H8 showed a morphology in which partially formed droplets were coagulated in large regions of the hydrogel that lack porosity (Fig. 2e, 2f).

**Fig. 2** Scanning electron micrographs of (a) PHEMA H1 (Cu-free PHEMA, no heating in hydrazine solution), (b) PHEMA H2 (Cu-free PHEMA, heated in hydrazine hydrate solution), (c) Cu-PHEMA H1 (synthesized using 4.8 mol% of Cu(II) ions relative to HEMA), (d) Cu-PHEMA H4 (synthesized using 9.7 mol% of Cu(II) ions relative to HEMA), (e) and (f) Cu-PHEMA H7 hydrogel (synthesized using 9.7 mol% of Cu(II) ions relative to HEMA, in the absence of DPAP initiator)
3.2 Characterization of Cu NPs inside the hydrogels

The size, shape, and distribution of Cu NPs within Cu-PHEMA hydrogels were examined by TEM. Cu-PHEMA hydrogels were embedded in Procure-Araldite epoxy resin prior to analysis. In Fig. 3-5, the lighter regions in the TEM images represent the polymer matrix while the slightly darker regions represent pores filled with resin, and the black spots of represent the metal particles. In all samples, the Cu NPs were distributed on the surfaces of polymer droplets. We suggest that during the polymerization of HEMA, as the growing PHEMA polymer separates into polymer droplets, the aqueous medium containing the Cu(II) ions is excluded from the less hydrophilic region within the polymer droplets. The resulting PHEMA sponge is a porous network of PHEMA droplets, with Cu(II) ions in an aqueous solution filling the pores. After reduction of the Cu(II) ions to Cu(0), Cu NPs formed and become absorbed to the surface of polymer droplets, where they are stabilized by PHEMA chains.

3.2.1 Effect of Cu(II) concentration on the size of nanoparticles

The size of Cu NPs increased with increasing concentration of Cu(II) ions in the polymerization mixture. When the concentration of CuSO_4·5H_2O was increased from 4.8 wt% (Cu-PHEMA H1) to 9.7 wt% (Cu-PHEMA H4), but with all other parameters unchanged, the size of nanoparticles increased from 20-140 nm (Fig. 3a) to 50-240 nm (Fig. 3b), respectively. This result is consistent with a previous report [35] showing that increasing the concentration of copper sulfate in an aqueous formulation results in the formation of larger nanoparticles.
Fig. 3 Bright field transmission electron micrographs of (a) Cu-PHEMA H1 and (b) Cu-PHEMA H4, synthesized from formulations containing respectively 4.8 mol% and 9.7 mol% of CuSO₄ relative to HEMA

3.2.2 Influence of reducing agent concentration on the size of nanoparticles

Cu-PHEMA hydrogels H4, H6, and H7 were prepared under identical conditions except that the concentration of N₂H₄·H₂O used during the reduction step was increased from 20.0 mg/mL to 41.2 mg/mL to 61.8 mg/mL across the series. The increasing concentration of N₂H₄ resulted in a decrease in the size of Cu NPs, from 50-240 nm (Cu-PHEMA H4, Fig. 4a) to 30-140 nm (Cu-PHEMA H5, Fig. 4b) to 15-100 nm (Cu-PHEMA H6), with Cu-PHEMA H6 showing regions of isolated nanoparticles (Fig. 4c) and regions of clusters of nanoparticles (Fig. 4d). These results may be rationalised in terms of rates of nucleation—when the concentration of reducing agent increased, copper nuclei are generated at higher rates, leading to faster nucleation of the copper nanoparticles, resulting in the formation of many smaller nanoparticles rather than fewer large ones. Similar findings have been reported from studies of Cu NPs formed by reduction of Cu(II) by sodium hypophosphite [36]. When numerous small
nanoparticles form, the large surface area of nanoparticles may exceed the ability of the nearby PHEMA chains to stabilize the nanoparticles, resulting in agglomeration.

**Fig. 4** Bright-field transmission electron micrographs of Cu-PHEMA samples prepared from formulations containing 9.7 mol% CuSO$_4$ relative to HEMA, but using different concentrations of N$_2$H$_4$·H$_2$O in the reduction step. (a) Cu-PHEMA H4, 20.0 mg/mL N$_2$H$_4$·H$_2$O; (b) Cu-PHEMA H5, 41.2 mg/mL N$_2$H$_4$·H$_2$O; (c) and (d) Cu-PHEMA H6, 61.8 mg/mL N$_2$H$_4$·H$_2$O. Images (a) and (b) are representative of the whole of sample Cu-PHEMA H4 and sample Cu-PHEMA H5 respectively. Cu-PHEMA H6 contained regions with low density of small, isolated nanoparticles (as in c) and other regions with aggregates of small nanoparticles (as in d).
3.2.3 Influence of temperature on the size of nanoparticles

The size of Cu NPs increased as the temperature used during the reduction stage was increased. Thus, when PHEMA hydrogels containing Cu(II) ions were heated in N$_2$H$_4$ solutions at 40 °C, 60 °C, or 80 °C, the size of Cu NPs increased from 20-50 nm (Cu-PHEMA H2) to 30-120 nm (Cu-PHEMA H3) to 50-240 nm (Cu-PHEMA H4) respectively (Fig. 5). Changes in the size of nanoparticles with temperature can be explained by LaMer’s model [37] [38], according to which the formation of nanoparticles involves two distinct separate steps: nucleation and growth. As the temperature is increased, Cu(II) ions are reduced over a shorter period of time and the concentration of copper nuclei increased, reached to a saturation concentration and later to the nucleation concentration. Subsequently, spontaneous nucleation begins, and sudden nucleation lowers the concentration of Cu(0) below the self-nucleation level. As a result, growth of particles becomes the dominant process, leading to an increase in the size of nanoparticles.

**Fig. 5** Bright field transmission electron micrographs of Cu-PHEMA hydrogels prepared from formulations containing 9.7 mol% of CuSO$_4$ relative to HEMA, but with the subsequent reduction of Cu(II) ions by N$_2$H$_4$ to form Cu NPs conducted at different temperatures. (a) Cu-PHEMA H2, reduction at 40 °C. (b) Cu-PHEMA H3, reduction at 60 °C. (c) Cu-PHEMA H4, reduction at 80 °C
The hydrogel samples Cu-PHEMA H1-H6 discussed above were all formed via polymerizations in which DPAP was used as photoinitator. We have also characterised Cu NPs formed in the control hydrogels Cu-PHEMA H7, for which photopolymerization was done in the absence of DPAP. In Cu-PHEMA H7, there was some agglomeration of Cu NPs (Fig. 6a), suggesting that they were not properly stabilized by PHEMA chains. This result may be due to the different porosity of the Cu-PHEMA H7 hydrogels compared to the others (Fig. 2). In Cu-PHEMA H1-H7, the polymer droplet morphology presents a large surface area of PHEMA chains to the aqueous medium, onto which Cu NPs can be adsorbed and stabilized by PHEMA chains. For Cu-PHEMA H7 hydrogels, the polymer morphology is more condensed, providing a lower surface area of PHEMA chains to stabilize Cu NPs.

For Cu-PHEMA H8 hydrogels, Cu(II) was not present in the original polymerization mixture, but was introduced into a pre-formed PHEMA hydrogel prior to the reduction of Cu(II) by N₂H₄. Examination of a sample of Cu-PHEMA H8 hydrogel by TEM showed Fig. 6b showed fewer Cu NPs were present compared to Cu-PHEMA H4, prepared under the same conditions as Cu-PHEMA H8 but with Cu(II) ions present in the initial polymerization formulation rather than added afterwards.
Fig. 6 Bright field transmission electron micrographs of (a) Cu-PHEMA H7 hydrogel synthesized in the absence of DPAP initiator and (b) Cu-PHEMA H8 hydrogel (Cu(II) ions incorporated into hydrogels by absorption in CuSO₄ solution in water and further reduce to Cu NPs)

HAADF-STEM was used to further characterize the Cu NPs inside a hydrogel sample (Cu-PHEMA H4). The sample used for TEM analysis, already embedded in resin, was coated with carbon (~25 nm) and the HAADF image was recorded (Fig. 7). In Fig. 7a, the darker area represents the matrix of PHEMA and resin, and the brighter spots are related to heavy metals. Energy-dispersive X-ray microanalysis (EDS) mapping of the sample confirmed that the brighter spots are related to copper (Fig. 7b). EDS spectra were also recorded for two different areas and shown in Fig. 7a: area 1, selected as a background where no bright spots were observed, and area 2, selected as a region where a bright spot was present. The EDS spectrum of area 1 (Fig. 7c) indicated peaks at 0.27 keV, 0.52 keV, and 7.47 keV, which are evidence of carbon, oxygen, silicon, and nickel, but no peak for copper recorded. Carbon and oxygen are from both PHEMA and the resin (used for sample preparation for TEM analysis) and silicon is from the resin, while nickel is from the grid used as a sample support for TEM and HAADF-
STEM analysis. However, the spectrum in area 2 (Fig. 7d) showed signal at 8.0 keV, which is evidence of copper.

**Fig. 7** STEM analysis of Cu-PHEMA H4. (a) High angle annular dark field (HAADF) image of the sample. (b) Cu element analysis map. (c) EDS spectrum of area 1 (selected in image a). (d) EDS spectrum of area 2 (selected in image a). Note: Ni signals arise from nickel grid used as the sample support and the silicon signal arises from resin used for sample preparation.

### 3.3 X-Ray diffraction analysis

X-ray diffraction patterns were recorded for dry powders prepared from samples of Cu-PHEMA H2 hydrogel after storage in water for 1, 2, or 3 weeks at 6 °C. The patterns suggested that the copper nanoparticles contained crystalline copper. The peaks in the XRD (Fig. 8) were indexed using JCPDS files (JCPDS Card No 04-0836).
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The diffraction peaks at 2θ values of 43.4°, 50.5°, and 74.2° can be indexed as the (111), (200), and (220) planes of metallic copper. The XRD pattern of Cu NPs consistent with the spectrum of pure copper, and no peaks attributed to copper oxide was observed.

![XRD pattern of Cu NPs in Cu-PHEMA H2 hydrogels](image)

**Fig. 8** XRD pattern of Cu NPs in Cu-PHEMA H2 hydrogels. Pieces of Cu-PHEMA were stored in water for 1, 2, or 3 weeks, then dried and ground into powders for the XRD study.

3.4 Leaching experiments

Release of Cu from hydrogels (Cu-PHEMA H4) in water was investigated using ICP-OES. Copper leached into water over time, and the maximum amount of copper leached (7.8%) during reduction of Cu(II) ions to Cu(0) in the hydrazine solution. Loosely bound nanoparticles or nanoparticles on the surface of hydrogel can leach fast. The release of copper in water...
decreased with time, becoming very slow after one week. Overall, 9% copper leached in water during three months of carrying out the experiment. Release of copper from Cu-PHEMA H2 was only 3% in 10 days and reached to near zero by day 10.

![Graph showing concentration of copper leached from Cu-PHEMA H4 hydrogel.](image)

**Fig. 9** Concentration of copper leached from Cu-PHEMA H4 hydrogel. The points joined by red curve (referenced to the concentration axis on the left) shows a period of rapid leaching during the first 7 days, with the rate of leaching declining rapidly to near zero by day 10

### 3.5 Mechanical testing
Mechanical properties of the hydrogels were explored by compression testing. Samples were fully hydrated, then subjected to a compressive load. The samples became compressed to < 30% of their original height, and water was squeezed out of the samples during the compression. When the compressive load was released, the samples sprang back to >90% of their original height. The samples were soaked in water to ensure complete re-hydration before the compression test was repeated. None of the samples crumbled or were visibly fractured during compression testing. Results of compression tests are summarized in Table 2.
Table 2 Compressive characteristics of hydrated PHEMA and Cu-PHEMA H2 hydrogels$^a$

<table>
<thead>
<tr>
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<th>Compression</th>
<th>Compressive modulus (kPa)</th>
<th>Compressive strength at 70% strain (kPa)</th>
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<tbody>
<tr>
<td>PHEMA</td>
<td>1st</td>
<td>27.7 ± 2.5</td>
<td>276 ± 35</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>19.3 ± 1.2</td>
<td>206 ± 23</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>16.9 ± 0.5</td>
<td>157 ± 18</td>
</tr>
<tr>
<td>Cu-PHEMA H2</td>
<td>1st</td>
<td>27.8 ± 0.7</td>
<td>165 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>24.1 ± 2.0</td>
<td>108 ± 10</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>20.9 ± 0.2</td>
<td>122 ± 9</td>
</tr>
</tbody>
</table>

$^a$ The values represent mean ± standard deviation from two Cu-PHEMA H2 hydrogels and three PHEMA hydrogels.

The compressive modulus is a measure of how much a sample shrinks when subjected to a compressive force. PHEMA and Cu-PHEMA showed similar compressive moduli. This result is consistent with the macroscopic morphology (especially porosity) being similar for PHEMA and Cu PHEMA samples, with the major process occurring during compression being the squeezing out of water from the pores in the hydrogel. The compressive strength is a measure of the ability of the hydrogels to resist being compressed. PHEMA hydrogels showed ~30 % to ~90% higher compressive strength than Cu PHEMA hydrogels. This result presumably reflects differences in the mechanical properties of the polymer matrix in the two materials, the matrix in the PHEMA hydrogels having greater strength than in the Cu PHEMA hydrogels. It may be that the Cu(II) present in the reaction mixtures leading to Cu-PHEMA hydrogels caused chain transfer reactions that resulted in lengths of PHEMA chains being
shorter in Cu PHEMA compared to PHEMA hydrogels, resulting in weaker mechanical strength of the polymer matrix in the Cu PHEMA hydrogels. Matyjaszewski et al. have shown that Cu(II) can induce atom transfer radical polymerization (ATRP), a process that involves chain transfer events.[32]

3.6 Cytotoxicity of hydrogels

Cytotoxicity study of Cu-PHEMA hydrogels were conducted to see if release of Cu NPs is low enough that it might be tolerated by HCE-T cells. PHEMA is well known for its biocompatibility.[23] The incorporation of Cu NPs within PHEMA hydrogels can affect their cytotoxicity. Extracts of PHEMA and Cu-PHEMA H2 hydrogels in culture medium were used to study cytotoxicity towards HCE-T cells. Extract of PHEMA was pink while extract of Cu-PHEMA H2 was purple suggests that not only lots of Cu released but also dissolution of as Cu(II) in the medium. According to the results of a MTT assay, Cu NPs that likely were leached from Cu-PHEMA H2 hydrogel showed significant cytotoxicity; the percentage of viable cells were 96 ± 13% and 4 ± 2% for HCE-T cells treated with extracts of PHEMA and Cu-PHEMA H2 hydrogel, respectively. According to ICP-OES results leaching of Cu NPs in water was very slow, however their leaching in cell culture medium can be different as it depends on pH and may be affected by the reaction of copper with amino acids [39]. It has been reported that copper-polymer nanocomposites are cytotoxic and cytotoxicity depends on both concentration of copper and characteristics of polymer matrix [40].

Changes in the morphology of transformed human corneal epithelial (HCE-T) cells grown on tissue culture plastic were evaluated after incubation with cell culture medium with or without PHEMA or Cu-PHEMA H2 extract for 24 h. Cells grown in the presence of control medium (Fig. 10a) and PHEMA extract (Fig. 10b) showed typical corneal epithelial cell morphology. On the other hand, cells became round in shape and did not spread when incubated
with Cu-PHEMA H2 extracted medium (Fig. 10c). These results suggest that the Cu NPs leached from Cu PHEMA are toxic to the HCE-T cells.

**Fig. 10** Morphology of HCE-T cells grown in (a) control medium, (b) PHEMA extract, and (c) Cu-PHEMA H2 extract

### 3.7 Antibacterial properties

Antibacterial activity of PHEMA and Cu-PHEMA H2 hydrogels was investigated using broth culture test. PHEMA and Cu-PHEMA H2 discs were placed into *E. coli* and *S. aureus* bacterial suspensions and incubated at 37 °C for 24 h (Fig. 11).

**Fig. 11** Photographs of *E. coli* in TSB culture medium incubated with (a) PHEMA and (b) Cu-PHEMA H2, and *S. aureus* in TSB culture medium incubated with (c) PHEMA and (d) Cu-PHEMA H2 hydrogels. The photograph was taken after 24 h of incubation
Both *E. coli* and *S. aureus* bacterial suspensions incubated with PHEMA discs turned turbid (Fig. 11a and 11c, respectively) which indicated growth of bacteria. On the other hand, *E. coli* and *S. aureus* bacterial suspensions incubated with Cu-PHEMA H2 discs were only slightly turbid (Fig. 11b and 11d, respectively), which indicated that Cu NPs restricted growth of bacteria.

4. CONCLUSIONS

PHEMA hydrogels doped with Cu NPs can be synthesized in a two-step process starting from aqueous formulations containing HEMA, CuSO$_4$, a crosslinker, and a photoinitiator, where the first step is a photoinitiated polymerization to form a PHEMA sponge, and the second step is a reduction of Cu(II) to Cu(0) by hydrazine. The NPs in the resulting hydrogel are located over the surface of the polymer droplets that make up the PHEMA sponge and, as indicated by XRD studies, contain crystalline Cu rather than CuO or Cu$_2$O. Cu-PHEMA sponges are synthesized more conveniently by this two-step procedure, and have a more uniform distribution of Cu NPs, than Cu-PHEMA sponges synthesized by introduction of CuSO$_4$ into a pre-formed PHEMA sponge followed by a hydrazine reduction of Cu(II) to Cu(0). The PHEMA chains at the surface of the polymer droplets appear to exert a stabilizing effect for the Cu NPs, preventing their agglomeration. The size of Cu NPs depends on the concentrations of Cu(II) ions and of reducing agent the temperature at which the reduction is carried out. The presence of Cu NPs inside hydrogels does not have a significant impact on their mechanical strength. Cu NPs are slowly leached from the Cu-PHEMA hydrogels and exert antibacterial activity against *E. coli* and *S. aureus*, but also exert cytotoxicity towards HCE-T cells.
5. REFERENCES


Chapter 4 – PHEMA Sponges Doped with Cu NPs


Chapter 5.

Summary, Conclusions, and Suggestions for Further Work

1. Summary and Conclusions

Polymer scaffolds have been used in tissue engineering applications. A polymer scaffold should possess certain characteristics in biomedical applications such as biocompatibility, porous, biodegradable, and mechanical integrity. It also necessary that scaffold can inhibit the attachment or growth of bacteria on its surface. PHEMA is well-known for its biocompatibility and can be rendered biodegradable by the incorporation of a bio-susceptible crosslinker. Porous PHEMA can be easily synthesized by polymerization-induced phase separation of HEMA in water. Polymers containing certain functional groups such as biguanide groups and phenol derivatives within the polymer backbone can prevent the attachment of bacteria on the surface of hydrogels. Such methodology requires the synthesis of functionalized monomers, and the additional functionality can complicate the course of polymerization-induced phase separation.

An alternative approach to synthesizing a polymer with antibacterial properties is the incorporation of metal nanoparticles into the polymer matrix. Metal nanoparticles are not stable and tend for aggregation, but a range of polymers have been used to stabilize metal nanoparticles within their matrix. Polymer stabilized metal nanoparticles have been used in catalytic applications. The work presented in this thesis aimed to synthesize porous PHEMA hydrogels doped with silver, copper, and gold nanoparticles to develop this area further. The incorporation of silver nanoparticles into PHEMA hydrogels was explored in Chapter 2. PHEMA hydrogels doped with silver nanoparticles were prepared in a one-step process by
photochemically-induced phase separation polymerization of HEMA in water containing silver ions as a source of nanoparticles. The formation of hydrogels and the formation of nanoparticles occurred simultaneously. The resulting PHEMA hydrogels had a polymer droplet morphology with a 3D-interconnected porous network. The size and distribution of the Ag NPs inside hydrogels was characterized by TEM and HAADF-STEM analysis. The size of the Ag NPs depended strongly on the concentration of silver ions in the polymerization mixture. Silver nanoparticles slowly leached from hydrogels and showed antibacterial activity against *E. coli* and *S. aureus* bacteria. Furthermore, the silver nanoparticles were not toxic towards HCE-T cells. The incorporation of silver nanoparticles did not change the mechanical integrity of the PHEMA hydrogels.

PHEMA hydrogels doped with gold nanoparticles were synthesized in a one-step process by either thermally- or photochemically-induced phase separation polymerization of HEMA in water containing Au(III) or Au(I) ions as a source of nanoparticles (Chapter 3). Polymerization of HEMA and photoreduction of gold ions occurred concurrently. A series of experiments were conducted to understand the mechanism of the formation of Au NPs inside PHEMA hydrogels. During photochemical polymerization, DPAP photoinitiator played a significant role in the reduction of Au(III) ions to Au(0), while HEMA itself appeared to play only a minor role. The morphology of the PHEMA hydrogels changed from a polymer droplet morphology to a honeycomb morphology as the concentration of Au(III) ions in the polymerization mixture increased. In thermal polymerization, the PHEMA polymer chains appeared to be involved in the reduction of Au(III) ions to Au(0), with HEMA having only a minor effect. The size of Au NPs depends largely on the Au(III) ion concentration in the reaction mixture. As the concentration of Au(III) was increased in thermal polymerizations, the size of polymer droplets in the resulting hydrogel increased slightly, as indicated in SEM and LCMS images.
The work described in Chapter 4 involves the synthesis of porous PHEMA hydrogels doped with copper nanoparticles. Cu-doped PHEMA hydrogels were synthesized in a two-step process. In the first step, PHEMA sponges doped with Cu(II) ions were synthesized by a photoinitiated polymerization of HEMA in an aqueous solution containing a crosslinker, CuSO_{4}, and a photoinitiator. In the second step, the Cu(II) ions were reduced to Cu(0) by hydrazine and resulting Cu NPs were located on the surface of polymer droplets. The size of the Cu NPs was dependent on the concentration of Cu(II) ions, concentration of reducing agent, and reduction temperature. XRD studies showed that Cu NPs were present in pure metallic form without having impurities of CuO and Cu_{2}O. Cytotoxicity study indicated that Cu NPs are toxic towards HCE-T cells and abnormal cell growth observed. Cu NPs slowly leached from hydrogels and showed antibacterial activity against \textit{E. coli} and \textit{S. aureus}.

Two alternative routes to PHEMA sponges doped with Cu NPs were also explored. In the first alternative route, a three-step route, an aqueous solution containing Cu(I) ions were introduced into the pores of a pre-formed and dried PHEMA sponge, and the now Cu(II)-containing sponge was exposed to hydrazine to reduce the Cu(II) ions to Cu NPs. This route resulted in a PHEMA sponge with a less uniform distribution and lower stability of Cu NPs than was obtained using the two-step route described above. The second alternative route was the same as the original two-step route but without any DPAP photoinitiator in the polymerization formulation. In this case, there was some polymerization of HEMA to from PHEMA, but the PHEMA sponges did not have the desired polymer droplet morphology. In this case, polymerization was likely photo-initiated by Cu(II) ions, but insufficient initiation events to produce a PHEMA sponge of the required morphology.

This research demonstrated that a simple one-step process could synthesize porous PHEMA hydrogels doped with silver and gold nanoparticles. Nanoparticles can easily incorporate inside polymer droplets as well as on the surface of polymer droplets when the
formation of PHEMA hydrogel and formation of nanoparticles occur concomitantly. Polymer chains can stabilize metal nanoparticles. This simple one-step process did not work for synthesis of PHEMA doped with copper nanoparticles. Cu-PHEMA hydrogels were synthesized by two step process. This work is a significant advance in the field of hydrogels for biomedical applications.

2. **Suggestions for Further Work**

(a) **Biodegradable PHEMA hydrogels doped with metal nanoparticles**

Biodegradable tissue engineering materials with antibacterial properties might be useful, and extension of the work in this area would be worthwhile for tissue engineering applications. Ag NPs could be released from Ag NP-PHEMA as biodegradable PHEMA degrades, and at the same time, cells of regenerating material will proliferate and take up the space vacated by degrading PHEMA.

Biodegradable Ag NP-PHEMA could be synthesized by using a biodegradable crosslinker instead of the non-degradable crosslinker EGDMA used in the polymerisations described in Chapter 2. Incorporation of biodegradable crosslinkers\(^1\) such as disulfide,\(^2\) peptide,\(^3-7\) poly(N-vinylpyrrolidinone),\(^8\) carbohydrate,\(^9\) polylactide\(^10\) instead of EGDMA in PHEMA-based materials should render PHEMA materials biodegradable. Figure 1 shows a schematic representation of degradation of a crosslinked PHEMA polymer network. Non-degradable PHEMA polymer chains are interconnected through biodegradable crosslinkers, which contain labile bonds. Crosslinkers can be degraded by chemical, thermal, and enzymatic processes and which can lead to degradation of the PHEMA network into linear PHEMA chains. Previous studies have been carried out in this area indicating that PHEMA with a
molecular weight lower than 1200 Da are water-soluble,\textsuperscript{11} and it would be easy to remove these fragments through renal excretion because they would be soluble and carried away to the kidneys by the circulatory system.

In cases where a PHEMA material degrades into longer linear chains (\(> 1200\) Da) that would normally be insoluble, solubility (and hence degradability) can be enhanced by incorporating a solubilising monomer, such as poly(ethylene glycol) methacrylate, into the PHEMA.\textsuperscript{12-13}

![Figure 1](image.png)

**Figure 1.** Rendering PHEMA materials degradable by incorporation of biodegradable crosslinkers.

(b) **PHEMA hydrogels doped with Au NPs for applications in catalysis and surface-enhanced Raman spectroscopy**

Work with PHEMA hydrogels doped with gold nanoparticles could be extend by testing Au-PHEMA as a catalyst, for example for the reduction of \(p\)-nitrophenol\textsuperscript{14} in aqueous solution.
According to findings in Chapter 3, gold nanoparticles slowly leaching from hydrogels. For catalytic longevity, Au-PHEMA prepared with high crosslinker content may worth exploring as a way of minimising leaching of Au NPs from the Au-PHEMA.

Au-PHEMA hydrogels could be used as substrates for the surface-enhanced Raman spectroscopic (SERS)\textsuperscript{15-17} analysis to detect compounds present in very low quantities, such as biomarkers and other biomolecules in physiological fluids, or contaminants in pharmaceutical products, food, or environmental matrices.

(c) **One step synthesis of PHEMA hydrogels doped with copper nanoparticles**

In the work described in Chapter 4, PHEMA hydrogels doped with copper nanoparticles were synthesized in a two-step process. In the first step, Cu(II) ions were incorporated into the hydrogel, followed by the reduction of Cu(II) ions to Cu(0) using hydrazine. Hydrazine is toxic to cells. It can be hard to remove hydrazine from hydrogels even after several washes.

Work with PHEMA hydrogels doped with Cu NPs could be extended by using Cu complexes rather than simply aqueous Cu\textsuperscript{2+} ions, as precursors of Cu NPs. An interesting aspect here would be the search for Cu complexes that would undergo photoreduction\textsuperscript{18} to form Cu NPs, without the need for hydrazine or other toxic reducing agents. Such complexes should permit formation of Cu-PHEMA in a one-step process. Moreover, by avoiding the use of toxic reducing agents, this route to Cu-PHEMA might produce materials that have the potential for some degree of biocompatibility.

(d) **Polymer stabilized nanoparticles for colorimetric sensor applications.**

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Gold nanoparticle-based colorimetric biosensing assays have recently attracted attention because of their simple, cost-effective fabrication and their simple sensing mechanism that can sometimes be evaluated simply by naked-eye observations.\textsuperscript{19-20} Use of Au NPs in colorimetric sensors involves two steps: dispersion of Au NPs and aggregation of Au NPs. Dispersed Au NPs have burgundy colour, while after aggregated Au NPs are blue.

The work in Chapter 3 could be extended to explore the use of Au PHEMA for sensing organic volatiles. PHEMA doped with Au NPs is a nice substrate for colorimetric sensing because the PHEMA is porous, facilitating the diffusion of the analyte through the material and to the Au NPs. Analytes that are able to disrupt the interaction between Au NPs and PHEMA chains may mobilise the Au NPs and lead to their aggregation and a colour change.
3. REFERENCES


Thank You.