Stimuli-responsive hydrogels are appealing systems for tissue engineering and regenerative medicine applications due to their high water content mimicking the natural extracellular matrix, the mechanical properties resembling those of living soft tissues and the capability to adapt their properties in response to external stimuli (e.g., temperature, pH, light). Particularly, their stimuli-responsiveness makes them smart systems, able to play an active role in the application they were designed for. For instance, they have been widely explored as advanced drug delivery hydrogels, providing payload protection, localised administration, and stimuli-responsiveness for an accurate control over drug release kinetics. Alternatively, stimuli-responsiveness has been also widely investigated to design bio-inks for additive manufacturing able to meet several requirements, such as tuneable rheological and mechanical properties, as well as biomimicry. Currently, to comply with all these characteristics, bio-inks are often complex formulations obtained from polymers synthesised from combination of monomers of different origins or additives (e.g., plasticisers). However, multi-functional bio-inks are generally awesome engineered systems lacking of likewise impressive final performances.

In this scenario, this work aimed at providing a step forward in the design of multi-functional hydrogels with reduced complexity by exploiting a unique polymeric component bearing different functional moieties to provide stimuli-responsiveness. This was achieved by combining the chemical versatility of poly(urethane)s with *in bulk* or surface functionalisation techniques (i.e., plasma treatment and carbodiimide chemistry). Such stimuli-responsive hydrogels were engineered to function as drug-delivery bio-inks.

Hence, exploiting the high versatility and workability of poly(ether urethane) (PEU)-based hydrogels, **the final purpose of this Ph.D. project was the design of an innovative wound dressing able to efficiently treat chronic infected wounds, promoting tissue healing**. Infections represent one of the most important factors of impaired healing, requiring local or systemic administration of high doses of antibiotics. However, this approach could in turn bring to bacteria resistance. Moreover, uncontrolled drug release mechanisms could require high doses of therapeutic agents with potentially low overall effectiveness. Thus, the design of smart and antibiotic-free wound dressings locally treating infections in response to clinical conditions (e.g., wound exudate pH) represents an engaging challenge in the biomedical field. Furthermore, the low effectiveness of currently available commercial wound dressings can be also attributed to the lack of personalised treatments, i.e., dressings with patient-specific shape and drug content. To face the lack of shape personalisation, few attempts have been recently reported towards the design of personalised wound dressings exploiting complex technologies, such as *in situ* bioprinting or electrospinning. However, these approaches are considered to be too complicated for clinical translation. An alternative approach to develop wound dressings able to perfectly fill the wound cavity while exploiting a simple technology consists in the combination of 3D wound bed scanning and 3D patch printing. In this scenario, the design of a 3D printed smart and patient-specific patch based on customised stimuli-responsive hydrogels aims at addressing drawbacks of commercial wound dressings, promoting speeded up healing of hard-to-close wounds.

Specifically, such personalised patch was here designed through the **sequential printing of two different bio-inks combining different functions in two adjacent patch sides**: (i) the bio-ink used to print layers in contact with the wound bed (Gel A) should be able to provide a moist environment and release encapsulated drugs via a pH-controlled mechanism triggered by exudate absorbance; (ii) the bio-ink used to print upper layers (Gel B) should be able to further support the maintenance of a moist wound environment and provide ulcer coverage for several days. Figure 1 illustrates the approach exploited to develop the two multi-functional bio-inks.

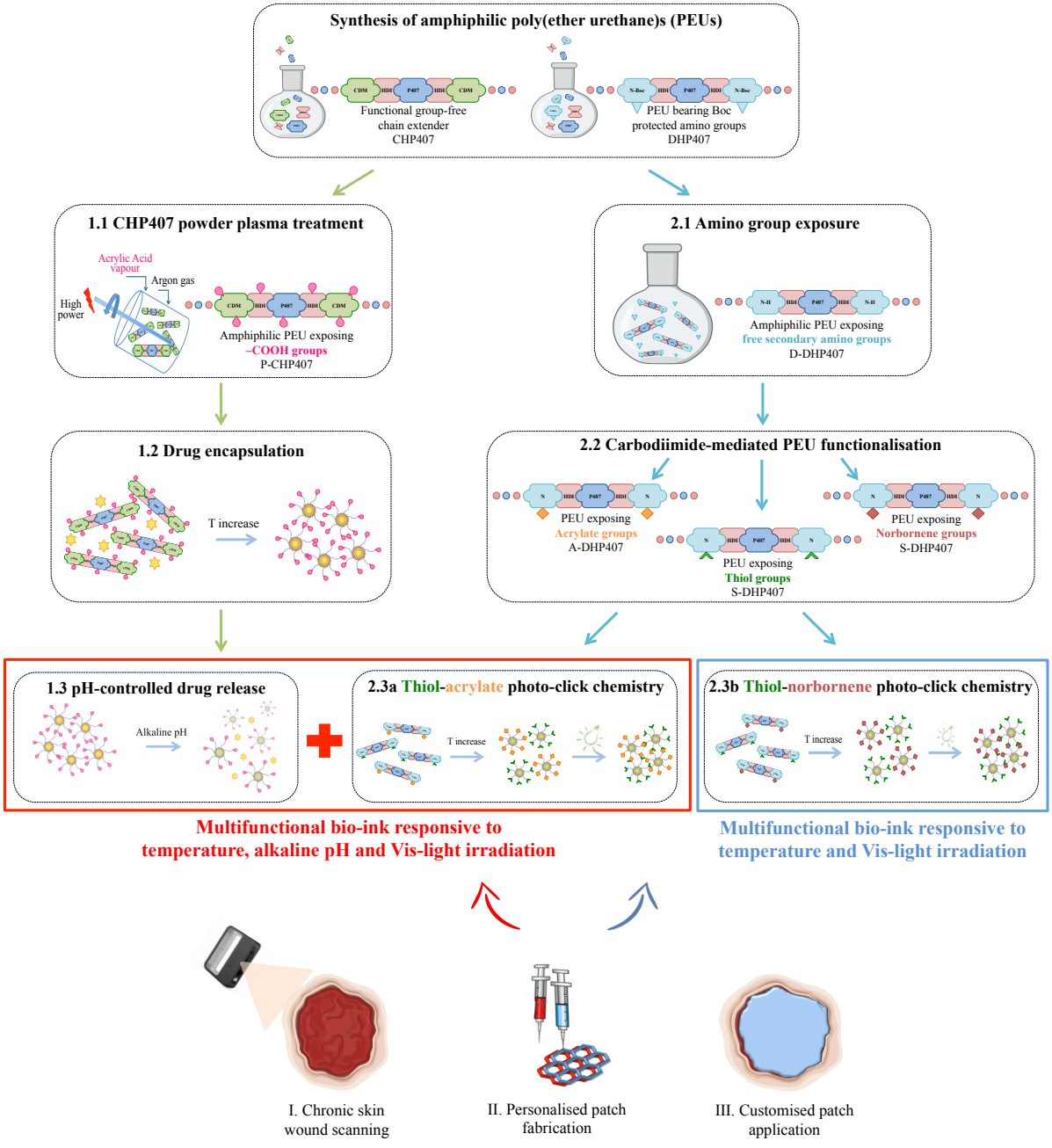

Figure 1. Schematic representation of the approach exploited to design the multi-stimuli-responsive bio-inks.

Synthesis of two amphiphilic poly(ether urethane)s (PEUs) based on a common pre-polymer (i.e., Poloxamer® 407 –P407– and 1,6-hexamethylene diisocyanate - HDI) and differing for their chain extender, i.e., 1,4-cyclohexanedimethanol (CDM) and N-Boc diethanolamine (N-Boc) giving CHP407 and DHP407, respectively. (1.1) Plasma treatment performed on CHP407 powders (P-CHP407) to expose alkaline-pH-responsive carboxylic acid groups, (1.2) temperature-driven drug encapsulation in P-CHP407-based hydrogels and (1.3) alkaline-pH-triggered drug release. (2.1) Acidic treatment performed on DHP407 to expose secondary amino groups along polymeric chains (D-DHP407) and (2.2) carbodiimide-mediated D-DHP407 functionalisation with photo-sensitive moieties: acrylates (A-DHP407), thiols (S-DHP407) and norbornene molecules (NB-DHP407). (2.3) Optimisation of two different thermo-sensitive and Vis-light-responsive thiol-ene formulations: thiol-acrylate (2.3a) and thiol-norbornene (2.3b). Optimisation of the two bio-inks: mixture of P-CHP407, S-DHP407 and A-DHP407 polymers to develop a thermo-, pH- and Vis-light-responsive bio-ink to 3D print the layers in contact with the wound bed (Gel A, 1.3 and 2.3a); mixture of S-DHP407 and NB-DHP407 to develop a thermo- and Vis-light-responsive bio-ink to 3D print the upper layers of the engineered patch (Gel B, 2.3b).

To design multi-functional bio-inks fully complying with application requirements (i.e., thermo-/pH-/photo-responsive Gel A and thermo-/photo-responsive Gel B), two PEUs differing for their chain extender were first synthesised (Figure 1). Specifically, the two polymers were based on a common thermo-responsive pre-polymer obtained from the reaction between an amphiphilic triblock copolymer belonging to the Poloxamer® family (i.e., Poloxamer® 407) and an aliphatic diisocyanate (i.e., 1,6-hexamethylene diisocyanate). Subsequently, PEU pre-polymers were chain-extended with two low molecular weight diols, i.e. 1,4-cyclohexanedimethanol and N-Boc diethanolamine exposing no functional groups or Boc protected secondary amines, respectively (referred to with the acronym CHP407 and DHP407, respectively). The success of the syntheses was then assessed through infrared spectroscopy and size exclusion chromatography (50 kDa and 30 kDa Weight Average Molecular Weight $\overline{M}_w$, respectively). Specifically, **both synthesis procedures were optimised to obtain PEUs with the target properties**, i.e., high molecular weight, marked thermo-responsiveness and, in the case of N-Boc diethanolamine-based PEU, high number of available functional groups.

Concerning the PEU containing the functional group-free chain extender (i.e., CHP407), the influence of Poloxamer® 407 macrodiol purity on the resultant polymer chemical properties and thermo-responsiveness was investigated. Macrodiol purification, performed to remove low molecular weight diblock copolymers, caused a reduction in CHP407 polydispersity index from 1.6 to 1.4 and a slight decrease in the onset temperature (from 14.6 °C to 12.7 °C) of the sol-to-gel transition of its aqueous solutions. In addition, a highly organised gel network was finally achieved. However, swelling/stability tests in simulated physiological conditions evidenced an initial lower absorbance capability of hydrogels (with 15% w/V polymer concentration) based on CHP407 with purified macrodiol, while detrimental dissolution/erosion phenomena started after 7 days of incubation (93% dissolution at 14 days). In addition, by adapting for the first time to micellar hydrogels an innovative nano-scale characterisation technique, i.e., Low Field Nuclear Magnetic Resonance spectroscopy, a thorough investigation of micelle packing into an organised hydrogel network was performed. Specifically, such nano-scale characterisation highlighted the formation of a more organised but less interconnected network upon macrodiol purification, leading to worsened

hydrogel properties (i.e., lower capability to absorb fluids from the surrounding environment, resist against dissolution/erosion and withstand to applied strain). Based on the above results, **CHP407 was synthesised from non-purified Poloxamer® 407, resulting in additional advantages such as faster and environmentally friendly synthesis procedure**.

Subsequently, CHP407 hydrogels suitability as drug delivery system was explored by using drugs with different hydrophobic/hydrophilic nature (i.e., Ibuprofen -IBU- and Ibuprofen Sodium Salt -IBUSS- as hydrophobic and hydrophilic drug, respectively). Specifically, the interactions occurring between drug molecules and polymer chains undergoing temperature-driven arrangement into micellar structures were investigated by studying changes in the micelle average diameter upon drug content increase. Dynamic Light Scattering measurements highlighted the formation of slightly bigger PEU micelles in the presence of IBU compared to IBUSS (e.g., at 37 °C the average hydrodynamic micelle diameter was measured to be 58.2 ± 4.7 nm and 37.3 ± 2.1 nm, respectively), thus suggesting **different cargo localisation in the micellar hydrogel network: hydrophobic IBU was probably encapsulated into the micelle core, while hydrophilic IBUSS was included in the interstitial space among micelles**. These hypotheses were further supported by lower sol-gel transition temperatures observed for hydrogels encapsulating IBU, suggesting increased hydrophobicity, and by the different drug release kinetics and mechanism as assessed through the application of the Korsmeyer-Peppas model.

CHP407 powders were also plasma treated in the presence of acrylic acid vapour to expose carboxyl groups for an additional control over payload release from hydrogels through an alkaline pH-triggered mechanism. A thorough investigation of process conditions (i.e., Ar gas flow rate between 10 and 50 sccm) allowed a **selection of the optimised treatment parameters** (i.e., 10 sccm Ar flow) **for the exposure/grafting of 5.3×10^{18} ± 5.5×10^{17} –COOH units/g of polymer** (colorimetrically quantified through Toluidine Blue O assay) **while preserving polymer chemical integrity ($\bar{M}_w$ 50 kDa) and thermo-responsiveness** (Figure 1.1). Subsequently, swelling tests performed in contact with buffers at different pH values (i.e., pH 5 and 8) proved hydrogel capability to actively transmit pH changes from an external alkaline environment to their core, through –COOH group deprotonation and thus, through the formation of electrostatic repulsive forces among polymer chains. In addition, hydrogel network broadening driven by alkaline pH was further assessed at the nano-scale through an innovative technique, i.e. LF-NMR analysis, highlighting a re-arrangement of water molecules respect to micelles in the presence of carboxylated ions. Lastly, proof of concept results of pH-triggered drug delivery were obtained by analysing **IBU release** from hydrogels, which **resulted significantly higher at each analysed time point ($p < 0.0001$) when hydrogels were in contact with an alkaline buffer at pH 8 compared to an acidic medium at pH 5** (Figure 1.2 and 1.3).

Concerning the PEU bearing Boc-protected amino groups in its chain extender (i.e., DHP407), synthesis procedure was investigated as a function of initial macrodiol concentration (ranging between 10% and 20% w/V) and first and second step reaction times (varying between 30 min - 150 min and 1 h and 20 h, respectively) to obtain a more stoichiometric polymer. Specifically, such optimisation was carried out to synthesise **low molecular weight pre-polymers and highly**

extended PEU, thus maximising the number of available amino groups along each chain for further functionalisation. The optimal synthesis conditions (i.e., 15% w/V concentrated macrodiol, 45 min I step and 2 h II step reaction times) were selected based on quantification of amino groups ($4.5\times10^{20} \pm 1.8\times10^{19}$ units/g of polymer) through Orange II Sodium Salt colorimetric assay and Nuclear Magnetic Resonance (NMR) analyses.

Furthermore, the acid treatment performed to expose secondary amines did not affect polymer chemical integrity ($\bar{M}_w$ 30 kDa) and thermo-responsiveness ($T_{gelation}$ = 27 °C within 7 min for 18% w/V concentrated system) (Figure 2.1). Subsequently, **to provide PEU with responsiveness to Vis-light irradiation, secondary amino groups were exploited to graft photo-sensitive moieties** (i.e., thiol, acrylate and norbornene groups) **through water-based carbodiimide chemistry** (Figure 2.2). Each coupling reaction was optimised in terms of time and pH to maximise the grafting yield while preserving the functionality of photo-sensitive groups. Based on infrared and NMR data and the colorimetric Ellman's test, the highest number of exposed thiol groups (i.e., **$1\times10^{19} \pm 1.3\times10^{18}$ thiol units/g of polymer**) was achieved by setting pH 4 and 6 h as reaction parameters. Furthermore, shelf life studies evidenced the need of inert storage conditions (i.e., storage under nitrogen) to avoid thiol oxidation into disulphide bonds over time. Regarding functionalisation with acrylate and norbornene groups, the highest grafting yields (i.e., **$2.6\times10^{19} \pm 1.5\times10^{18}$ acrylate units/g of polymer** and **$8.1\times10^{17} \pm 2.6\times10^{16}$ norbornene units/g of polymer**) were obtained by setting as reaction conditions pH 7 and 9, respectively, and 6 h. Irrespective of the grafted molecules, although slight changes were observed in polymer thermo-responsiveness at the micro-scale, **carbodiimide chemistry did not affect hydrogel thermosensitivity at the macro-scale**.

Hydrogel responsiveness to Vis-light was achieved by exploiting thiol-ene photo-click chemistry, thus by blending PEUs exposing thiol/acrylate (Figure 2.3a) and thiol/norbornene (Figure 2.3b) at 1:1 functional group molar ratio. A preliminary investigation through UV/Vis spectroscopy was first carried out on control virgin molecules (i.e., the same molecules grafted to PEU chains to expose functional groups) to select the optimal photo-initiator concentrations (i.e., Eosin Y 0.5 mM and 1 mM for thiol/norbornene and thiol/acrylate, respectively). Furthermore, the thiol/acrylate system also evidenced the need of a co-initiator (i.e., triethanolamine) at 7.5 mM concentration to improve the efficiency of photo-induced reaction. Based on such photo-irradiation conditions, **the success of Vis-light-induced thiol-ene photo-click reaction in hydrogels was first chemically assessed through** NMR analysis, which indicated a **simultaneous consumption** (although not complete) **of both thiol/acrylate and thiol/norbornene functional groups**. Then, rheological tests performed on photo-irradiated hydrogels and solutions showed their improved properties such as the prevalence of elastic over viscous behaviour in a wider frequency range and gel superior resistance to applied deformation. In addition, the comparison between thiol/acrylate and thiol/norbornene rheological behaviours evidenced **higher gel strength in thiol/norbornene compared to thiol/acrylate hydrogels** (as suggested by the critical deformation value of 11.6% *vs* 7.2%). Thus, thiol/norbornene hydrogel was selected as thermo-/photo-responsive bio-ink in the perspective printing of patch upper layers (Gel B, Figure 2.3b). **Proof of concept results of thiol-ene hydrogel printability were obtained through temperature-controlled layer-by-layer deposition** according to different CAD models (i.e., square grid and honeycomb mesh). Furthermore, the success of the

photo-induced reaction step, exploited **to provide 3D printed construct with secondary stability**, was demonstrated by the higher layer resolution and shape maintenance over time compared to not photo-irradiated systems.

Lastly, to design a multi-functional bio-ink able to release its payload in response to alkaline environment (i.e., Gel A, Figure 1.3 and 2.3a) in the perspective fabrication of personalised wound patches, thiol/acrylate hydrogels were blended with the thermo-/pH-responsive system at different weight ratios (i.e., 75/25 wt, 50/50 wt and 25/75 wt). Frequency and strain sweep tests evidenced the formation of weaker gel networks by decreasing the concentration of the photo-sensitive counterpart (i.e., decreased frequency range for prevalent elastic behaviour and lower storage/loss moduli differences measured at 37 °C and 0.01% strain). On the other hand, pH measurements upon incubation with pH 8 buffer showed improved alkaline pH transmission capability by increasing thermo-/pH-responsive polymer content (e.g., $\Delta pH_{@10min}$ = 1.5, 2.5 and 2.7 for 25%, 50% and 75% wt thermo-/pH-responsive polymer content, respectively). Hence, **the blend obtained by mixing thermo-/photo-responsive and thermo-/pH-responsive PEUs at 50/50 wt. ratio was the most promising formulation ensuring enhanced mechanical properties, while keeping pH transmission capability**. Lastly, pH-triggered IBU release tests from the optimised blend composition revealed extremely higher amounts ($p < 0.0001$) of drug released at pH 8 compared to pH 5 milieu.

The here-developed hydrogel formulations could thus represent **a significant step forward in the design of patient specific patches to treat hard-to-close chronic wounds, allowing a high degree of personalisation** in terms of shape and drug content, which could be finely optimised according to wound biochemical properties and geometrical features acquired through imaging techniques. Nevertheless, by exploiting the wide versatility of formulation components, the developed hydrogel platform could be easily adapted to effectively face many other applications in the tissue engineering/regenerative medicine field.