

# Rapid prototyping of polymeric hydrogels for biomedical applications

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## Abstract

Rapid prototyping techniques have been investigated for the production of biomedical devices that perfectly fit the patient's tissue defect (e.g. for bone and dental applications) and/or reproduce the microstructure of the tissue or organ of interest. The possibility to create patient-specific devices has been recently exploited for the creation of tissue engineering scaffolds, i.e. porous, resorbable matrices, which stimulate cell functions and induce tissue regeneration by providing cells with appropriate physical, mechanical and biochemical cues. Poly(ethylene glycol) (PEG)-based hydrogels, although intrinsically non-biodegradable and non-bioactive, show great promise as tissue engineering scaffolds, due to their ability to be covalently linked to bioactive and/or degradable moieties, that elicit specific cell responses, and to their fast and biocompatible formation under ultraviolet (UV) exposure. In this work, poly(ethylene glycol)-based hydrogels, containing bioactive moieties, were photopolymerized and characterized in terms of mechanical, swelling and degradation properties. The production of hydrogels possessing a complex shape was finally investigated by means of stereolithography, a rapid prototyping technique which is able to build a three-dimensional object, starting from the CAD model, by guiding an ultraviolet laser beam on the surface of a photosensitive solution. The results demonstrated that the developed hydrogel formulations allow the creation of biomimetic constructs with complex shapes, which might be useful as platforms for tissue engineering or as tissue mimicking phantoms.

## INTRODUCTION

Polymeric hydrogels are three-dimensional

macromolecular networks which are able to absorb great amounts of water solutions [1], and retain or release the absorbed water according to the environmental conditions (e.g. temperature, pH). Due to their high water content, hydrogels are highly biocompatible, possess mechanical and diffusive properties similar to those of soft tissues, and are usually non-adhesive for cells, with the exception of those hydrogels containing extracellular matrix proteins (e.g. collagen) [2-3]. These features make hydrogels particularly interesting for the development of biomedical devices for a number of *in vivo* applications, e.g. devices for controlled drug release, anti-adhesive barriers for soft tissues, and platforms for the design of tissue engineering scaffolds [2-3]. With regard to *in vitro* applications, hydrogels represent useful tissue mimicking phantoms, which can be exploited to simulate the response of soft tissues to given external stimuli (e.g. mechanical deformation, ultrasound propagation, diffusion of drugs, etc) [4].

Among synthetic hydrogels, those based on poly(ethylene glycol) (PEG) are particularly attractive for *in vivo* use. This is due to the intrinsic resistance of PEG to protein adsorption, which makes it an ideal substrate or platform for the creation of biomaterials with specific biological properties [5-11]. Indeed bioactive and degradable moieties, either in the macromolecular form or as short peptide sequences, can be covalently attached to the PEG network in order to obtain biomimetic hydrogels able to stimulate specific cell functions [5-11]. The formation of hydrogels from acrylate derivatives of PEG is particularly straightforward. Aqueous solutions with low polymer concentrations can be easily photo-stabilized through exposure to UV light for a few minutes. Such a photocrosslinking reaction is carried out at mild conditions, so that cells and biomolecules can be entrapped in the hydrogel network while crosslinking, without compromising their viability and bioactivity

[3, 12-13].

Recently the production of PEG-based hydrogels by means of stereolithography (SLA) has been investigated [14-16], in order to create biomimetic constructs which reproduce customized tissues and/or organs. SLA is a rapid prototyping technique where the object of interest is produced layer by layer, according to its CAD model, by photostabilizing a reactive solution through an UV laser beam. Starting from diagnostic images (e.g. nuclear magnetic resonance and computed tomography), it would be possible to realize patient-specific devices, which not only do fit the size and shape of the tissue of interest, but also reproduce the microstructure of the tissue itself. The suitability of a resin to be processed by means of SLA is defined by two parameters [17], which are related to the laser energy according to the following:

$$C_d = D_p \ln \frac{E_o}{E_c} \quad (1)$$

where  $E_o$  is the energy dose at the liquid surface,  $C_d$  is the thickness of gelled resin, and the working parameters  $D_p$  and  $E_c$  are the penetration depth and critical energy respectively.  $E_c$  is the minimum value of energy required to polymerize the resin, while  $D_p$  is the gelled thickness when  $E_o = E_c \cdot e$ . A good resin should have low values of  $E_c$  and high values of  $D_p$ , in order to have high cured thicknesses with low energy doses.

Rarely, studies on the production of PEG-based hydrogels via SLA report the characterization of the polymeric solutions in terms of  $D_p$  and  $E_c$  [15]. Nevertheless, the SLA of a number of hydrogel formulations can be feasible, even when not using the optimal values of  $D_p$  and  $E_c$ .

In this study, we synthesized several hydrogels based on poly(ethylene glycol) diacrylate (PEGDA) and methacrylated hyaluronic acid (mHA). HA is a glycosaminoglycan diffused in the extracellular matrix of animal and human tissues. In addition to biodegradability, biocompatibility and immunoneutrality, HA is also reported to possess angiogenic properties when starting degrading in the vivo environment [18]. Due to these attractive features, HA was considered as a candidate bioactive moiety able to confere biodegradability and bioactivity to PEGDA-based hydrogels. Following methacrylation of HA, hydrogels with different percentages of PEGDA and mHA were synthesized and characterized in terms of swelling capability, in different water solutions, and mechanical stiffness. The presence of HA in the hy-

drogel network was further assessed through *in vitro* degradation experiments. Finally, the production of hydrogels with complex shapes was attempted by means of SLA.

## MATERIALS AND METHODS

The materials employed in this study were purchased from Sigma-Aldrich and used as received, unless otherwise stated.

### *Methacrylation of hyaluronic acid*

Hyaluronic acid (HA, MW 150 kDa, Fidia Advanced Biopolymers, Italy) was methacrylated by means of a glycidyl methacrylate grafting reaction, according to a protocol described in the literature [19]. Briefly, HA (1% w/v) was dissolved in 50 ml distilled water. Subsequently, triethylamine (TEA, 1.8 ml), glycidyl methacrylate (GDM, 1.8 ml) and tetrabutyl ammonium bromide (TBAB, 1.8 g) were added in this order to the HA solution. The reaction mixture was then kept under magnetic stirring (about 500 rpm) for 24 hours at room temperature. The reaction product was precipitated and washed in acetone (at least 20 times the volume of HA solution), redissolved in distilled water and finally lyophilized for 24 hours in order to get methacrylated hyaluronan (mHA) in the dry state. The degree of methacrylation for mHA is reported to be 11% [19].

### *Hydrogel synthesis*

Poly(ethylene glycol) diacrylate (PEGDA, average  $M_n=700$ ) and mHA were used to produce biodegradable and biomimetic hydrogels by means of UV light-induced crosslinking reaction. PEGDA and mHA were dissolved in distilled water, with different weight ratios, according to Table 1.

As a suitable photoinitiator, DAROCUR 1173 (BASF), which is soluble in acrylate-based systems, was then added to the polymer solution in a 3  $\mu$ l/ml amount. Hydrogel disk-like samples were obtained by photostabilizing 1.5 ml of the above solution in a

Sample code	PEGDA conc. (% w/v)	m-HA conc. (% w/v)
PEGDA5_HA1	5	1
PEGDA5	5	0
PEGDA10_HA1	10	1
PEGDA10	10	0

Table 1. Hydrogel formulations synthesized in this study.

35-mm diameter Petri dish, through the exposure at 365 nm for 5 min, at an intensity of  $\sim 2$  mW/cm<sup>2</sup>. The UV lamp adopted for the hydrogel formation is a medical device routinely used for diagnostic purposes in dermatology (Jelosil Srl, Italy).

Following UV exposure, the hydrogel samples were washed in an excess amount of distilled water overnight, in order to remove unreacted chemicals.

### Swelling ratio measurements

The hydrogel swelling capability in distilled water was assessed by measuring the mass swelling ratio, defined as follows:

$$SR = \frac{M_{sw} - M_d}{M_d} \quad (2)$$

where  $M_{sw}$  is the mass of the swollen hydrogel sample and  $M_d$  the mass of the dried sample. For the swelling measurements, each hydrogel type was synthesized in triplicate, and from each sample four 5-mm diameter disks were punched out. After washing in distilled water, both at room temperature and 37°C, the weight of the disks was measured to determine  $M_{sw}$ . Before weighing, the hydrogel samples were gently blotted with soft paper to remove excess water from their surface. The samples were then air-dried at room temperature under a chemical hood for 24-48 hours, and weighed again to determine  $M_d$ .

In order to verify whether the hydrogel formulations were sensitive to ionic strength variations, the swelling ratio of the hydrogels was also assessed at room temperature in water solutions at different NaCl concentrations ( $10^{-5}$ ,  $10^{-4}$ ,  $10^{-3}$ ,  $10^{-2}$ ,  $10^{-1}$ , 1 M). Results were averaged over three independent measurements.

### Mechanical tests

After swelling in distilled water for 24 hours, hydrogel disks were subjected to uniaxial compression at 20°C, by means of a parallel plate rheometer (ARES, Scientific Rheometric). Such tests were carried out to assess the mechanical properties of the samples and to verify if the theory of rubber elasticity could be applied to evaluate their crosslink density.

In case of a perfect polymer network subjected to small uniaxial deformations (i.e. for negligible volume changes), the following equation derived by Flory [1] might be used to estimate the elastically effective degree of crosslinking:

where  $\sigma$  is the uniaxial stress,  $R$  is the universal gas constant,  $T$  is the absolute temperature,  $\rho_{xe}$  is the crosslink density,  $V_{2s}$  is the polymer volume fraction

$$\sigma = RT\rho_{xe}V_{2s}^{1/3}V_{2r}^{-1/3}\left(\alpha - \frac{1}{\alpha^2}\right) = G\left(\alpha - \frac{1}{\alpha^2}\right) \quad (3)$$

in the swollen state, i.e. the inverse of the volume swelling ratio,  $V_{2r}$  is the polymer volume fraction in the relaxed state (i.e. in the reactive mixture),  $\alpha=L/L_i$  is the deformation ratio, with  $L$  the actual thickness of the deformed sample and  $L_i$  the initial thickness of the swollen sample ( $\alpha>1$  for elongation and  $\alpha<1$  for compression, respectively) and  $G$  is the shear modulus of the swollen polymer. Therefore, for a crosslinked rubber-like polymer and for  $\alpha\rightarrow 1$ , the plot of  $\sigma$  against  $(\alpha-1/\alpha^2)$  is linear, with a slope that defines the shear modulus  $G$ .

In this study, thin disks of swollen samples (25 mm diameter,  $n=3-4$  for each hydrogel type) were accurately cut and positioned between the parallel plates of the rheometer. The gap was adjusted starting from the original sample height (between 1 and 1.5 mm). A constant velocity equal to 0.001 mm/s was then imposed to the upper plate to start the measurement. The evolution of the normal force was recorded as a function of the gap between the plates, in the range of  $\alpha$  values between 0.9 and 1.

### In vitro degradation experiment

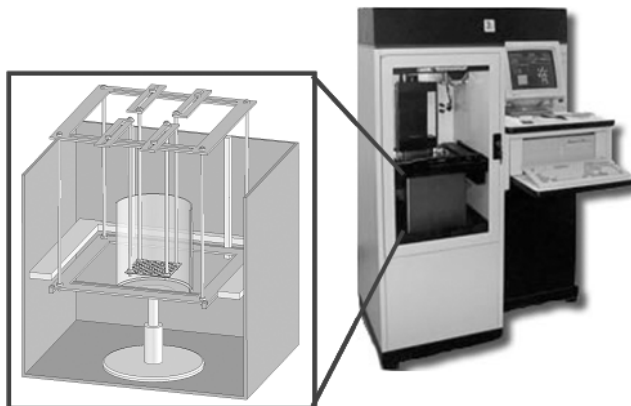
The *in vitro* degradability of the hydrogels was assessed by means of gravimetric measurements. Hydrogel disks of 5 mm diameter were swollen in phosphate buffered saline (PBS, pH=7.4) at 37°C. After swelling, their initial weight  $W(t_0)$  was recorded. The samples were then incubated in 1 ml of 15 U/ml hyaluronidase solution in PBS, at 37°C. At fixed time points, up to 180 hours, the samples were weighed to measure their time-dependent weight,  $W(t)$ . The degradability of the samples resulting in mass loss was evaluated throughout the study by considering the ratio between  $W(t)$  and  $W(t_0)$ . During the experiment, every 24 hours the hyaluronidase solution (1 ml) was replaced with an equal amount of fresh solution, in order to ensure the enzyme bioactivity for the entire time length of the study.

### Stereolithography of PEGDA-mHA hydrogels

An SLA device (3D System, Valencia, CA), operating with a He-Cd laser emitting at 325 nm, was modified with a custom-designed elevator-driven build table, as described in detail elsewhere [4] (Fig. 1). This system allowed to use a reduced volume (from 0.3 up to 3 kg) of polymeric solution, compared to that of the standard SLA tank (43 kg). The

tank was replaced with a small glass beaker fixed on an adjustable support. The recoating process was disabled due to the low viscosity of the PEGDA-mHA solutions. Hydrogels with several shapes and porous patterns were then constructed layer-by-layer, at fixed values of the working parameters  $D_p$  and  $E_c$ , equal to 0.11 mm and 6 mJ/cm<sup>2</sup> respectively. Such values had been measured for similar PEGDA-based hydrogels investigated in a previous study [4].

## RESULTS AND DISCUSSION



**Fig. 1.** Stereolithography equipment (right) and custom design of an elevator-driven build table, suitable to process low volumes of polymeric solutions (left).

### Swelling ratio measurements

The results of the swelling measurements in distilled water, both at 22 and 37°C, are reported in Table 2. The measurements confirmed that, as expected, the hyaluronan was covalently attached to the PEGDA network, so that polyelectrolyte hydrogels were formed, due to the carboxylic groups brought by HA. Indeed, for each PEGDA concentration and test

Sample code	SR 22°C (g water/g dry polymer)	SR 37°C (g water/g dry polymer)
PEGDA5_HA1	39.16 ± 6.99	28.20 ± 4.35
PEGDA5	21.32 ± 8.71	27.27 ± 4.96
PEGDA10_HA1	9.91 ± 0.87	9.60 ± 2.26
PEGDA10	6.72 ± 0.18	6.25 ± 0.09

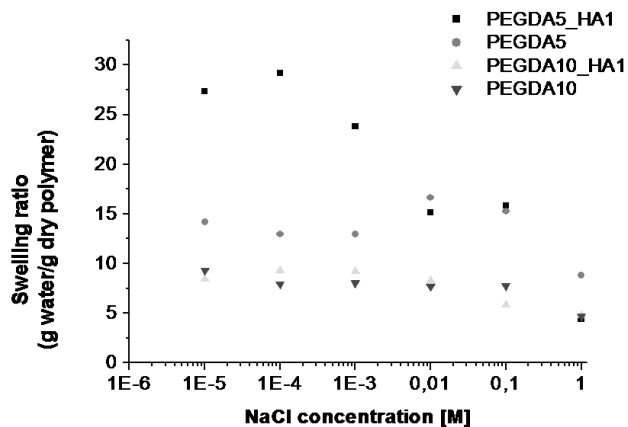
**Table 2.** Swelling ratios in distilled water. Results were averaged over 12 measurements and are expressed as mean ± the standard deviation.

temperature, the swelling ratio of PEGDA-mHA hydrogels was significantly higher than the one reported for the PEGDA samples. The electrostatic charges that were anchored to the PEGDA-mHA network enhanced the swelling capability due to the combined effect of electrostatic repulsion and, most of all, the so-called Donnan equilibrium [1].

The Donnan contribution to hydrogel swelling is related to the higher concentration of counterions which are present within the gel to ensure the macroscopic neutrality. Such an unbalance of counterions between the inside and the outside of the gel causes more liquid to enter the network, thus increasing the swelling capability.

It is also clear that the increase of the swelling ratio is dependent on the concentration of fixed charges within the gel. This is why higher swelling ratios were obtained for those samples having the lowest ratio between PEGDA and mHA (i.e. samples PEGDA5\_HA1). However, the difference in swelling capability between samples PEGDA5 and PEGDA5\_HA1 was not found to be significant at 37°C. Such temperature-dependent behaviour seemed to be related to the PEGDA/mHA ratio of the hydrogels, since the swelling ratios attained for samples PEGDA10\_HA1 and PEGDA10 did not change sharply when increasing the temperature from 22 to 37°C.

In addition to possess higher swelling capability, polyelectrolyte hydrogels are sensitive to environmental stimuli, such as pH and ionic strength of the external solution [1]. In order to verify the environmental sensitivity of the PEGDA-mHA hydrogels, their swelling ratio was assessed in water solutions with different ionic strength (Fig. 2). As expected, hydrogels based on PEGDA alone were not particularly sensitive to ionic strength variations, although a slight increase of the swelling ratio when



**Fig. 2.** Swelling ratio in water solutions at different ionic strength. Average results are reported (n=3-4).

lowering the ionic strength could be detected. On the contrary, polyelectrolyte PEGDA-mHA hydrogels displayed high sensitivity to environmental ion concentration, and this finding was more evident for those samples containing the higher amount of mHA compared to PEGDA (i.e. samples PEGDA5\_HA1). Therefore, hydrogels based on PEGDA and m-HA can be considered as smart materials, as they are able to respond to changes in the external environment by swelling/deswelling transitions. It is worth noting that changes in the pH and/or ionic strength are particularly relevant in the physiological environment. Therefore, the PEGDA-mHA hydrogels synthesized in this study show potential to be used as sensitive materials in biomedical applications.

### Mechanical tests

Uniaxial compression tests on hydrogel disks were carried out to assess the mechanical properties of the hydrogels, as well as to estimate their degree of crosslinking or crosslink density [1] (Table 3).

Sample code	Shear modulus G (kPa)	Crosslink density $\rho_{xc}$ (mol/cm <sup>3</sup> )
PEGDA5_HA1	2.58 ± 6.66	2.14 ± 0.71
PEGDA5	6.83 ± 2.18	6.48 ± 2.07
PEGDA10_HA1	20.3 ± 5.89	9.05 ± 2.62
PEGDA10	20.4 ± 2.38	8.53 ± 0.99

**Table 3.** Results from uniaxial compression tests. Reported values were averaged over 3-4 independent measurements and are expressed as mean ± the standard deviation.

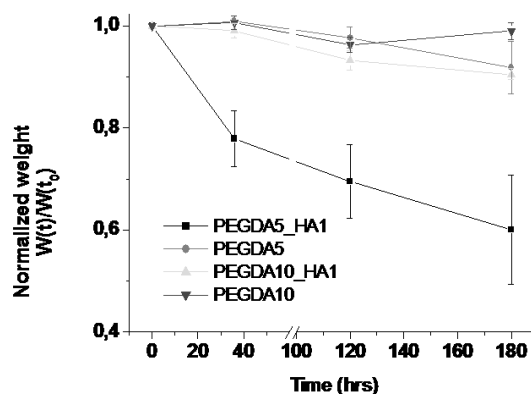
For a PEGDA concentration of 5% w/v, the results showed that the crosslinking of mHA with PEGDA led to a significant decrease of the shear modulus of the samples and, as a consequence, of the estimated elastically effective crosslink density. This might be ascribed to the fact that the hyaluronan molecules, possessing a much higher molecular weight compared to PEGDA ones (150 kDa vs. 700 Da), act as a spacer within the polymer network, thus limiting the crosslinking reaction that would be attained among PEGDA molecules themselves. This could also explain why the negative effect of mHA on the mechanical properties and crosslink density of the hydrogels was not relevant, when doubling the PEGDA concentration (10% w/v) at fixed mHA content. The

ratio between PEGDA and mHA thus seems to be a strategic design variable, as it is able to modulate the mechanical stability of the hydrogels, as well as their swelling behaviour. Further studies should be carried out in order to find the optimal PEGDA/HA ratio for which suitable environmental sensitivity of the hydrogels is attained, without compromising their mechanical properties.

### In vitro degradation experiment

Incubation of the hydrogels in 15 U/ml hyaluronidase solution at 37°C allowed to detect the sample biodegradability, by measuring their progressive weight loss.

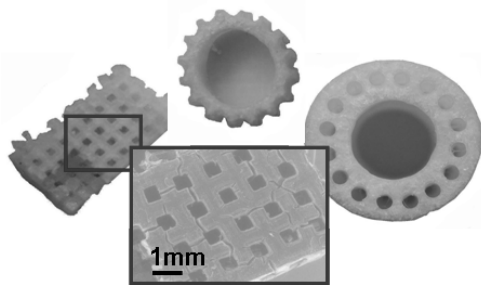
As reported in Fig. 3, the PEGDA/mHA ratio used during the hydrogel synthesis was found to significantly affect the degradation behaviour of the samples. As expected, the highest degradation rate was displayed by samples with the lowest PEGDA/mHA ratio (i.e. samples PEGDA5\_HA1), which were found to lose about 40% of their initial weight, after 180 hours of incubation in the hyaluronidase solution. It is worth noting that samples containing only PEGDA also showed a slight degradation (less than 10%) during the time length of the study, which can be ascribed to the hydrolysis of the ester linkage present on the PEGDA backbone [20-21]. Samples PEGDA10\_HA1 were found to degrade with a rate quite similar to that of PEGDA hydrogels.



**Fig. 3.** Degradation of hydrogels in 15U/ml hyaluronidase solution at 37°C. Reported results are expressed as mean ± the standard deviation (n=3).

### Stereolithography of PEGDA-mHA hydrogels

Hydrogel constructs with complex shapes were successfully built by means of stereolithogra-



**Fig. 4.** Examples of hydrogel constructs built by means of stereolithography. Cross-sectional photographs (x-y plane) of the hydrogels are reported. The inset micrograph shows that pores with a size of about 500  $\mu\text{m}$  could be obtained in the final constructs.

phy (SLA) (Fig. 4). The constructs displayed mechanical properties suitable for handling and smooth surfaces. In particular, constructs with particular pore designs were built in order to evaluate the resolution of the SLA process on the tested hydrogel formulations. The smallest pore size attainable by means of SLA was about 500  $\mu\text{m}$ , which is a limit due not only to the starting beam diameter, but also to possible scattering phenomena between the incident beam and the PEGDA and/or mHA macromolecules. However, a certain extent of dimensional shrinkage, following the photocrosslinking reaction, was detectable in the x-y plane (the shrinkage was instead negligible along the z axis). Such a shrinkage might contribute to the formation of pores with a slightly lower diameter than that dictated by the dimensional resolution of stereolithography. As expected, the x-y plane shrinkage induced also the formation of tensions within the hydrogel constructs and, as a result, a curling effect.

Although further studies should be carried out in order to find the optimal curing parameters,  $D_p$  and  $E_c$ , for the specific hydrogel formulations tested, the SLA process was found to be suitable for the production of PEGDA-mHA hydrogels with complex shapes and microstructures. The dimensional resolution, which is particularly relevant for the design of microporous scaffolds for regenerative medicine, can be improved by properly reducing the laser beam size. Typical scaffolds employed in regenerative medicine possess pore sizes in the range 10-400  $\mu\text{m}$ , with the largest pores particularly recommended for bone regeneration [22-23].

## CONCLUSIONS

In this study, biomimetic hydrogel formulations based on poly(ethylene glycol diacrylate) (PEGDA) and methacrylated hyaluronic acid (mHA) were synthesized through a photostabilization reaction, starting from PEGDA-mHA solutions with different weight ratios between PEGDA and mHA. The presence of mHA in the hydrogel network led to the formation of polyelectrolyte hydrogels, which were sensitive to environmental changes (i.e. ionic strength of the external solution). In particular, the reported results show that the swelling capability and sensitivity of the hydrogels, as well as their mechanical and degradation properties, could be controlled and modulated by changing the PEGDA/mHA ratio. Furthermore, hydrogel constructs with complex shapes could be built by means of stereolithography. Although the resolution of the process should be improved for a successful implementation of the hydrogels as tissue mimicking scaffolds, this study demonstrates that PEGDA-mHA hydrogels are attractive candidates for the future development of patient-specific biomedical devices.

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