Dual-reactive hydrogels functionalizable using "Huisgen click" and "Schiff base" reactions

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Abstract: Hydrogels incorporating different reactive groups are important platforms for the fabrication of functional materials through the conjugation of diverse molecules. In this study, a dual-reactive hydrogel system was designed utilizing aldehyde and azide groups containing methacrylate monomers. Hydrogels were obtained in the presence of a dimethacrylate crosslinker with a combination of hydrophilic PEG-based monomers via free-radical polymerization. The azide and aldehyde sites of the hydrogel network are reactive towards alkyne and amine functional groups, respectively. The advantage of the different reactivities of these functional groups was demonstrated through the attachment of two different dye molecules onto the hydrogel platform via the "Huisgen click" and "Schiff base" reactions to obtain a sensing platform for various applications, such as indicating change in pH of the environment.

Key words: Dual-reactive hydrogel, "Huisgen click", "Schiff base"

1. Introduction

Three-dimensional, insoluble macromolecular polymeric structures that have the ability to swell and hold water are defined as hydrogels [1,2]. The design and development of these polymeric networks are crucial since they exhibit many useful properties such as biocompatibility, softness, biodegradability, responsiveness, and reactivity towards various ligands and molecules [3,4]. Due to these versatile functions, hydrogels are widely employed in many biological, environmental, or industrial applications such as drug [5] or protein delivery [6], sensing applications [7–9], contact lenses [10,11], or cell scaffold materials [12,13].

Hydrogels can be synthesized either through covalent [14–16] or noncovalent crosslinking [17] strategies from various types of monomers and polymers. Noncovalent crosslinking strategies include Van der Waals forces, hydrophilic-hydrophobic or ionic interactions, and hydrogen-bonds, though crosslinked networks obtained through these interactions are not always sufficient to make the hydrogel stable. Covalent crosslinking strategies used to design three-dimensional networks comprise covalent bonds which can either be obtained via a thermally-initiated radical polymerization, photopolymerization [18–21], or click-type reactions [22–26].

Actually, "click" reactions, not only enable efficient synthesis of hydrogels but also allow their modification [27] which is of significance in terms of conjugation of molecules of interest to the network. These reactions [28–35] are known to be facile, selective, versatile, and efficient reactions which were first defined by Sharpless [36]. Some of the click reactions that have been applied for the functionalization of polymeric materials are Huisgen 1,3-dipolar cycloaddition [37], Michael addition [38], Diels–Alder [39], radical-mediated thiol-ene [40] and thiol-yne [41], and Schiff base reactions [42]. The conjugations can be carried out through "click-type" reactions among the reactive groups of hydrogels and the complementary reactive groups of the molecule of interest to be attached. Traditional hydrogels which do not contain reactive functional groups are deficient in terms of some application areas, especially enzyme and drug delivery systems or sensing systems. Reactive hydrogels can be an effective remedy for shortcomings of traditional hydrogel systems which limit their applications. For example, Santander-Borrego et al., described the synthesis of PHEMA based hydrogels using HEMA and GMA in the presence of EGDMA crosslinker with a thermal free-radical initiation method [43]. Epoxide groups of the network were reacted with a specific amount of azido alcohol to obtain azido functionalized hydrogel which would be available to achieve covalent immobilization of alkyne-bearing peptide for cellular response. In another

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study, Becker and colleagues demonstrated the preparation of reactive hydrogels via oxime chemistry by mixing bis-aldehyde functionalized PEG with either a pendant azide or alkene functioanal bis-aminooxy crosslinker. Then, the azide groups were conjugated with alkyne-peptide and alkene units were conjugated with thiol-peptide for tissue engineering applications. Both examples are different in terms of synthesis strategies of hydrogels, but postgelation functionalization is based on "click-type" reactions. In the first example, reactive groups were not affected by the hydrogel synthesis procedures and were available for further modifications [45–47]. In the latter example, the remaining reactive groups after "click-based" crosslinking strategies were used for postmodification. To our surprise, despite many reactive hydrogel examples [48,49], or some dual-reactive polymers [50–52] and polymeric materials [53] in the literature, we did not find dual-reactive hydrogels bearing more than one type of functionality to attach different molecules in an orthogonal fashion. To address this shortcoming, in this study, we exploited monomers that are unreactive towards each other, for the synthesis of dual-reactive hydrogels.

Herein, utilizing free-radical polymerization technique synthesis of dual-reactive hydrogels from azide- and aldehyde-containing methacrylate monomers, a commercially available PEG-based dimethacrylate crosslinker and a PEG-based hydrophilic monomer is described (Figure 1). To demonstrate the dual-functionalization ability of these hydrogels, the azide group of the hydrogel was first functionalized via "Huisgen click" reaction with 1-ethynylpyrene, a blue fluorescent dye. The blue-fluorescent dye attached hydrogel was then modified with a red fluorescence rhodamine-amine dye using the accessible aldehyde units of the hydrogel network through the "Schiff base" reaction. Rhodamine is well-known to give colorimetric responses to acidic conditions or specific metal ions [54]. For example, Kim et al., synthesized hydrogels incorporating fluorescein and rhodamine moieties to accomplish fluorescence changes in various pH ranges [55]. Recently, we also utilized a metal-catalyst-free amine-epoxy "click" reaction to fabricate hydrogels, and the remaining epoxide groups were functionalized with rhodamine-amine for a colorimetric response. Both pH and metal ion responsiveness were successfully demonstrated for this hydrogel system [26]. Pyrene molecule is another well-known fluorescent sensor. Triazole-linked pyrenyl systems were known to be used for the selectivity of some metal ions, or for exhibiting fluorescence properties [56,57]. Hence, we believe that our dual-reactive hydrogel system can be a good candidate for multi-responsive sensing systems.
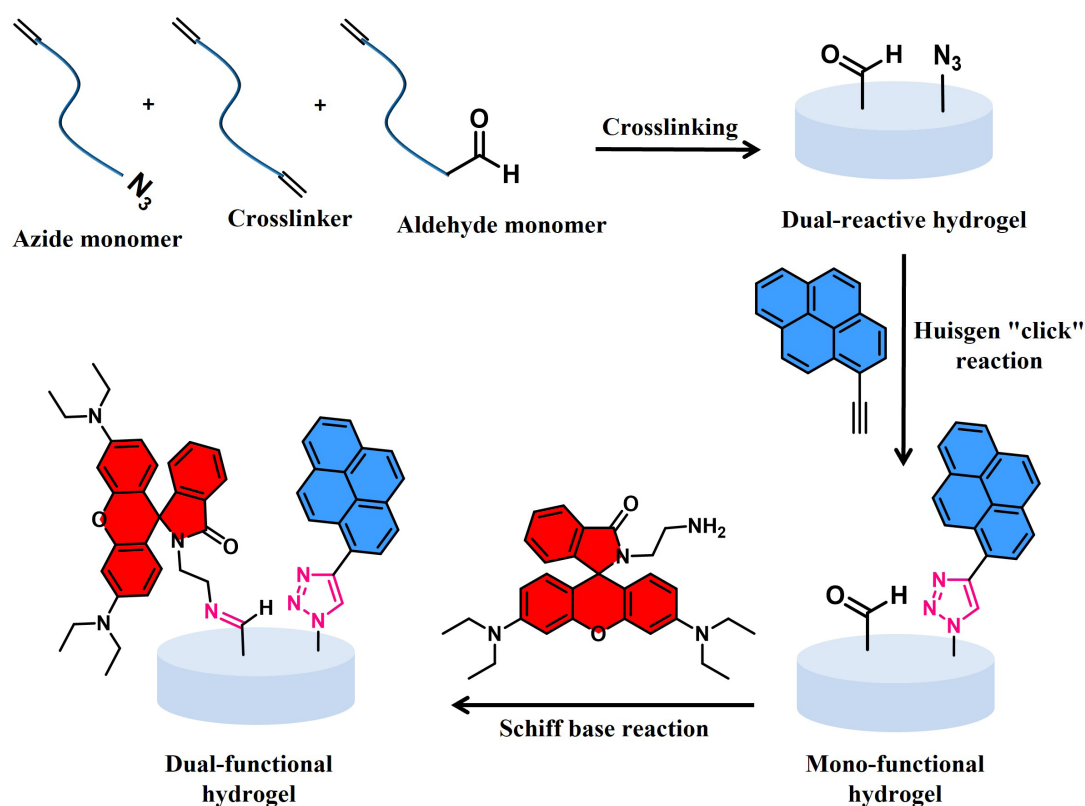

Figure 1. Hydrogel synthesis and dual-functionalization through "Huisgen click" and "Schiff base" reactions respectively.

2. Experimental section

2.1. Materials

6-Azidohexyl methacrylate (AHMA) [51], 6-oxohexyl methacrylate (OHMA) [58], and rhodamine-NH$_2$ (RHB-NH2) [59] were synthesized as described in literature procedures. Poly(ethylene glycol) methyl ether methacrylate (PEGMEMA, M_n = 300 g/mol^{-1}), rhodamine B, poly(ethylene glycol) dimethacrylate (PEGDMA, M_n = 330 g/mol^{-1}), 1-ethynylpyrene, copper(I) bromide (Cu(I)Br), azobisisobutyronitrile (AIBN), potassium hydroxide (KOH), N", N"-pentamethyldiethylenetriamine (PMDETA), sodium azide (NaN$_3$), methacryloyl chloride (MAC), ethylenediamine, and 1,6-hexanediol were obtained from Sigma-Aldrich. 6-chloro-1-hexanol was purchased from TCI. Tetrahydrofuran (THF), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), dichloromethane (DCM), triethylamine (Et$_3$N), and pyridinium chlorochromate (PCC) were obtained from Merck. Prior to use, PEGMEMA and PEGDMA were filtered through a short plug of basic alumina to remove the inhibitors. 1H-NMR spectroscopy characterization of the monomers and dye was conducted by using a Varian 400 MHz instrument. Infrared analyses were carried out using a Bruker Vertex 70 FTIR spectrometer. Fluorescence microscopy images were captured using a Leica CytoVision microscope. Scanning electron microscopy (SEM) images of hydrogels were recorded with QUANTA FEG-250, field emission scanning electron microscope, with low vacuum detector.

2.2. Hydrogel synthesis

In a glass vial, AHMA (0.024 g, 0.114 mmol), OHMA (0.021 g, 0.114 mmol) monomers and PEGDMA (0.019 g, 0.057 mmol) crosslinker, were dissolved in MeOH (60 μL) and carefully purged with nitrogen for 15 min at room temperature. AIBN radical initiator (0.005 g, 0.0284 mmol) and MeOH (80 μL) were added to the precursor solution under nitrogen and the reaction mixture was heated at 65 °C for 2 h. The obtained hydrogel (H1) was washed with THF, MeOH, and deionized water, and dried under vacuum (yield 71%). The aforementioned method was also used for the synthesis of hydrogels H2 and H3, which also includes hydrophilic PEGMEMA (M_n = 300 g/mol^{-1}). The feed monomer ratios (mmol) of AHMA:PEGMEMA:OHMA are 1:0:1, 1:2:1, and 1:6:1 for H1, H2, and H3, respectively as described in Table 1. The ratio of crosslinker in mmol to total monomer in mmol is 0.25 for all procedures.

2.3. Water absorption (%) studies of the hydrogels

Hydrogels were first freeze-dried and measured to find initial weights. The samples were placed in deionized water and gently taken out periodically. In every period, the extra surface water was removed using wet filter paper; the mass of the swollen hydrogels was measured and submerged into water. The water absorption amount was measured until the equilibrium swelling. The experiments were conducted in triplicate, and water uptake capacities (%) were calculated using Equation 1 (Eq.1).

$$\% \, Water \, Absorption = \frac{100x(Wwet - Wdry)}{Wdry}$$

Eq.1

W_{wet}: Wet weight of hydrogel, W_{dry}: Dry weight of hydrogel

2.4. Modification of hydrogel H1 through "Huisgen click" reaction

To the sample of hydrogel H1 (27 mg) in a glass flask, Cu(I)Br (0.34.mg, 0.002 mmol), PMDETA (0.42 mg, 0.002 mmol), and 1-ethynylpyrene (11.00 mg, 0.049 mmol) were added with THF/DMF mixture (1: 0.5 mL) under nitrogen. The mixture was heated at 40 °C for 20 h. Pyrene-functionalized hydrogel (P-H1) was washed multiple times with THF, DMF, and deionized water to eliminate unreacted materials and dried in a vacuum.

Table 1. Monomer feed ratios and yields of synthesized hydrogels.

	Feed monomer ratio (mmol) AHMA:PEGMEMA:OHMA	AHMA (mmol)	PEGMEMA (mmol)	OHMA (mmol)	PEGDMA (mmol)	AIBN (mmol)	Yield (%)
H1	1:0:1	0.114	-	0.114	0.057	0.028	71
H2	1:2:1	0.054	0.109	0.054	0.054	0.022	85
H3	1:6:1	0.027	0.163	0.027	0.054	0.022	92

Total monomer concentration: 37% WP in MeOH, time: 2 h, Temperature: 65 °C.
%Yield = (W_m/W_h) × 100 (W_m = Weight of the total monomers and crosslinker, W_h = (Weight of the dry hydrogel).

2.5. Modification of hydrogel P-H1 through "Schiff base" reaction

Rhodamine-NH$_2$ (2 mg) was dissolved in DMSO:MeOH solvent mixture (1 mL, 1:1). Pyrene functionalized hydrogel (P-H1, 10 mg) was added to the dye-containing solution and heated at 50 °C for 18 h. Dual-modified hydrogel (PR-H1) was cleaned with DMF, MeOH, and deionized water to eliminate unreacted materials and dried in a vacuum.

3. Results and discussion

Aldehyde and azide functional groups containing methacrylate monomers OHMA and AHMA were synthesized according to the literature procedures [58,51]. Figure 2 shows the synthesis and 1H-NMR spectra of the monomers. The resonances of the vinylic protons appear at 6.07 and 5.54 ppm, and the aldehyde proton appears at 9.77 ppm for OHMA monomer in Figure 2a. For the AHMA monomer, peaks at 6.09 ppm and 5.55 ppm show the vinylic protons and 3.54 ppm shows the -CH$_2$ protons adjacent to azide moiety in Figure 2b. Monomers were also characterized using FTIR spectroscopy, and the azide stretching peak for the AHMA monomer was observed at 2093 cm^{-1}. Although no distinct peak for aldehyde was evident in the OHMA monomer, the 1H-NMR spectrum proved its existence beyond doubt. After the monomer synthesis, crosslinking of azide and aldehyde-bearing methacrylate monomers was achieved in the presence of PEGDMA crosslinker in MeOH through AIBN-initiated free radical polymerization at 65 °C, in 2 h to yield (H1). To evaluate the effect of hydrophilic monomer addition to the hydrogel formulation, PEGMEMA was also added to the precursor solution in different ratios (H2, H3), as depicted in Table 1. The molar concentration of PEGDMA crosslinker was adjusted as 25% of the total molar monomer concentration in all gel formulations. Figure 3 (top) shows the representative crosslinking reaction of monomers and crosslinkers through free radical polymerization.

After preparation, hydrogels were swelled in water to reach equilibrium and freeze-dried to analyse their morphologies. SEM images of networks were recorded, and it was observed that none of the hydrogels showed a porous structure (Figure 3, bottom). This nonporous physical image can be the result of both short chain crosslinker PEGDMA (M$_n$ = 330 g/mol) usage in the gel and the amount of crosslinker might be relatively high. Table 1 shows the monomer, crosslinker and initiator feed ratios of the hydrogels. For all hydrogel combinations, AHMA/OHMA reactive monomer ratios were kept constant to facilitate comparison. When we increased the amount of PEGMEMA, the yield of the hydrogel reactions was increased up to 92% as seen in Table 1 for hydrogel H3.
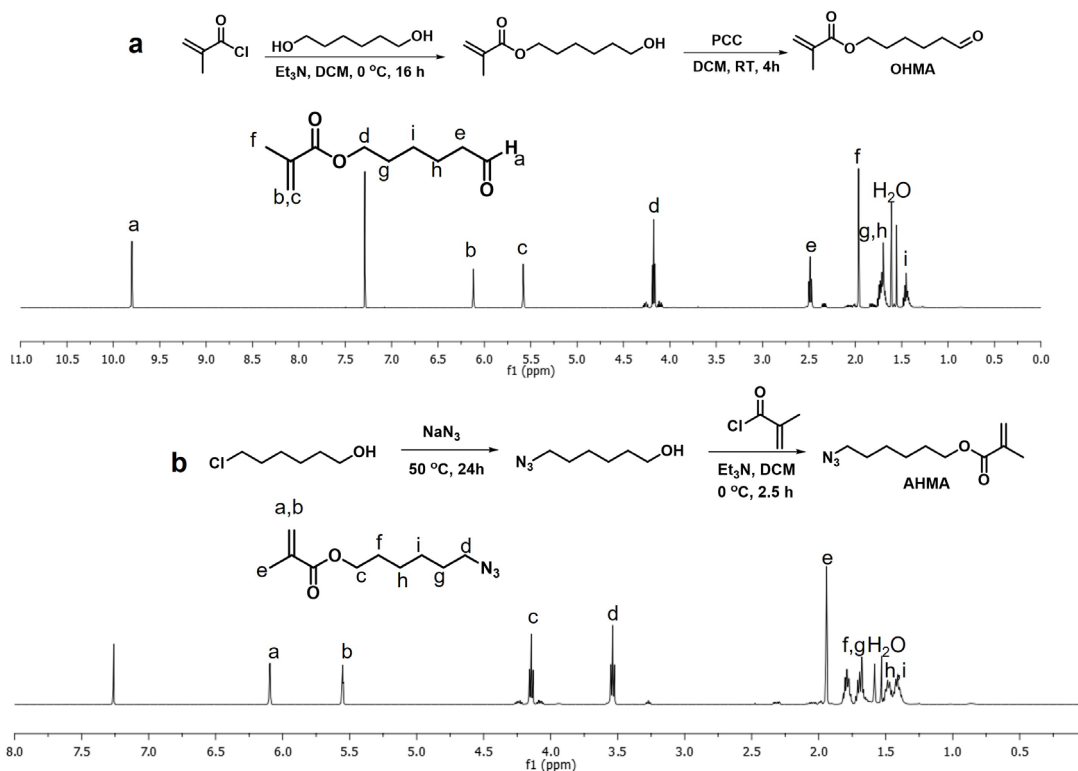

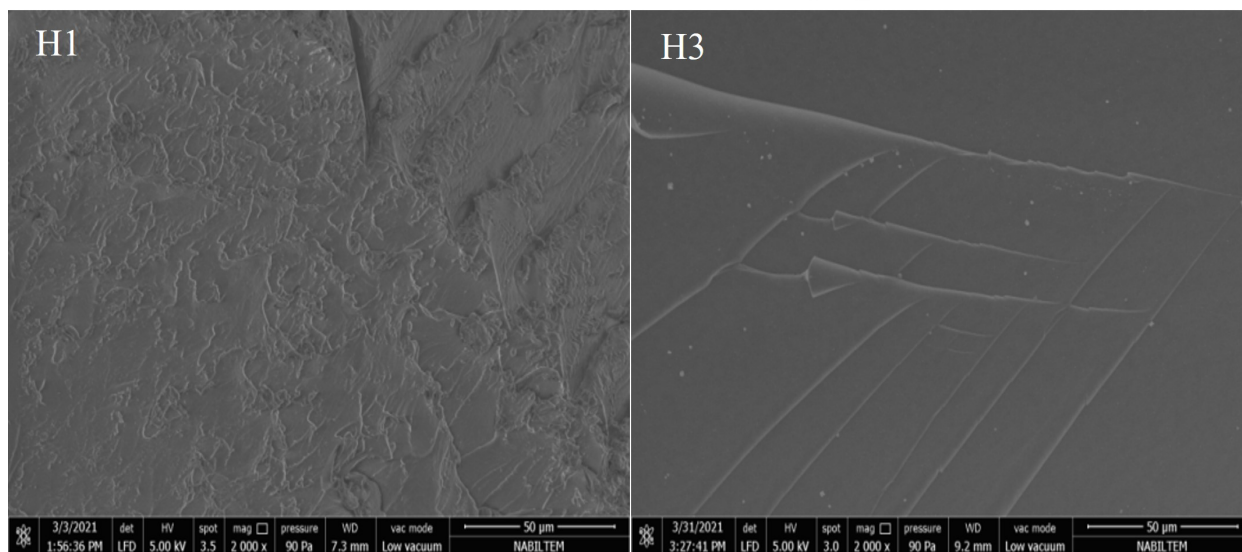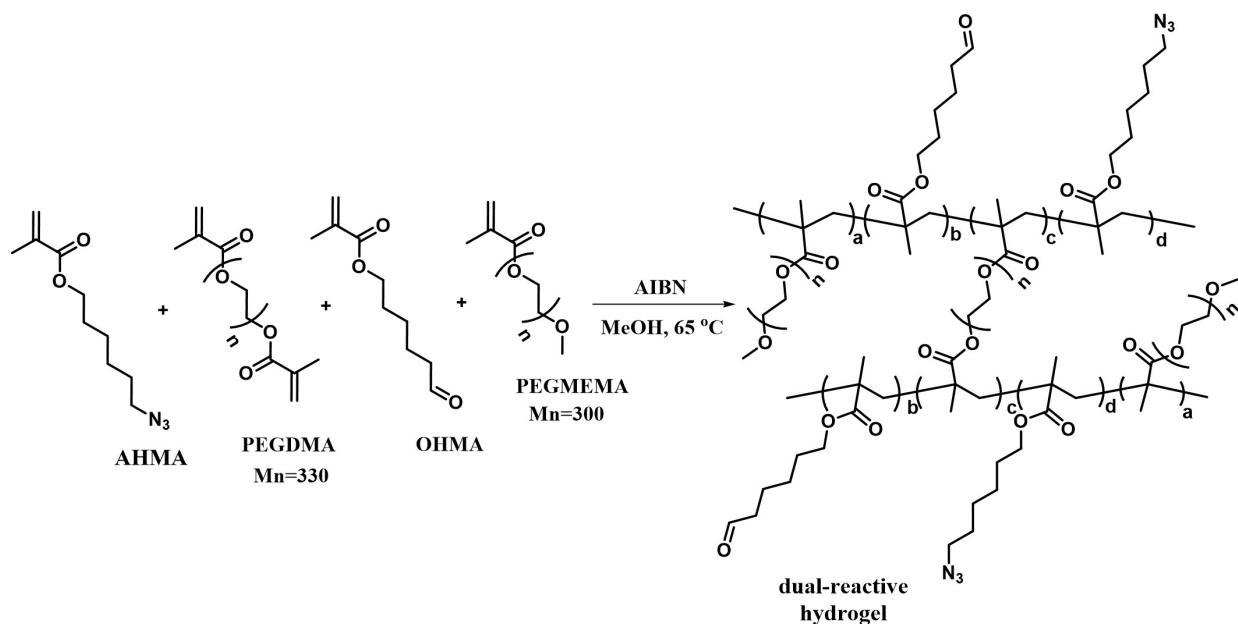**Figure 2.** Synthesis procedures and 1H-NMR spectra of OHMA (a) and AHMA (b).

Figure 3. Synthesis of hydrogel network (top), and SEM images of hydrogels H1 and H3 (bottom).

Water uptake capability is an intrinsic feature of hydrogels, where water diffuses inside the network, resulting in expansion of the hydrogel. The swelling behaviour of hydrogels was studied depending on time since this property can be useful for many (bio)applications such as drug delivery. To obtain the swelling profile of hydrogels, the gravimetric method was used immersing preweighed dry hydrogels in aqueous media and removing them at a certain time from the media, and measuring the weight again until the hydrogels reach equilibrium. Then, %water absorption capacity was calculated using Eq. 1. Figure 4 shows the water uptake profile of the hydrogels. As expected, H1 without hydrophilic monomer exhibited the lowest swelling capacity; 13%, while H2 including more PEG units showed 50% capacity, and H3 which has the highest amount of PEG units exhibited 55% water uptake capacity.

After the synthesis of hydrogels, the feasibility of orthogonal functionalization was demonstrated using two different reactive dye molecules through "Huisgen-type click" reaction and the "Schiff base" reaction. First, azide groups within hydrogel H1 were reacted via the Cu-catalyzed "Huisgen click" reaction in the presence of PMDETA and Cu(I)Br at 40 °C for 20 h with 1-ethynylpyrene which contains a reactive alkyne unit. Figure 5 shows the infrared spectra of the network before and after functionalization through the "Huisgen click" reaction (P-H1). For hydrogel H1, the absorption bands
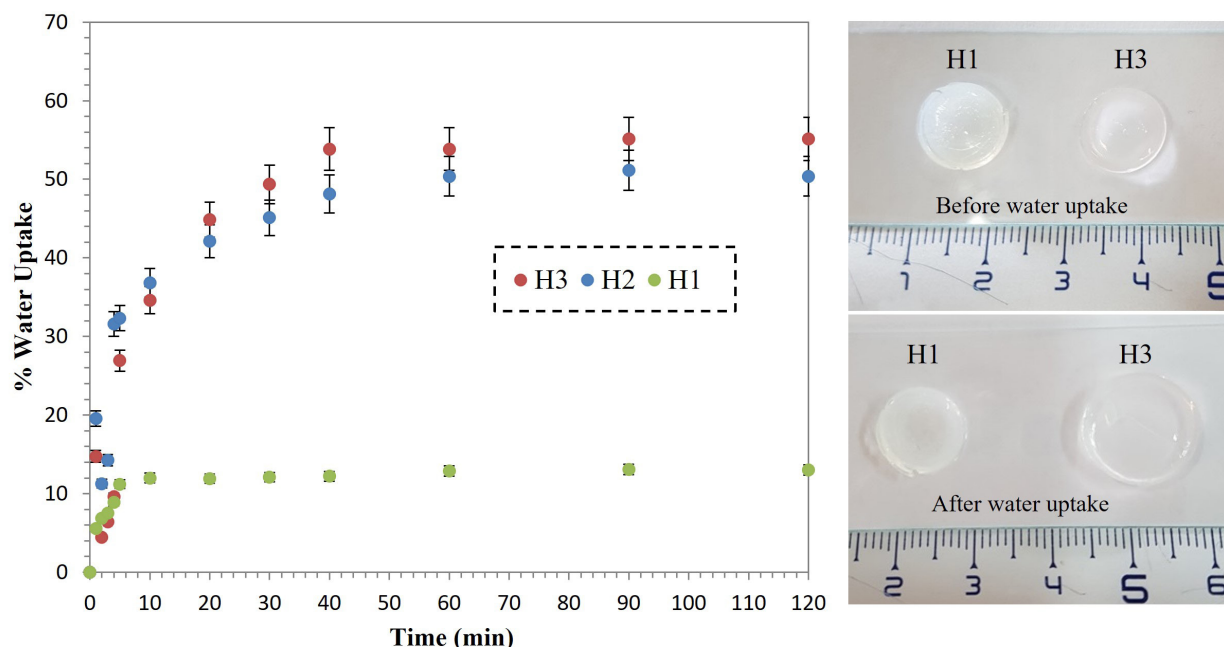
Figure 4. Percentage water uptake versus time graphic of H1, H2, and H3 (left), photographs of H1 and H3 before (right, top), and after swelling in water (right, bottom).

seen at 2095 and 1677 cm^{-1} correspond to the azide and aldehyde groups, respectively. P-H1 spectrum shows the successful attachment of 1-ethynylpyrene through the "Huisgen click" reaction since the azide peaks at 2095 cm^{-1} disappeared completely and new peaks belonging to the aromatic pyrene C=C stretching were observed starting around 1600 cm^{-1} and aromatic C-H bending around 1690 cm^{-1} which overlapped with the aldehyde peak. In addition, the broad peak at 1636 cm^{-1} belonging N=N stretch suggests formation of the triazole moiety [60,61]. Since N=N stretch of triazole ring and aromatic C=C bond stretching due to the pyrene group are expected as a broad peak around the same region, the most crucial evidence for click reaction is the disappearance of azide peak at 2095 cm^{-1}.

Pyrene attachment to hydrogel H1 was also proved with fluorescence microscopy. The blue fluorescence in Figure 6a results from the pyrene functionality after UV-excitation. The inset image 6b shows the fluorescence microscopy image of hydrogel H1 at the same excitation. As expected, hydrogel H1 which does not incorporate any pyrene group did not show fluorescence.

Dual functionalization of hydrogel H1 was shown by further functionalization of P-H1 to obtain PR-H1 through an aldehyde-amine coupling reaction. Hydrogel containing reactive aldehyde groups (P-H1) was reacted with rhodamine-NH$_2$ in DMSO:MeOH solvent mixture at 50 °C for 18 h. Then, the characterization of dual-modified hydrogel (PR-H1) was undertaken by FTIR spectroscopy (Figure 5). PR-H1 spectrum exhibits an imine peak at 1620 cm^{-1} which proves the successful functionalization of aldehyde groups with rhodamine-NH$_2$ through the Schiff base reaction. Also, characteristic absorption bands of rhodamine due to aromatic C=C stretching can be distinguished between 1600–1500 cm^{-1} [62]. A fluorescence microscopy image was also taken after rhodamine conjugated PR-H1. The red fluorescence in Figure 7a suggests the successful attachment of the rhodamine dye.

It is well-known that the rhodamine group can easily give response to acidic pH [63]. To observe the response, hydrogel PR-H1 was placed in acetate buffer (pH:5, 20 mM) for 5 min and then washed with deionized water and dried under a vacuum. Then, the fluorescence image of acid-treated hydrogel PR-H1-a was found to be considerably brighter compared to the nontreated one, as shown in Figure 7b. The increase in the fluorescence intensity before and after exposure to an acidic environment is quite distinguishable (Figure 7, bottom, right). Depending on the increasing conjugation of rhodamine unit in acidic pH (Figure 7, bottom, left), it is quite reasonable to obtain a higher fluorescence. The FTIR spectrum after acid treatment was also taken, and it was observed that specific rhodamine bands between 1600–1500 cm^{-1} and imine peak at 1620 cm^{-1} survived which proves utility of the system for different applications.
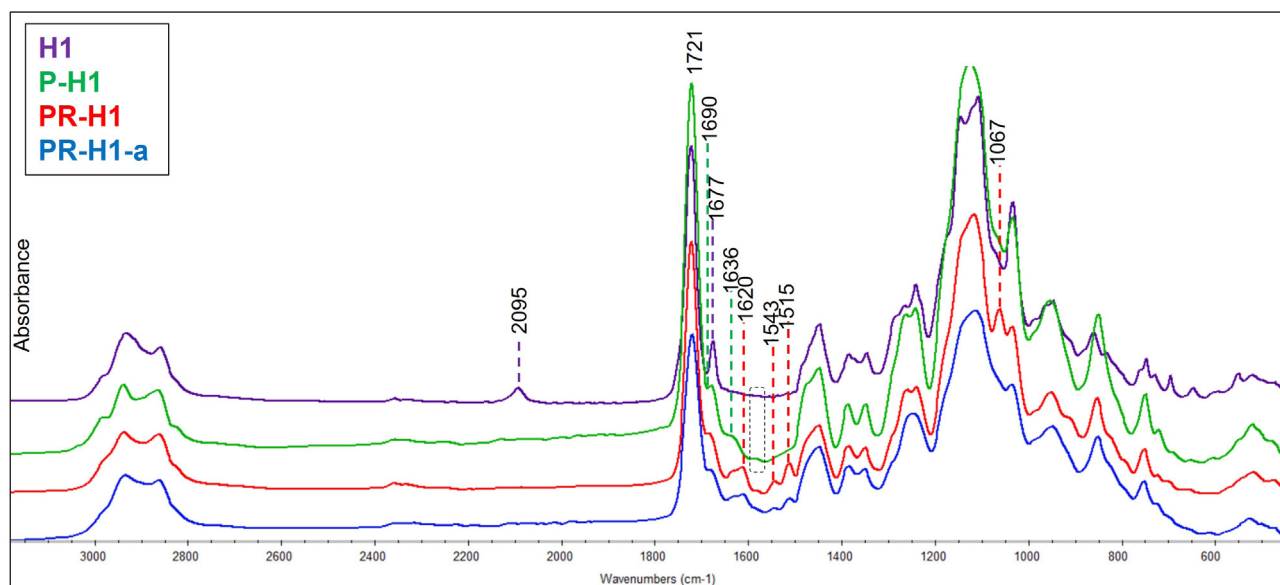
Figure 5. FTIR spectra of hydrogel H1, after functionalization through "Huisgen click" (P-H1), after dual functionalization through the "Schiff Base" reaction (PR-H1), and after treatment with pH 5 acetate buffer (PR-H1-a).
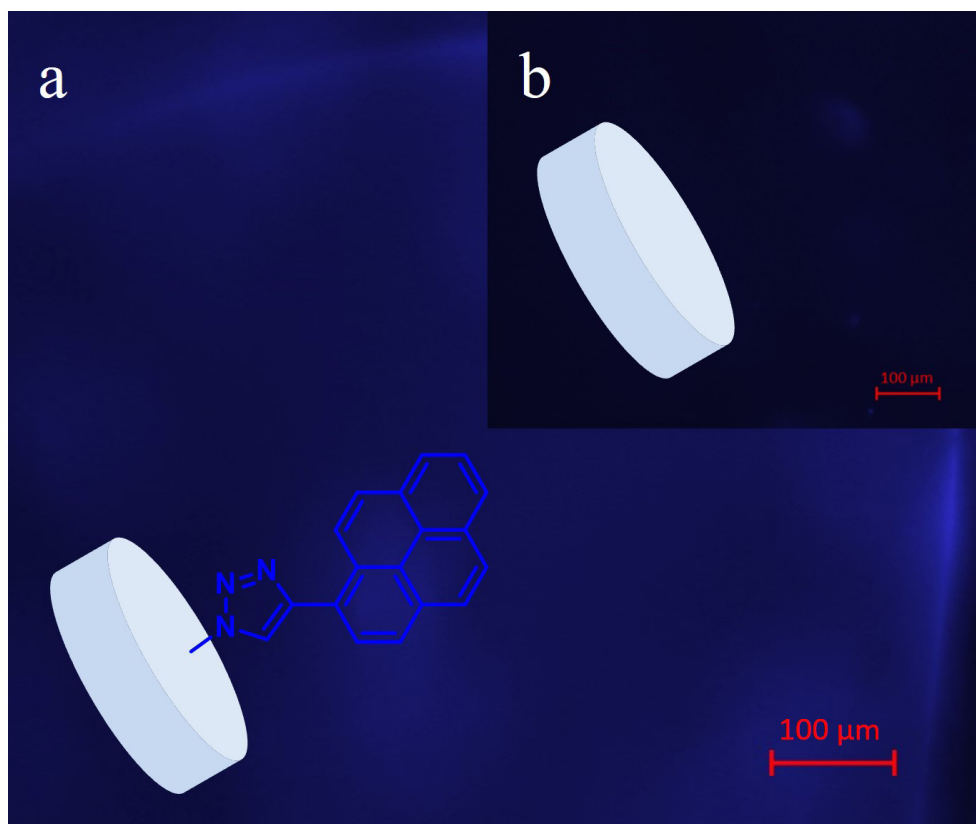

Figure 6. Fluorescence microscopy images of H1 (inset b), and P-H1 upon functionalization with 1-ethynylpyrene (a), Fluorescence images were taken with Hoechst/DAPI (UV); (Excitation BP 350/50, Emission BP 460/50) filter set.
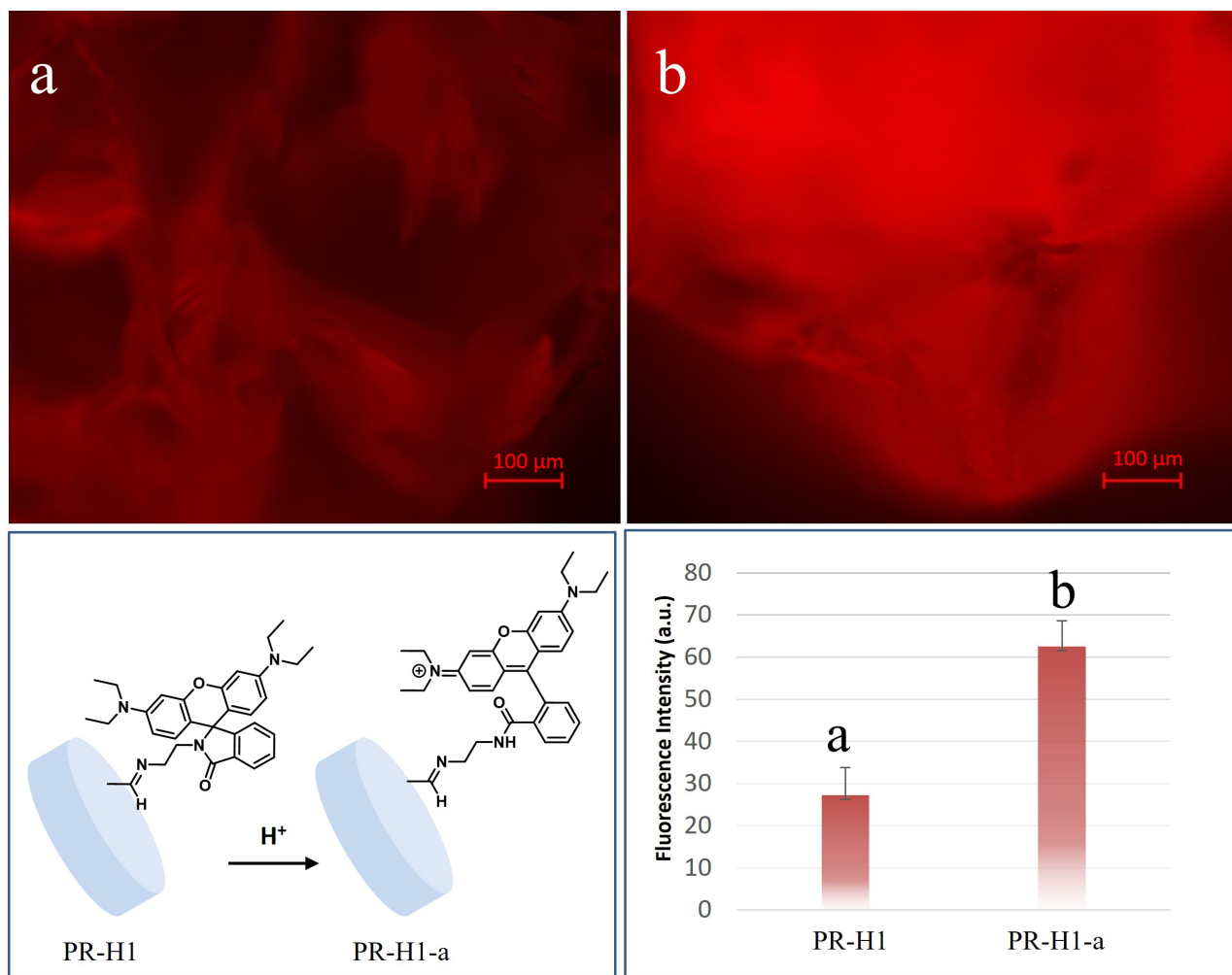
Figure 7. Fluorescence microscopy image of rhodamine functionalized PR-H1 (a, top), and fluorescence image of rhodamine functionalized PR-H1-a after treatment with acid (b, top). Schematic representation of rhodamine group response to acid for PR-H1 (bottom, left). Fluorescence intensity comparison of PR-H1 and PR-H1-a, before and after acid treatment, respectively (bottom, right). Fluorescence images were taken with AF647/CY5(Red); (Excitation BP 620/60, Emission BP 700/75) filter set.

4. Conclusion

In conclusion, a dual-functionalizable hydrogel was synthesized using aldehyde and azide bearing reactive monomers through AIBN-initiated free radical polymerization. The incorporation of hydrophilic monomer into the system enhanced the water uptake capacity of the hydrogel system. Facile orthogonal functionalization of the hydrogel with two different dye molecules via the "Huisgen click" reaction and "Schiff base" reaction was accomplished. One can foresee that such orthogonally functionalizable hydrogels can find applications in the fabrication of various sensing platforms.

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