European Polymer Journal 181 (2022) 111641

Contents lists available at ScienceDirect

European Polymer Journal

journal homepage: www.elsevier.com/locate/europolj

Characterization of self-healing hydrogels for biomedical applications

Jennika Karvinen *, Minna Kellomäki

Biomaterials and Tissue Engineering Group, Faculty of Medicine and Health Technology and BioMediTech Institute, Tampere University, Tampere, Finland

ARTICLE INFO

Keywords:
Hydrogel
Self-healing
Characterization
Reversible interaction

ABSTRACT

Self-healing hydrogels have become attractive biomaterials due to their ability to repair their initial structure and properties in response to damage. When designing ideal self-healing hydrogels the understanding of their properties but also the actual healing process is required. Even though there currently are different characterization methods used, the lack of standardization makes comparison of different hydrogels difficult. The challenges in standardization arise, for example, from the use of different healing methods (i.e. healing environments) or different testing equipments used. In order to help the comparison of hydrogels, a group of characterization methods should be chosen and the measuring parameters and results in the literature should be presented more consistently. The characterization should include methods suitable to determine the presence of reversible interactions and their reversibility study, to investigate the self-healability of hydrogels and to determine the healing efficiencies of hydrogels, not forgetting time dependence and dynamics of self-healing. More quantitative, as well as theoretical studies are recommended. In this review different general characterization methods, including different measuring parameters and environments, used for self-healing hydrogels are charted, but also additional methods suitable for injectable/3D-bioprintable and conductive self-healing hydrogels are discussed. Some challenges of each method and future aspects for self-healing hydrogels and their characterization are also given.

1. Introduction

Hydrogels have already shown great potential in different biomedical applications, for example, in drug delivery, cell therapy and tissue engineering. Regardless of their favorable properties, such as biodegradability and biocompatability, conventional hydrogels, however, may lose their original mechanical properties and the network structure may be affected when damaged, which may further limit their lifetime. [1] This is because they cannot self-heal and reform the broken bonds [2]. This kind of damage caused by an external mechanical force taken place *in vivo* would cause a risk of inflammation, if, for example, a burst release of drug from hydrogel would suddenly happen [3]. Due to these reasons, hydrogels with self-healing ability have been developed by mimicking the self-healing ability of the human body (e.g. wound healing) [4,5]. Self-healing hydrogels have a build-in ability to autonomously repair their initial properties and structure in response to damage. They can therefore have extended lifetimes, and the reliability and safety will be improved as they function in predetermined way. [6,7]

The self-healing in hydrogels can be initiated by an external stimulus, like temperature, light or pH, or the interactions of hydrogels can autonomously reform. In some cases, stimulus-based hydrogels may not be able to self-heal unlimited times due to the consumption of self-healing agent during the healing process. [8] Autonomously self-healed hydrogels can heal multiple times because the interactions can reform spontaneously [4]. Reversible bonds can be either chemical covalent (e.g. acylhydrazone bonds, imine bonds, Diels–Alder reactions, disulfide bonds, and boronate ester bonds) or physical non-covalent (e.g. hydrogen bonds, hydrophopic interactions, ionic interactions, host–guest interactions, metal–ligand coordination complexes and peptide self-assembling) interactions [4]. The network of chemical self-healing hydrogels is reformed through dynamic covalent bonds (higher bond energy compared with physical hydrogels), whereas, the network of physically self-healing hydrogels is dynamically reformed through non-covalent interactions between polymer chains, oligomers or molecules. In both systems, the functional groups must exist in such form that the reformation of bonds in the damage site is possible. [1] The degree of self-healing ability, as well as the mechanical properties and stability of hydrogel, are determined by the number of bonds and how strong the moieties used in the bonds are [9].

There are two steps in the mechanism of healing process in

* Corresponding author.
E-mail address: jennika.karvinen@tuni.fi (J. Karvinen).

https://doi.org/10.1016/j.eurpolymj.2022.111641
Received 19 August 2022; Received in revised form 4 October 2022; Accepted 13 October 2022
Available online 26 October 2022

hydrogels, which are independent of the interaction type between the polymer chains. In the first step, long polymer chains are interdiffused through the damaged surface. The rate of this interdiffusion is determined by the molecules' free chain length and the temperature. The longer the free chain length, the stronger the interaction and deeper the interpenetration. Further, at higher temperature, the diffusion is facilitated. Due to the polymer chains' large mobility facilitated by the high water content of hydrogels, interdiffusion is a rapid process. This "mobile phase" formed around the cracks in damage site is needed for the initiation of the healing process. In the second mechanism step, the bonds between the polymer chains reform. [4,1]

Ideal self-healing hydrogel would autonomously, rapidly, efficiently and repeatably respond to damage in micro- and macroscales, but would also retain the original mechanical and rheological properties, as well as the morphology after the healing process [7,9]. In general, the design of self-healing hydrogels should always be done according to the application and in addition to the properties listed above, they should also meet other requirements, such as biocompatibility or tissue-mimicking mechanical properties if required by the application [7]. In order to be able to design these ideal self-healing hydrogels, their self-healing properties along with the general ones need to be known. When planning the characterizations, there are at least three aspects that should be considered. First is the timescale of healing. The healing can take seconds or even days and depends on the interdiffusion's dynamics described earlier, as well as on the time that is needed for the sufficient recovery of bonds. Second is the time dependence of self-healing. It has been shown that after a long separation, most hydrogels cannot heal. Third is the efficiency of healing. Ideally the mechanical properties would be completely restored, but unfortunately this is not the case in reality. [4]

There are currently multiple characterization methods that are based either on qualitative or quantitative evaluation, and examine the self-healing ability either in macro- or microscale (morphology and topology). Starting from the analysis of reversible interactions using, for example, spectroscopic and X-ray-based methods, and continuing with the testing of reversibility of interactions with rheology-based method. The macroscopic self-healability of hydrogels has been studied using gel block fusion test, while smaller scale healing can be followed using microscopic methods. The healing efficiency calculated based on the mechanical properties of hydrogels together with time related studies also gives information, for example, for the time dependence of the healing process. [6,4,1,7]

Even though we already have some more or less generally used methods, there are some limitations and points that should be considered. For example, the requirements for the sample preparation or the data quality, as well as the high water and low solid contents, bond strength and dynamic properties of hydrogels can add challenges to the characterization [10]. Further, the different measuring parameters used in same method can make the comparison of results impossible [4]. Also, since many method is still based on qualitative evaluation, more quantitative methods would be needed as well as those performed *in situ* and non-destructively [1]. The kinetic study should not either be forgotten [11]. The measuring conditions can also affect to healability of the material [9]. Overall, the comparison of different studies is difficult at the moment, since there does not exist fully standardized methods for the characterization of self-healing hydrogels [4].

In this review, the characterization methods of self-healing hydrogels are divided in four main sections and two additional sections for specific cases that are also presented in Fig. 1. Chapters 2, 3, 4 and 5 present different methods suitable for the determination of the presence of

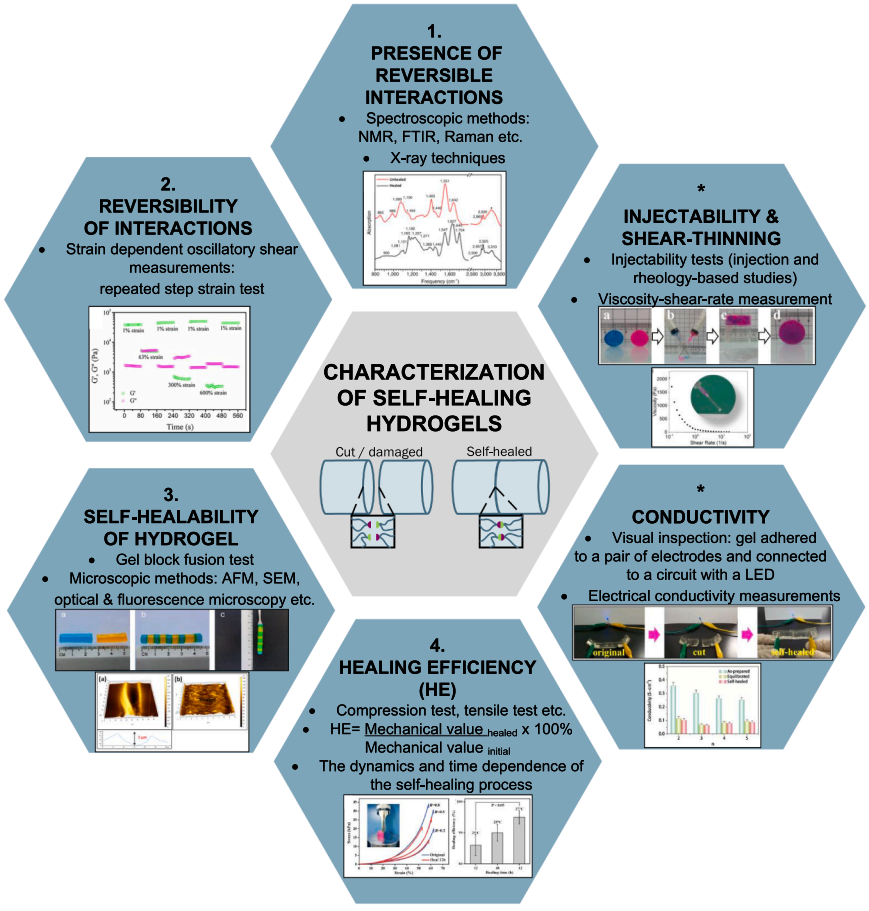

Fig. 1. Characterization steps (1–4) of self-healing hydrogels. Additional steps (*) are needed for applications requiring injectability of the self-healing hydrogel (e.g. 3D-bioprinting), or for conductive self-healing hydrogels. Reprinted with permission from Phadke et al. (2012) [12] Copyright ©2012 PNAS, Xiao et al. (2019) [13] Copyright ©2019 Elsevier, Li et al. (2015) [14] Copyright ©2015 ACS Publications, Khamrai et al. (2019) [15] Copyright ©2019 Elsevier, Wei et al. (2015) [16] Copyright ©2015 Wiley, Wei et al. (2016) [17] Copyright ©2016 Scientific Reports, Zhang et al. (2017) [18] Copyright ©2017 Royal Society of Chemistry, Yuan et al. (2018) [19] Copyright ©2018 Elsevier, Shao et al. (2018) [20] Copyright ©2018 ACS Publications.

reversible interactions, to study the reversibility of these interactions, to investigate the self-healability of hydrogels and to determine the healing efficiencies of hydrogels, respectively. Last chapter also discuss about the characterization of time dependence and dynamics of self-healing. The example cases from the literature presented in the previous chapters are also collected in Supplementary tables (Table S1-S5) with more detailed information. In conclusion, we chart the different characterization methods, including different measuring parameters and environments, used for self-healing hydrogels. We focus on the general methods suitable for all self-healing hydrogels, but we also shortly discuss about the additional methods suitable for injectable/3D-bioprintable and conductive self-healing hydrogels (chapters 6 and 7, respectively). In addition, we show some challenges of each method and give some future aspects for self-healing hydrogels and their characterization. The purpose of this article is to search a group of suitable characterization methods (to be standardized) in order to be able to compare the self-healability of different hydrogels more easily.

2. Determining the presence of reversible interactions in hydrogels

The presence of reversible interactions in self-healing hydrogels can be monitored using different analysis methods, such as spectroscopic and X-ray-based techniques, as well as some less frequently used methods, like thermal analysis methods, which are not presented in this article. These methods basically show that the experimental production of hydrogel has lead to a correct outcome and intended reversible interactions have been created. However, especially in the case of more complex structures, one method alone may not give enough information about the structure, and a combination of different techniques are needed [21].

2.1. Spectroscopic methods: FTIR, NMR and Raman

The reversible chemical bonds can be analyzed using spectroscopic methods, such as Fourier transformation infrared spectroscopy (FTIR) [21], Nuclear magnetic resonance spectroscopy (NMR) [22] and Raman spectroscopy [23]. The electrons and nuclei response to irradiation, so the bonding situations and chemical structures can be obtained. Morphological information is also possible to get, because spectroscopic methods are non-destructive and therefore the measured sample can be recovered after the online analysis. Spectroscopic methods can display how chemical bonds response to environmental changes. In case of dynamic covalent bonds, spectroscopic measurements can show the dynamic nature of the bond by showing the transition between the bonded states (the bonding-debonding process). The signal-concentration correlation is also possible to generate to get information about the extent of the reaction. Redeeming feature of these methods is that they are quantitative. [11]

FTIR is a nondestructive, fast and sensitive method, with a simple sample preparation [10,24]. FTIR in transmission mode can be used for solid or thin enough samples, whereas the attenuated transmission reflectance (ATR-FTIR) mode is better for powder, liquid and coated film samples [10]. The specific chemical functionalities involved in chemical bonds can be identified and followed using FTIR spectroscopy [10]. FTIR can be used to study the presence of reversible interactions (specific

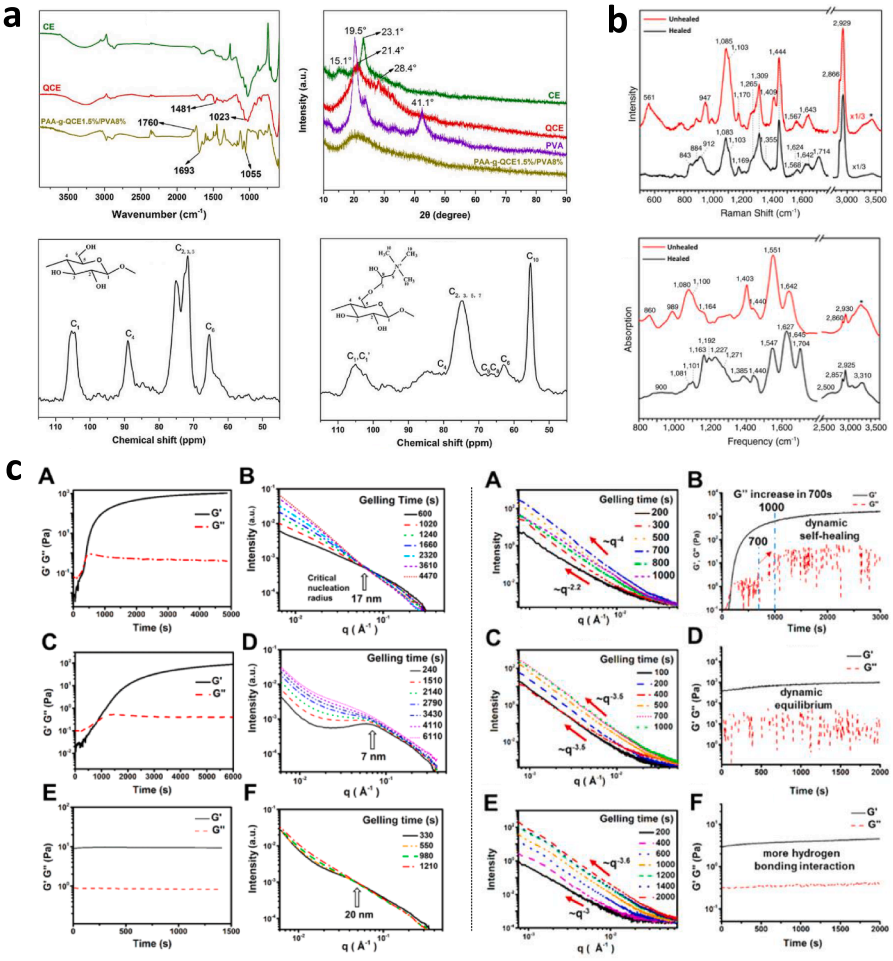

Fig. 2. Spectroscopic and X-ray-based methods used to study self-healing hydrogels and their precursors. (a) FTIR, XRD and ^{13}C NMR analyses of cellulose (CE), quaternized cellulose (QCE), and polyacrylic acid-grafted quaternized cellulose (PAA-g-QCE) 1.5%/PVA8% samples [25], (b) FTIR and Raman analyses of A6ACA hydrogels [12], and (c) *in situ* SAXS and coherent X-ray scattering (CXS) combined with a rheometer analyses of N-carboxyethyl chitosan-based (CEC) hydrogels [32]. Reprinted with permission from Wang et al. (2017) [25] Copyright ©2017 Elsevier, Phadke et al. (2012) [12] Copyright ©2012 PNAS, and Lin et al. (2019) [32] Copyright ©2019 ACS Publications.

chemical groups) and formation of the bonds (Fig. 2 (a)) [25–28,5,29,30], but also the actual bonding-debonding process. For example, to quantitatively study the kinetics of reversible covalent bonds, the changing vibrational bands can be compared to those staying constant during the process [11]. In case of supramolecular self-healing hydrogels, FTIR can be used to study the formation of hydrogel since it can show how the molecular scale building blocks assemble and it allows to detect the non-covalent interactions responsible for gelation [31]. Fig. 2 (b) shows an example of FTIR-ATR (and Raman) study, where Phadke et al. [12] studied the role of hydrogen bonding in self-healing of acryloyl-6-aminocaproic acid (A6ACA) hydrogel by measuring the spectra for both unhealed (high pH) and healed (low pH) samples. The results showed an IR band typical for hydrogen-bonded terminal carboxylic-acid group (1704 cm^{-1}). The analyses given by both spectroscopic measurements also indicated two types of hydrogen bonding across the interface: carboxyl groups directly interact with the amide groups of the opposing pendant side chain in an interleaved configuration (1627 cm^{-1}), and a smaller fraction of carboxyl groups interact with the opposing carboxyl groups in a face-on configuration. Basically, this analysis showed the reversibility of the healing, meaning that by changing the pH the healing can be switched off and on. [12]

In NMR, the peak shift and intensity in the resulting spectra gives information about the polymer composition, but also about interactions, molecular organization, morphology sequence distributions and molecular weight. The internal mobility of the chemical component or group can be indicated by the width of the peaks, for example, gel like samples have broader peaks. [10] NMR requires only a small amount of sample, the sample preparation is easy and they can be measured as prepared [10]. The NMR samples are usually measured in liquid or solution state, but investigation of solid samples like hydrogels is also possible using solid-state NMR [11,33]. In the case of self-healing hydrogels with reversible covalent bonds, NMR can show the reversibility of the network by showing the chemical structures of debonded and bonded states, but also can give the concentration ratios between these states [11]. In terms of stability of these networks, the structural properties of the components and areas that participate in the interactions affecting to it, can also be provided by the NMR spectra [31]. NMR is also suitable for the characterization of the supramolecular hydrogels formed through non-covalent bonds, for example, because the observed nuclei's relaxation times are relatively long [31]. If the reaction kinetics is slow or a trigger is needed for the bonding-debonding process, *ex-situ* NMR analysis can be used. Even though the characterization is done in temporal isolation from the debonding event, quencing or trapping the reaction in debonded state can give false resonance ratios due to the bonding-debonding during the cooling. Also, the remained solvent has to be removed before redissolving in suitable deuterated NMR solvent. For more rapid reaction, *in situ* NMR analysis with no trapping needed is the only possibility. There the possible trigger is applied to the sample in the device. [11] Specific tests, for example, temperature- and concentration-depended studies can also be done with NMR [31]. Additionally, self-assembly processes can be studied using Diffusion ordered NMR spectroscopy (DOSY) that gives not only the aggregates' hydrodynamic dimensions (size and shape), but also some thermodynamic parameters of the process. Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR), on the other hand, gives more information about the molecule compared with traditional 1D NMR. For example, 2D NOESY can be used to study host–guest complexes, i.e. the relative positions of their building components. [31]

Raman is a fast method, with simple sample preparation and the samples can be measured as prepared [10,24]. Raman can used to study the molecular interactions, giving also information about the structure of water in hydrogels or the strength of the molecular bonds [34,10]. Fig. 2 (b) shows an example how Raman can be used together with FTIR-ATR to study the role of hydrogen bonding in self-healing of A6ACA hydrogel. In addition to previously shown results, Raman bands at 1714 and 1624 cm^{-1} (weak) related to hydrogen-bonded terminal carboxylic-acid and strongly hydrogen-bonded amide groups, respectively, supported the findings given by FTIR and suggested the pH-mediated self-healing mechanism. [12]

2.2. Other spectroscopic methods

Ultraviolet–visible spectroscopy (UV–vis) and fluorescence spectroscopy are types of electromagnetic spectroscopy, because both method work in the region of electromagnetic spectrum where the molecules undergo electronic transitions, in absorption from ground state to exited state, and in fluorescence vice versa. [31] UV–vis is easier than NMR, a rapid method with broad temperature range and sensitive to electronic transitions that are common for different functional groups, only depending on suitable solvent. However, in case of self-healing hydrogels, the functional group's absorption characteristics that associates with the bonding-debonding event have to be suitable without the interference of other molecular electronic transitions, for example, no aromatic $\pi - \pi^*$ or σ-bond transitions occur at transitions above 320 nm. Further, UV–vis can be used, for example, to study the *in situ* kinetics of different stages of the self-healing reactions. [11] Supramolecular self-healing hydrogels can also be characterized with UV–vis, i.e. hydrophobicity changes of the surroundings of a group can be seen and the non-covalent interactions identified [31].

2.3. X-ray techniques

X-ray scattering methods are indirect and non-destructive methods that provide information about the hydrogel sample's chemical composition, crystal structure and physical properties from as-prepared samples [10,31]. X-ray and neutron scattering methods (e.g. Small-angle X-ray scattering (SAXS), Wide-angle X-ray scattering (or powder diffraction), Small-angle neutron scattering (SANS)) give the data in the form of 2D scattering curves. Atomic scale wavelength of x-rays give high resolution information from nanometer to atomic scale range. [10] Further, scattering methods together with microscopy can give more complete information of hydrogel structure and morphology [10]. The sample preparation is easy and they can be measured as prepared (*in situ* and *ex situ*) [10,24]. Another X-ray-based method, X-ray diffraction (XRD), is also a nondestructive method that is used to characterize crystalline materials giving information about the structure and structural parameters, like crystallinity, at the atomic or molecular level [35]. In case of self-healing hydrogels, for example, the weak intermolecular forces, responsible for crystal stability of self-healing supramolecular materials, can be understood better by studying the molecular crystal's structural motifs using X-ray techniques [31]. Additionally, Fig. 2 presents two examples of how SAXS and XRD have been used to study self-healing hydrogels. Lin et al. [32] (Fig. 2 (c)) have used *in situ* SAXS and coherent X-ray scattering (CXS) combined with a rheometer to study the structural dynamics and gelation mechanism of self-healing N-carboxyethyl chitosan-based (CEC) hydrogels. They managed to show the nucleation and growth mechanism for the gelling process for self-healing hydrogel system for the first time. They also showed that self-healing ability and gelation rate are influenced by the critical nucleation radius (CNR) with different interactions. Also, based on the continuous time-resolved CXS profile and rheology, the dynamic behavior of hydrogels in mesoscale could be seen. [32] XRD (Fig. 2 (a)) on the other hand, has been used to show how the macromolecule network structure is transforming from crystalline state to a amorphous state when comparing diffraction peaks of gel components to diffraction peaks of formed hydrogel [25,29,2].

3. Studying the reversibility of interactions in hydrogels

Strain dependent oscillatory shear measurements are used to study whether the interactions verified with previous methods are truly

reversible. This means that, we study if, after breaking at large strains, the self-healing hydrogel can reform at small strains relatively fast. [4] Since hydrogels are soft viscoelastic materials, their static and dynamic viscoelastic response can be characterized using oscillatory shear tests i. e. rheology [10]. Specific theoretical background for rheology is given by, for example, Chen et al. [36] and Mezger et al. [37].

Basic amplitude sweep (variable amplitudes, constant frequency) and frequency sweep (constant amplitude, variable frequencies) measurements can be conducted for the hydrogel in order to determine so called linear viscoelastic region (LVR) and hydrogels's breaking behavior, or to study behavior of moduli in the LVR, respectively. Time related measurements can also be conducted and therefore rheology permits, for example, the study of gelation behavior and kinetics. In addition, degree of crosslinking, structural property (homogeneity/heterogeneity) and shear-thinning can also be studied. [10,38]

Rheology can be used to study the reversibility of the interactions of self-healing hydrogels, using so called alternate step strain (or stress) measurements [9], also called strain-relaxation experiments [9], repeated step strain measurements [4], continuous step-strain measurements [7], or dynamic strain amplitude cyclic test [39]. Also, other kind of rheological measurements have been used to study the reversibility of the interactions, for example, modified reversible amplitude sweep measurement [40] or modified time sweep measurement with altered strain value [41].

Alternate step strain measurements can show how material responds to a damage in terms of viscoelasticity. In order to monitor the reversibility of the process, the sample is, in a stepwise manner, exposed to a constant deformation at a constant angular frequency. In the linear viscoelastic range, a small strain is applied (gel state, G' > G"). Then, at high strain, the G' will be decreased indicating the structure breakdown, i.e. the interactions will be cleaved (sol state, G' < G"). [9,4,7] Shear-thinning and self-healing materials are able to recover the interactions (and G') if the strain is returned back to the original value [4]. The time-periods of the steps can be kept constant and same for both high and low strain steps. Typically the time-periods are from 60 s [42] to 300 s [39], or something in between. Alternatively, the time-periods can be different in high and low strain step. For example, Qian et al. [28] used longer time-period (250 s) for low strain (0.1%) and shorter time-period (50 s) for higher strain (1000 %), but it can also be other way. The step-times can also be altered during the test while keeping the strain value constant, for example, in the study of Xiao et al. [13] the moduli remained constant despite prolonging the step-time from 80 s to 240 s (at 300%), and returned to original level without any loss [13]. Different initial and increased strain values have been used between different studies, usually based on the breaking strain given by the amplitude sweep measurement done prior testing [13]. Most often the initial strain values are from 0.1% [43] to 10% [39], and increased strain values from 100% [43] to 1000% [28], or something in between. Other possibility is to alter the high strain or stress values after each step, usually by increasing the value. For example, Sun et al. [5] used stress values of 10 Pa (initial), 700 Pa, 1060 Pa and 2000 Pa, respectively, by keeping them for 2 min. After each cycle the G' returned back to the original level indicating rapid recovery. The used stress values were chosen based on the stress amplitude sweep measurement done earlier so that 1060 Pa was the sample's breaking stress. [5] In addition, the number of loading/unloading cycles varies between different studies. For example, typically the number of cycles is between 3 and 5 [28,5,2], but it can even be over 20 [39]. The limitation for the number of cycles is the possible drying of the sample during the test if the cycle steps are long. One example of the alternate step strain measurement is shown in Fig. 3 (a). In case of a typical self-healing hydrogel, at low strain the G' is higher than G", whereas near the breaking strain the G' and G" are approximately the same, and at larger strains the G' goes under the G", but they return back to the original levels again when low strain is applied. More examples and detailed information about the measurements (e.g. parameters) can be found from Supplementary information (Table S2).

In different studies, different measuring parameters and equipments are used. The geometry used are either plate-plate [43] or cone-plate [40] geometries. The size of the plates can be, for example, 50 mm [27], 40 mm [43], 20 mm [42], or even small as 12 mm [40] or 15 mm [17] even though the smaller plate sizes are known to be less suitable for hydrogel type samples. Also, the gap size varies from tens of micrometers to around 1 mm [39,28], giving more reliable results with gap sizes larger than 0.5 mm. The different measuring temperatures (room temperature [43] or 37°C [28]) used also affect to the results.

Alternate step strain test gives quantitative information about the

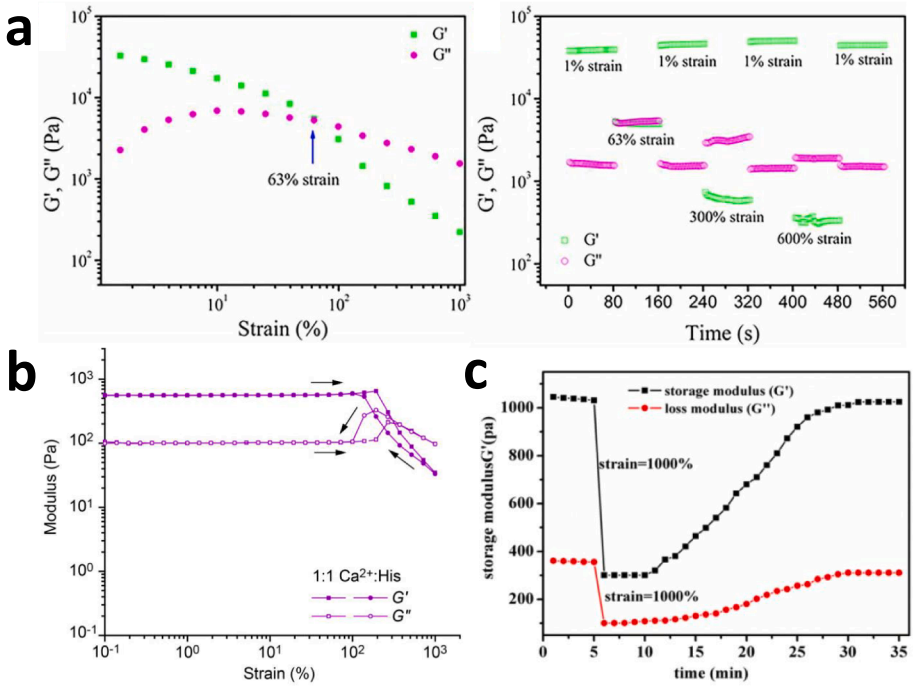

Fig. 3. Studying the reversibility of interactions using (a) amplitude sweep and alternative step strain test for dialdehyde cellulose nanocrystals/acylhydrazine-terminated polyethylene glycol hydrogels [13], (b) modified reversible amplitude sweep measurement for poly(ethylene glycol)-based hydrogels crosslinked with His-metal coordination sites-containing heterodimeric coiled coil (CC) peptides [40], and (c) modified time sweep measurement for chitosan/modified amino acid (acryloylphenylalanine)/ammonium persulfate hydrogels [41]. Reprinted with permission from Xiao et al. (2019) [13] Copyright ©2019 Elsevier, Tunn et al. (2019) [40] Copyright ©2019 MDPI, and Sharma et al. (2018) [41] Copyright ©2018 Elsevier.

self-healing properties of hydrogel, i.e the time scale of healing, recovered G' compared with original G' and how many cycles the hydrogel can bear [7,9]. If the moduli do not recover, material's interactions are not reversible. This is true (at least) in the time scale of the test, i.e. hydrogels with reversible gel-sol response at high strain may have reversible bonds that are needed for self-healing, but the test might not prove that there happens interpenetration of the polymer chains through the interface. [4]

The reversibility of the interactions can be studied also by conducting a modified reversible amplitude sweep measurement. Tunn et al. [40] measured first amplitude sweep from 0.1 to 1000 % showing typical linear viscoelastic region and failure of the sample's crosslinks around 100 %. When gradually lowering the strain back to 0.1 %, the original viscoelastic properties recovered. The cycles could be repeated few times to show the same self-healing behavior. [40] Another alternative test could be a modified time sweep measurement shown by Sharma et al. [41], where first the hydrogel deformation point was determined based on the amplitude sweep measurement and then this strain value (1100%) was momentarily applied to hydrogel showing first a decrease in moduli values, but within 30 min the moduli returned back to their original levels. [41] Examples of these both methods are shown in Fig. 3 (b) and (c), respectively. Additionally, a simple frequency sweep measurement can be used to measure the samples prior and after the healing process in order to verify the self-healing property as done, for example, by Qian et al. [28].

As Taylor et al. [9] have nicely shown, the variation between the mechanical properties provided and the use of different measurement parameters by different studies can make the comparison of materials difficult. They, however, found a correlation between the self-healing mechanism and storage modulus (G'): covalent bonding > ionic bonding > hydrogen bonding ≈ hydrophobic bonding > supramolecular interactions. [9]

4. Investigation of self-healability of hydrogels

Easiest way to determine if the hydrogel is self-healable, is to cut the hydrogel in half, rejoin it and observe the possible healing process by eye or by using microscopic methods. This kind of method, also called a gel block fusion test, verifies the healing in a macroscopic level. How perfectly the fusion takes place can indicate the amount of interactions between the cut pieces, but also about the mobility of the crosslinking network. However, gel block fusion test is only a qualitative method that does not really give details, such as the restoration of the crosslinks, extent of healing, or the horizontal and longitudinal width or depth of the healing interfaces. [7]

4.1. Gel block fusion test

In a simple gel block fusion test the examined hydrogel sample is cut in half using a razor blade or similar, followed by rejoining the pieces back together, and observing the healing process visually. [11,9] Most often the number of pieces is two [2], but rejoining more pieces is also possible, for example, multiple small pieces can be joined together in a mold [44,42], but also rejoining of lower number of pieces, such as 11 [14], 8 [16], or 4 [45,27] have been tested. The pieces can be different shapes and joined in different ways, for example, by putting cylindrical pieces together [27,14] or making a ring from cylindrical pieces [14]. Third option is to make hydrogel discs (same or different colors) that are grounded into particles and mixed to self-heal and form integral hydrogel [46,47]. Fourth option is to test the self-healing by making a small hole in the center of the sample and follow its possible disappearance over time that indicates self-healing [48,18]. In order to help the visualization, usually the pieces are dyed in different colors, such as rhodamine B [48,5,47], methylene blue [39,47,46], congo red [30], methyl orange [48,46], trypan blue [49], or by using food colorants [2]. This way also the possible diffusion through the rejoined interfaces can be seen [5,48,47].

The environment during the healing process is also important. In most cases, there is no intervention used during the testing and the tests have been done at room temperature [39,48]. However, there are also tests done under humidity (in a desiccator or similar) at certain temperature (also different from room temperature) depending on the healing method [43,47,50]. For example, Maity et al. [43] have used moisture saturated air (at room temperature) because it is helping to initiate the healing process, but humidity environment also helps to minimize the water evaporation [20].

The time of the healing process is also important to know. Depending on the healing process, the rejoining time of the pieces before visualization can be from seconds to hours or even days [14,39,42,44,19]. On the other hand, it should be also noted that in most cases only the freshly cut surfaces can heal and the healing ability deteriorates as the separation time of the pieces increases. The time-dependence of the healing process is caused by the hydrophobic rearrangement of the hydrogel surfaces. After cutting, in order to minimise the surface energy, the hydrophobic groups move to the surface forming a hydrophobic barrier even though the hydrophilic groups have been initially exposed to the air. When the surfaces are brought into contact, this barrier prevents the interdiffusion of the polymer chains. The ratio of hydrophobic and hydrophilic groups as well as polymers' segmental mobility determine the extent and rate of this surface reorganization. Self-healing can also be affected by the rearrangement of the reversible interactions. Therefore, the self-healing ability will be reduced after a longer separation time since there are only few active groups left to reform the linkages. [4]

The observation of the healing process can be done by eye and taking digital photos (like in most of the studies) and/or by using microscopic methods that are presented more closely in the next chapter. This way the results are, however, only qualitative. The self-healed sample can also be subjected to stretching [47,2,39,51] or bending tests [14] using tweezers or by hands [45] in order to show that the cracks stay closed and to evaluate roughly their mechanical stability [11,7]. For stronger hydrogels, additional winding while stretching [29,42], or knotting [42] can be made. The ability of hydrogel to support its own weight (gravity test) is also a common test to be done after the healing process [27,39]. Some previously presented examples of gel block fusion test are shown in Fig. 4. Table S3 in the Supplementary information gives more detailed information about the examples given here and presents few others as well.

4.2. Microscopic methods

Microscopic methods help to visualize the healing process by showing how the cracks are closing. Methods such as electron microscopy and atomic force microscopy (AFM) show this in nanometer scale, whereas optical microscopy and surface profilometry work at milli- and micrometer scales. [11] All these methods provide real time images of the structures [10].

4.2.1. Electron microscopy

Electron microscopy methods, scanning electron microscopy (SEM, resolution up to 2 nm, field of view about 1 mm, measures few micrometer depths) and transmission electron microscopy (TEM, resolution up to 0.2 nm, field of view 100 nm, very thin samples (100 nm thick)), can be used to study the morphology of hydrogels at atomic, nano and micro scale. The structure can be magnified up to 500000X. The sample images are taken in a low-pressure chamber, which is why the samples need to be dried before the experiment, therefore they cannot be measured directly. The samples also need to be (made) conducting, if not, the images will be blur with bad image quality. [10] SEM has many advantages, for example, it is widely available and relatively cheap, it produces very high resolution images and has a large depth of field, either the whole image can have a low magnification or the detailed structures of the samples can have a high magnification, and because of

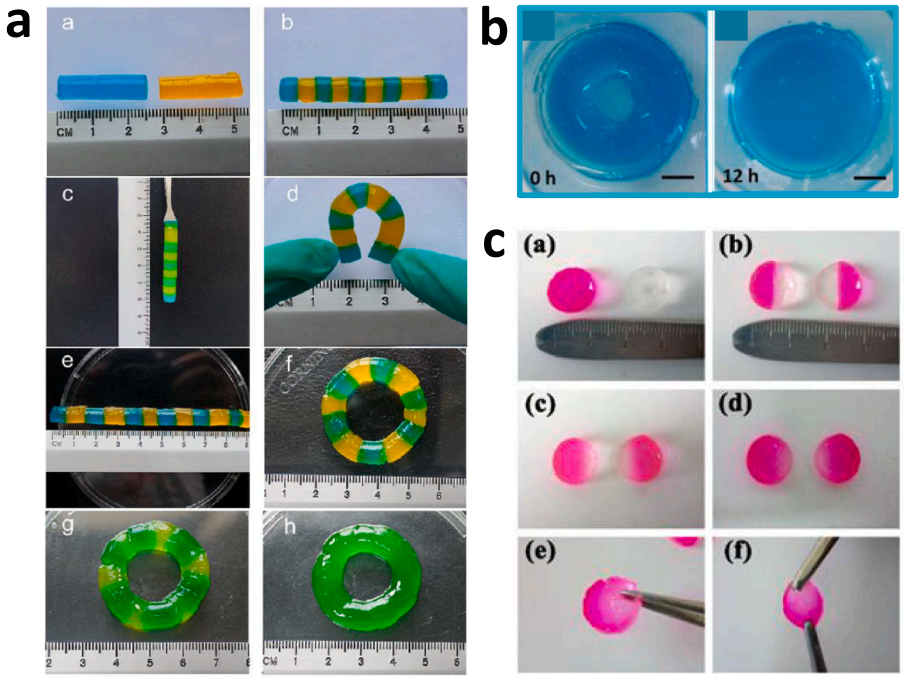

Fig. 4. Gel block fusion tests of (a) Poly(L-glutamic acid) $(PLGA)_{30250}$ hydrogel [14], (b) supramolecular hydrogel composed of ABA triblock copolymer containing a central poly(ethyl-ene oxide) block and terminal poly(N-isopropylacrylamide) (PNI-Pam) block with ureido pyrimidinone (Upy) moieties [18], and (c) poly(N-isopropylacrylamide) (PNIPAM) and alginate-based Gel-10–3.5 [5]. Reprinted with permission from Li et al. (2015) [14] Copyright ©2015 ACS Publications, Zhang et al. (2017) [18] Copyright ©2017 ACS Publications, and Sun et al. (2019) [5] Copyright ©2019 Elsevier.

its high depth of focus the three-dimensional (3D)- sample images can be obtained. [31] TEM, on the other hand, is a high resolution method providing subnanometer scale observation of molecules [31].

SEM can be used to study the self-healing of hydrogels by showing the changes in framework and crosslinking density before and after the healing process [7]. SEM can also be used together with other techniques, for example, optical microscopy, TEM, AFM or scanning tunneling microscopy (STM) in order to get the full understanding about the sample morphology [31]. For example, Ding et al. [26] used SEM together with optical microscopy to study the self-healing at different time-points. Optical microscopy showed a gradual healing of a crack in 10 min and full healing at 60 min. SEM confirmed this and provided

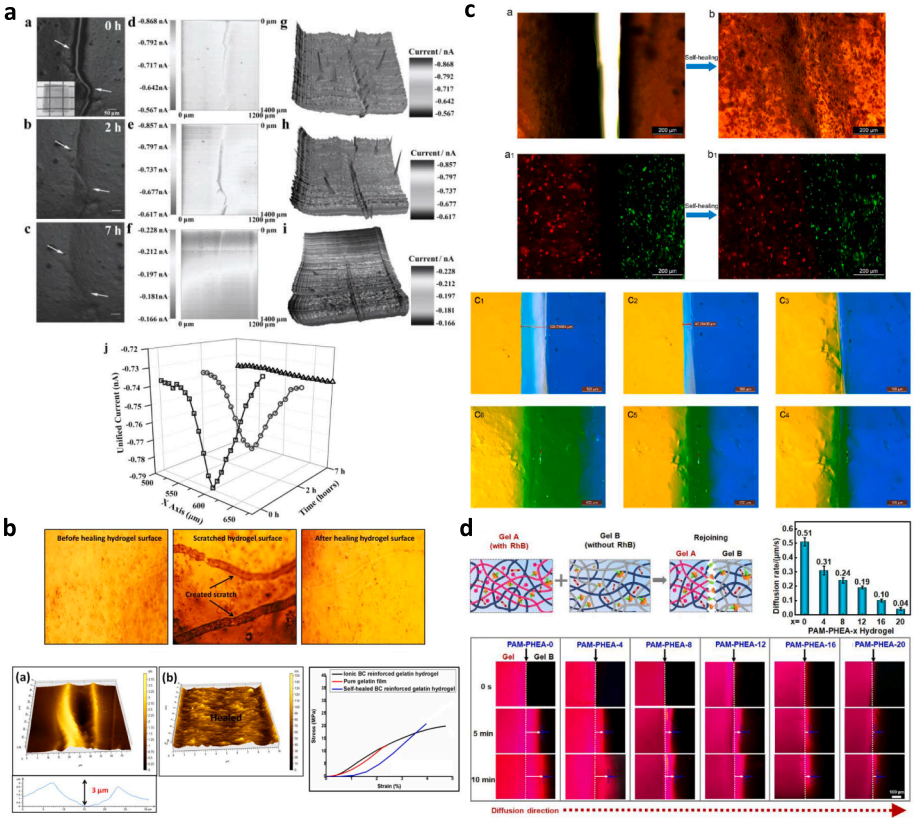

Fig. 5. Visualization of self-healing in hydrogels using (a) SECM & optical microscopy for fulvene-modified dextran/dichloromaleic-acid-modified poly(ethylene glycol) hydrogels [26], (b) AFM & optical microscopy for Curcumin entrapped gelatin/ionically modified self-assembled bacterial cellulose (iBC) hydrogels [15], (c) optical microscopy and fluorescence microscopy for cholesterol (Chol)-modified triblock poly(L-glutamic acid)-block-poly (ethylene glycol)-block-poly(L-glutamic acid) ((PLGA-b-PEG-b-PLGA)-g-Chol)/ β-cyclodextrin (β-CD)-modified poly(L-glutamic acid) (PLGA-g-β-CD) hydrogels [14], and (d) fluorescence microscopy for polyacrylamide (PAM)/poly(hydroxyethyl acrylate) (PHEA) hydrogels [53]. Reprinted with permission from Wei et al. (2013) [52] Copyright ©2013 Wiley, Khamrai et al. (2019) [15] Copyright ©2019 Elsevier, Li et al. (2015) [14] Copyright ©2015 ACS Publications, and Hai et al. (2022) [53] Copyright ©2022 Elsevier.

further information about the newly formed fibrils after 10 min referring to rebuilding network. After 60 min there were no visual difference between the undamaged and healed area. [26]

In order to study the self-healing process of hydrogels both qualitatively and quantitatively, also so called scanning electrochemical microscopy (SECM) has been used. Since the temporally and spatially resolving electrochemical signals can be detected with SECM, the healing process can be tracked *in situ* providing 3D images and topography data of the sample. [52,1,7] The scratch area can be illustrated and the healing efficiency can be calculated based on the width and depth of the scratch area at different time points, like for example Wei et al. [52] have done (Fig. 5 (a)) [52].

4.2.2. Atomic force microscope

Atomic force microscopy (AFM) can be used to study the sample's local structure in wet conditions, allowing also to draw 3D-images. The magnification of 1000000X is provided with horizontal resolution of 0.2 nm and vertical resolution of 0.05 nm. The field of view of AFM is hundreds of micrometers. [10] The method is non-destructive and the sample can be measured directly [10,31]. The image quality and resolution are dependent on the instrument settings but also about the cantilever tip used [10]. The advantages of AFM are, for example, high-resolution images and 3D-surface profile provided, its usability in gaseous to liquid environments, no special treatment (possibly damaging the sample) needed for sample, and possibility to measure supramolecular assemblies' attractive interaction energies or forces. [31]

AFM can be used to study self-healing processes of hydrogels so that a scratch is induced on the hydrogel surface and then the topographic changes (width and depth of crack) on a nanometer to micrometer scale are monitored throughout the whole healing process [1,6]. The degree of healing is also possible to assess with AFM, since the mechanical properties of the surface can be evaluated with AFM. Fig. 5 (b) shows one example of AFM study of self-healing hydrogels made by Khamrai et al. [15]. A scratch and heal method was used for polyelectrolyte modified bacterial cellulose reinforced gelatin film and the efficiency of healing was monitored using AFM depth profilometry. The scratch depth was monitored before and after healing. Figure shows that 3 μm scratch healed after addition of buffer solution. Optical microscope was also used in this study prior AFM to study the healing. [15]

4.2.3. Other microscopic methods

Optical microscopy allows the measurement of the as prepared hydrogel sample, but has microscale limit for the resolution. Optical microscopy provides magnification of 100X with resolution of 0.2 to 0.5 μm. The thickness the light can penetrate the sample has limitations and therefore thick samples are difficult to image. [10] In fluorescence microscopy, on the other hand, fluorescence is used to generate the image. Optical microscopy is widely used to study the self-healing of hydrogels. For example, Li et al. [14] have used optical microscopy to study self-healing process by taking images at various time-points. Fig. 5 (c) shows how the 126.7 μm crack between two hydrogel surfaces is narrowed and healed over time (c1 0 s, c2 5 s, c3 10 s, c4 20 s, c5 60 s and c6 5 min). The images also show some diffusion of coloring agents through the surfaces which is important, for example, for the delivery of bioactive agents. Diffusion of the same gel is also shown in Fig. 4 (a). Li et al. also took some fluorescence microscopy images (Fig. 5 (c)) of the healing process without and with adipose-derived stem cells (ASCs). Two cell-encapsulated hydrogel pieces were stained with 1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiL) or 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) and were put together. The images showed perfect integration of the cells between two parts and self-healing of surfaces without any interference of the process by the cells. [14] Hai et al. [53] also used fluorescence microscopy to directly visualize polymer chains diffusion across the interfaces and to quantitatively study healing dynamics by calculating diffusion speeds (Fig. 5 (d)). Rather than using free stained molecules, the fluorescent molecules were incorporated into the network to show real chain diffusion. The results showed that the constrain network suppressed polymer diffusion, which showed the mechanism of crosslinker content (x)-dependent self-healing of the hydrogels in question. [53]

Another microscopic method to follow self-healing of hydrogels is to use interferometric microscope with enhanced resolution compared to optical microscopy. For example, Mohamadhoseini et al. [54] used Mirau interferometer 3D microscope to produce images of the self-healing of ALG-CD_2:ALG-Ad_2/ionic hydrogel on a microscopic scale. A 100 μm scratch was made on the surface of hydrogel and 3D-images of the healing process were recorded. In 10 min the scratch was completely repaired showing the self-healing ability of hydrogel without any external stimuli. [54]

5. Determining the healing efficiency of hydrogels

Previous chapters have shown how to study the reversibility of reversible interactions in hydrogels, and how to investigate the self-healability of hydrogels through morphological studies. This chapter presents how the self-healing ability of hydrogels can be studied through mechanical testing (compression and tensile testing), especially how so called healing efficiency can be determined. Basically, the healing efficiency reveals how well the mechanical properties of the hydrogel are restored after damage repair. Also, the time-dependence and dynamics of self-healing are to be considered.

5.1. Mechanical testing

For many applications, the mechanical properties are one of the most important design parameters. For example, in biomedical applications, mechanical properties, especially stiffness, has shown to affect to the cell behavior. Studying the mechanical properties of self-healing hydrogels is important not only for the application but also for the healing process. The mechanical testing reveals the possible translation of the microscopically observed reversibility onto the materials level. On the other hand, with these methods variable physical properties (for example, tensile strength, mechanical moduli, viscosity etc.) can be monitored as a function of different variables, such as time or temperature. The mechanical properties can be defined with time-independent rubber elasticity theory and time-dependent viscoelasticity theory. These theories also allow the correlation of the properties with their structural networks. [38]

The most common ways to measure the mechanical properties are tension and compression tests, as well as indentation and frequency-based tests [38]. In case of self-healing studies, either tension or compression testing are mainly performed, in addition to the rheology presented in the previous chapter. In these methods, the load-displacement–time or stress–strain–time data are usually collected [38]. Testing is usually done in the bulk state [11]. In order to understand also the mechanical behavior of cell-seeded hydrogels and to ensure their performance in biomedical *in vivo* and *in vitro* applications, the measurement at that state would be important [38].

5.1.1. Calculation of healing efficiency

Healing efficiency (HE) reveals how well the mechanical properties of the hydrogel are restored after damage repair. The HE can be determined by comparing the original mechanical properties of hydrogel with the healed ones [7,9,4]:

$$\text{Healing efficiency} = HE = \frac{\text{Mechanical value}_{healed}}{\text{Mechanical value}_{initial}} \times 100\%. \quad (1)$$

Here, the mechanical value can be achieved from tensile or compression tests and it can be, for example, a Young's modulus, compressive load at breaking point, fracture strength at breaking point, elongation at break,

tensile strength at break, fracture stress or strain, or toughness. All of these cases are described in more detail in the following chapters.

However, it should be noted, that the HEs determined in different ways (tensile or compression) and using different mechanical values are not comparable and more information about the material's healing process would be received if more different mechanical values were used to determine the HEs.

5.1.2. Compression tests

Compression testing is usually used for self-healing hydrogels that are too soft, fragile and flexible and cannot withstand the clamping of tensile testing or are too dynamic to shape into required shape needed for tensile testing [9,7]. The advantage of compression test over tensile test is the geometry of sample which is not limited to specified shape. The compression tests of self-healing hydrogels are usually performed in unconfined fashion: a wedge-shaped sample is compressed between two non-porous plates [38,7,1]. The sample contraction happens along the direction of the stress [55]. The compressive force and displacement are measured and used to form stress–strain curve for further analysis [38]. In case of using Young's modulus for the HE calculations, it should be noted that even though the stress–strain proportionality is usually linear (slope of the linear segment), for some materials like hydrogels it is not, meaning that the Young's modulus (also called stiffness, elastic stiffness constant or second-order elastic constant) cannot be described this way. Nevertheless, this linear-fitting method is used in most of the articles in our field. Karvinen et al. [56] have previously presented an alternative way to determine the stiffness for the materials by using a polynomial-based approach. More detailed description of the method is shown in [56].

For the self-healing testing, the sample is usually cut in half and rejoined for certain period of time (depending on the healing process) at specific environment (air or moisture environment) and temperature (room temperature or 37°C) similarly as done in the gel block fusion test. After this the stress–strain curve of healed sample is measured similarly as pristine sample. The measurement has been done using either normal plates [57] or a beamed-shape strain compression [16,58,59]. In most of the compression test-based studies the HE is calculated based on the fracture strain [57] or fracture stress (strength) [16,57,58], but there are also cases where HE is calculated based on the compressive load at breaking point [59] or Young's modulus [57]. Fig. 6 shows three examples of HE determinations based on compression tests. More detailed information about the examples given here are provided by the Table S4 in Supplementary information.

Fig. 6 (a) shows that Wei et al. [16] have performed a beam-shaped strain compression test for N-carboxyethyl chitosan (CEC)/oxidized sodium alginate (OSA)/adipic acid dihydrazide (ADH) hydrogel with

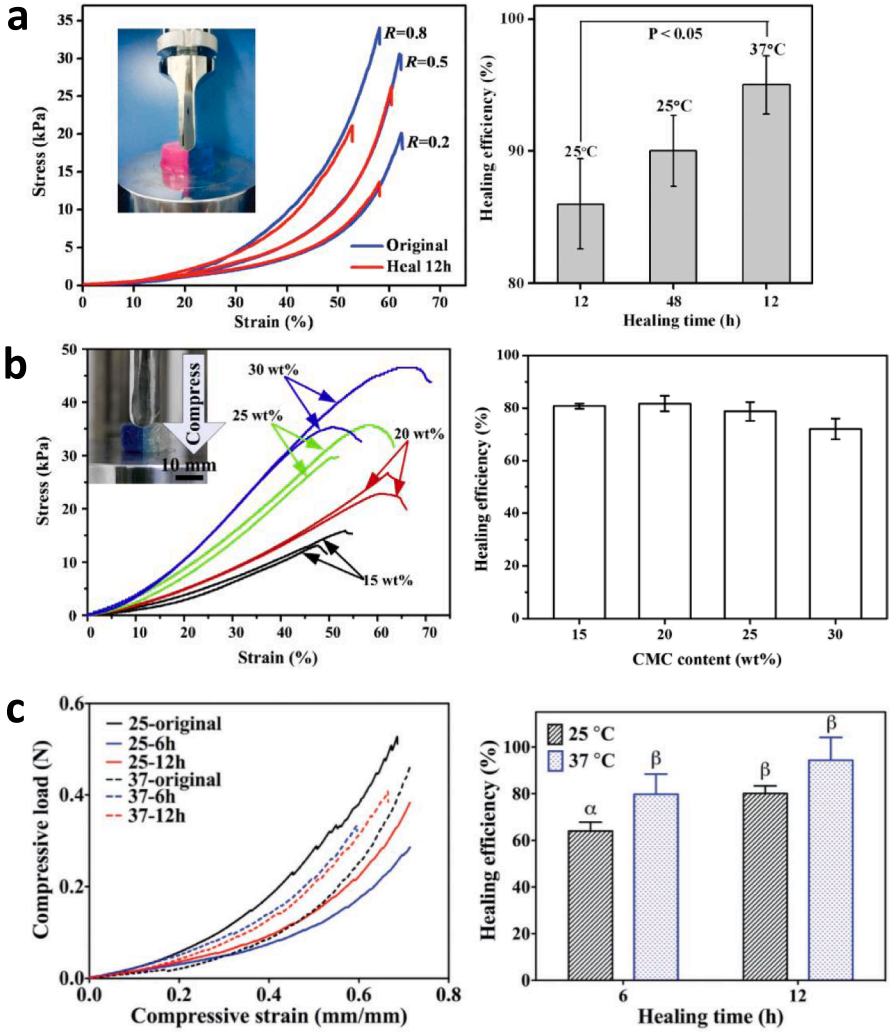

Fig. 6. Beamed-shape compression tests performed and HE calculated for (a) CEC-l-OSA-l-ADH hydrogel [16], (b) CMC hydrogels [58], and (c) CMC/PEG-BA hydrogel [59]. Reprinted with permission from Wei et al. (2015) [16] Copyright ©2015 Wiley, Zheng et al. (2015) [58] Copyright ©2015 Elsevier, and Huang et al. (2016) [59] Copyright ©2016 Wiley.

different CEC contents (R = 0.2, 0.5 and 0.8). The test was performed for original samples and healed samples. HE was calculated based on the healing strength at breaking point. The results showed that at suitable balance of mobile chains and crosslinking dynamics (R = 0.5) the best HE could be achieved. The HE could be further improved by increasing the temperature from 25°C to 37°C or prolonging the healing time from 12 h to 48 h. [16] Zheng et al. [58] have also performed similar beamed-shape compression test for carboxymethyl cellulose (CMC) hydrogels with different CMC content (15, 20, 25 and 30 wt%) (Fig. 6 (b)). HE was calculated based on the healing stress. The results showed that the HE decreased as the CMC content increased indicating decreased flowability of free polymer chains with higher CMC content. [58] Huang et al. [59], on the other hand, performed similar test for carboxymethyl chitosan (CMC)/benzaldehyde-terminated telechelic four-armed polyethylene glycol (PEG-BA) hydrogel (Fig. 6 (c)). HE was calculated based on the compressive load at breaking point. The results showed that the HE was increased when the healing time was prolonged from 6 h to 12 h or when the healing temperature was increased from room temperature to 37°C indicating enhanced dynamic kinetics of the bonds. [59] In the first two tests the healed sample was first cut in half and rejoined 12 h at 25°C [16,58] or also at 37°C [16] before the measurement. The third was healed either 6 h or 12 h at 37°C [59].

In rarer cases, the HE has been calculated based on the Young's modulus. For example, Bilici et al. [57] performed compression test using parallel plates for self-healing (and shape-memory) hydrogels consisting of poly(acrylic acid) (PAAc) chains containing crystallizable n-octadecyl acrylate (C18A) segments together with surfactant (SDS) micelles. The test was performed for original samples and healed samples. The Young's modulus was calculated based on the slope of the stress–strain curve (5–15 % compressions). The results showed complete HEs for hydrogels healed at 24 h at 80 °C. [57] As an additional note to this, it should be remembered that this kind of Young's modulus slope-based determination is rather vague due to the non-linear nature of the curve and it brings additional challenges to the comparability of results made by different research groups.

In addition to previous note, comparison of different compression test studies can be also hampered by the different measuring parameters used, for example, different loading velocity (e.g. 0.5 to 10 mm/min), different cross-sectional diameters (e.g. 5 to 25 mm) and heights (e.g. 5 to 20 mm) of the samples, different temperatures (room temperature or 37°C) used during the measurement, but also by different equipment used.

5.1.3. Tensile tests

Tensile test is mostly an uniaxial elongation measurement performed on samples at large deformations [7]. In tensile test, the sample is hold between two grips so that the other end of the sample is extended by different extension rates and loads. The shape of the sample can be cylindrical, dog-bone-/dumbbell-shaped strips or rings. [38,7,9] From the obtained stress–strain curve some mechanical parameters can be derived, for example, Young's modulus, yield strength, ultimate tensile strength, fracture stress or elongation at break [38,7,9]. For the self-healing testing, these mechanical parameters can be used to calculate the HE.

In the self-healing tensile test, the size of the cut has been varied and the hydrogel pieces have been pressed together for specific time periods (seconds to hours) and/or times. The healing conditions (light, temperature etc.) could also be varied. The tensile testing has been performed for pristine and healed samples. [9] Fig. 7 presents some examples of tensile-based self-healing tests and calculated HEs. The size and shape of the samples vary between different studies, or those have not been given. Also, the extension rate varies from 30 to 100 mm/min. [60,45,19,61] More detailed information about the examples given here are presented in Table S5 in Supplementary information.

Liu, Kang et al. [45] (Fig. 7 (a)) studied the HE of different OSA-PAM hydrogels as a function of OSA content (1–5) as well as healing time (1, 2, 4 or 6 h). HE was calculated based on the tensile strength at break. The results showed that HE increased with increasing OSA content indicating more binding sites between PAM and OSA (Schiff base and hydrogen bonding interactions able to heal). HE was also shown to increase with increased healing time up to 6 h, after which no significant increase were not seen. [45] Li et al. [60] (Fig. 7 (b)) calculated HE of thiuram disulfide-functionalized cellulose nanocrystals (CNC) hydrogels either based on the healing stress at breaking point (HE_s) or healing

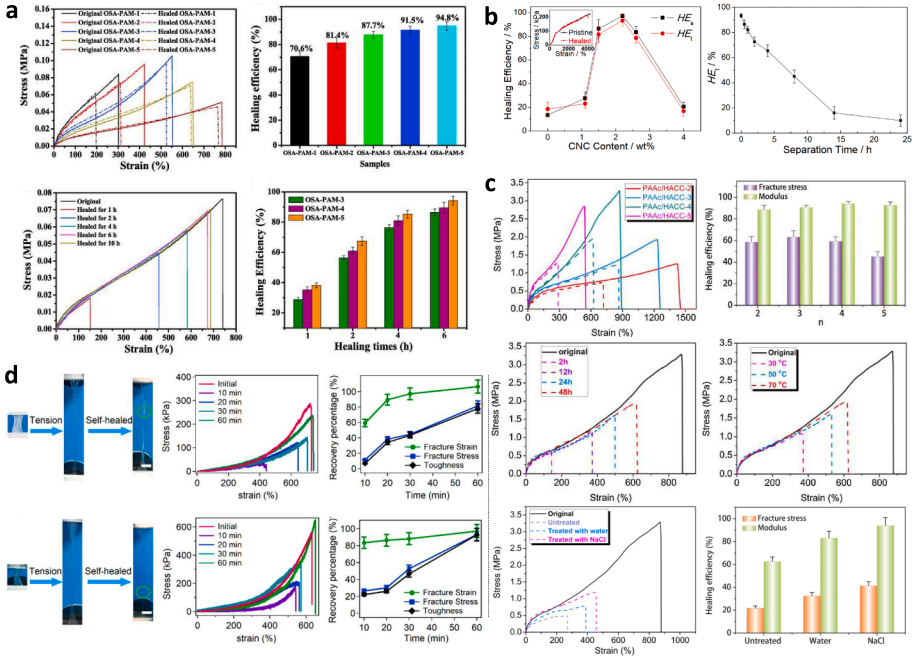

Fig. 7. Tensile tests performed and HE calculated for (a) OSA-PAM hydrogels [45], (b) CNC hydrogels [60], (c) PAAc/HACC-based hydrogels [19], and (d) GGH and GHHPH hydrogels [61]. Reprinted with permission from Liu, Kang et al. (2018) [45] Copyright ©2018 Elsevier, Li et al. (2018) [60] Copyright ©2018 MDPI, Yuan et al. (2018) [19] Copyright ©2018 Elsevier, and Zeng et al. (2019) [61] Copyright ©2019 MDPI.

strain at breaking point (HE_t). They studied HE_s and HE_t as a function of CNC content (0–4 wt%), but also HE_t as a function of separation time (of the pieces, 0–24 h). The results showed that HE_s and HE_t increased to almost 100 % as the CNC concentration increased from 0 to 2.2 %, but decreased again after that indicating possible inefficient chain movement and limited number of disulfide radicals on the surface. Overall, HE_s values were also slighly higher than HE_t values. The separation time was also studied so that the cut pieces were kept apart for different times and brought into contact for 2 min in visible light. The HE_t was shown to decrease when the separation time increased indicating that the separation time is a key factor for self-healing property. This means that gels that are separated for a long time cannot heal efficiently anymore. [60] Yuan et al. [19] (Fig. 7 (c)) compared different HEs based on fracture stress and modulus of different acrylic acid (AAc)/ 2-hydroxypropyltrimethyl ammonium chloride chitosan (HACC)-based hydrogels (n = 2, 3, 4 or 5). They also studied the HE at different healing time (2, 12, 24 or 48 h), as well as at different healing temperatures (30, 50 or 70°C). Additionally, they studied HEs as a function of different healing agents (water or NaCl). The results showed that HE calculated based on the fracture stress were lower than those calculated based on the modulus. Also, with fracture stress the HE was highest with n = 3 sample and lowered again, whereas with modulus the HE increased with n, so that n = 4 had the highest HE. HE was also shown to increase with increasing healing time and temperature indicating that the mobility of the chains is enhanced at higher temperatures and healing times. When compared different healing agents, NACl counterions were shown to have a shielding effect that enabled the diffusion of charged polymer chains from side to another. This was shown as increased HE. [19] Zeng et al. [61] (Fig. 7 (d)) used term recovery percentage as a synonym for HE, and it was calculated based on the fracture stress, fracture strain and toughness for Gly-Gly-His (GGH) and Gly-His-His-Pro-His (GHHPH) hydrogels. They studied the recovery percentage as a function of healing time (10, 20, 30 or 60 min). The results showed that the HEs based on all the parameters increased as the healing time increased for both gels. For GGH and GHHPH gels, the fracture strain could reach the initial level during the 60 min whereas fracture stress and toughness reached only 80 % and 90 %, respectively. The HE was slightly higher for GHHPH gel indicating possible higher cooperativity of the bonds between Zn^{2+} ions and GHHPH. [61]

5.2. Characterization the time dependence and dynamics of self-healing

The dynamics of the self-healing process can be determined by measuring the HE as a function of healing time [4]. In the previous chapters there are many examples of compression-based [58,59] and tensile-based studies [62,45,60,19,61] that have showed that HE increases when the healing time is prolonged. The HE can also be determined at different separation times of pieces in order to reveal the time dependence of the process as Li et al. [60] previously showed in Fig. 7 (b). They found out that the HE decreases when the separation time increases. This indicates that the separation time is a key factor for self-healing property, meaning that for gels that are separated for a long time the self-healing is not as efficient as for those that are newly separated. [60] The healing times also affect to the HE. For example, Yang et al. [62] found out that the HE started to drop after several times of healing indicating reduced molecular mobility in the fracture surface. [62] In addition, it has been shown that better self-healable hydrogels with higher HE can self-heal faster compared with only partially self-healable hydrogels. However, healing ability has shown to be inversely proportional to hydrogel's mechanical strength. Higher efficiencies can be achieved by decreasing reversible crosslink's lifetimes, whereas good mechanical properties can be achieved by making the crosslinks stronger. Currently self-healing hydrogels are still rather weak and soft which limit their use in applications bearing load and stress, although some improvement have been achieved by incorporating multiple crosslinking mechanisms into the system, for example in hybrid, interpenetrating network (IPN) or nanocomposite hydrogels. The crosslinking type has also shown to limit the healing ability. Many physically crosslinked hydrogels have 100 % HE, whereas, for example, physically and irreversibly chemically crosslinked IPNs do not. On the other hand, studies have shown that the healing time is affected by the sample and rupture sizes: larger sized samples and bulk ruptures require longer healing time compared with smaller sample sizes and microscopic ruptures. HE has also been found to be affected by the different sample geometries used, i.e. dumbbell or cylinder. [9]

6. Investigation of injectability and shear-thinning properties of self-healing hydrogels

Injectability is an important property for hydrogels used for biomedical applications. For example, cell transplantation using injectable hydrogels is a minimally invasive method where hydrogel can temporary support the delivery of cells [63]. The benefits of injectable self-healing hydrogels over conventional injectable hydrogels are that the injection is possible at the target site without gel fragmentation and they can be injected as bulk hydrogels [64]. Injectability is also important for other applications, such as 3D (and 4D) bioprinting, which is a growing field at the moment [38,9]. The ink used in 3D bioprinting should pass through the nozzle, but it also has to have such structural integrity that it can support the next layers [9]. Due to the reversible crosslinks in self-healing hydrogels, they have so called shear-thinning property, which is why they can be printed after the gelation and self-heal [9].

The injectability of self-healing hydrogels can been studied using basic injection tests. For example, Huang et al. [59] and Wei et al. [16] have used two syringes filled with preformed hydrogels colored with different dyes (Fig. 8 (a) and (b)). The hydrogels were injected through the needles on the bottom of a beaker, compressed and allowed to heal for suitable time at room temperature. Both studies showed that these hydrogels can be injected after gelation, meaning that small injected pieces could form an integral hydrogel that can be hold with tweezers and it can stand up by itself, even after immersion in PBS for few hours. [48,16] Huang et al. [59] performed additionally an extrusion test for carboxymethyl chitosan (CMC)/benzaldehyde-terminated telechelic four-armed polyethylene glycol (PEG-BA) hydrogel through a 20 G needle. As Fig. 8 (a) shows, smooth letters could be injected without clogging. [59]

Self-healing hydrogels can also be studied after injection using rheology. Wei et al. [17] made time sweep studies for CEC-l-OSA hydrogel after 24 h and 30 min of setting, and 5 min of self-healing after injection (Fig. 8 (c)). The results showed fast mechanical recovery of injected self-healing hydrogel compared with the control sample (24 h). Freshly prepared sample (30 min) using two-syringe system showed surprisingly lower modulus compared with both previous gels indicating more quick build up of mechanical modulus by the self-healing injectable gel. [17] Alternatively, Zhang et al. [65] designed a three-stage oscillation-shear-oscillation experiment (Fig. 8 (d)) to mimic the injection process and follow the healing after shear, but also used a three-step oscillatory rheological measurement to study hydrogels' self-healing [65].

For injectable and bioprinting applications, the shear-thinning property of self-healing hydrogels can also be studied using rheology. Fig. 8 (d) shows how Zhang et al. [65] studied the shear-thinning behavior of their xanthan gum (XG)-silk fibroin (SF)-sodium trimetaphosphate (STMP) hydrogels using rheological viscosity-shear rate measurement. The results showed typical decrease of viscosity as a function of increasing shear rate, which is also the definition of the shear-thinning. [65] Of course, if the material is, for example, temperature sensitive, additional temperature sweep measurements studying the dependence of moduli and/or viscosity on the temperature could be conducted [18,2]. More about the rheological characterization of hydrogel precursors intended for 3D-bioprinting (or injection)

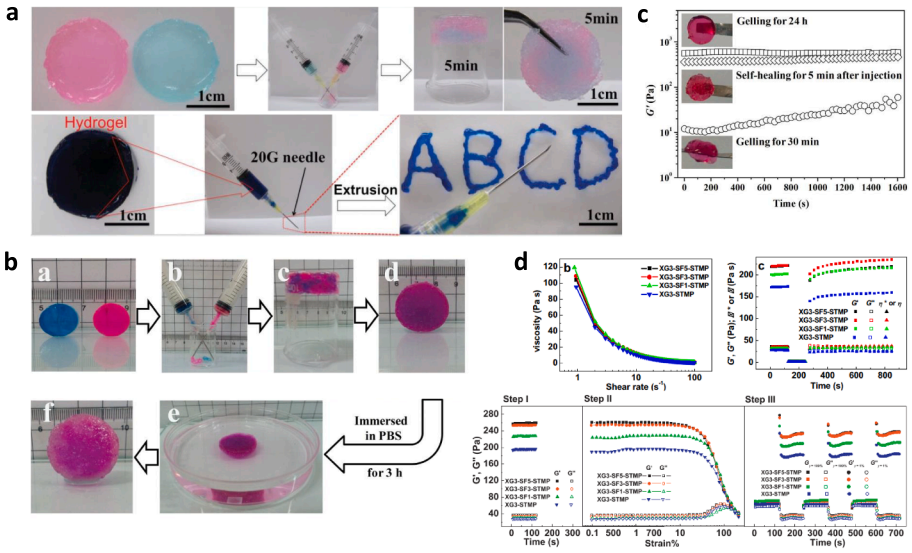

Fig. 8. Studying the injectability of self-healing hydrogels using basic injection tests for (a) CMC/PEG-BA hydrogel [59] and (b) CEC-l-OSA-l-ADH hydrogel [16], (c) rheological time sweep measurement 5 min of self-healing after injection for CEC-l-OSA hydrogel [17], and (d) viscosity-shear rate measurement, a three-stage oscillation-shear-oscillation experiment (step I: time sweep (initial), step II: steady shear flow (injecting), step III: time sweep (recovering)) and a three-step oscillatory rheological measurement (step I: time sweep, step II: strain sweep, step III: alternate step strain) for XG-SF-STMP hydrogels [65]. Reprinted with permission from Huang et al. (2016) [59] Copyright ©2016 Wiley, Wei et al. (2015) [16] Copyright ©2015 Wiley, Wei et al. (2016) [17] Copyright ©2016 Scientific reports, and Zhang et al. (2020) [65] Copyright ©2020 Elsevier.

applications can be found, for example, from the review-article of Townsend et al. [66]. Table S3 in Supplementary information collects all the examples presented above with additional information.

7. Characterization of conductivity of self-healing hydrogels

Conductivity of hydrogel is beneficial for many biomedical and electrical field applications, for example, in tissue engineering conducting components facilitate the environment for the electrical signal transmission of cells, thus promoting cell differentiation and proliferation. By knowing the benefits of self-healing hydrogels, by combining the self-healing ability and electrical conductivity, even better materials can be achieved. [8] For more information, Deng et al. [8] have nicely reviewed the preparation, properties and applications of self-healing conductive hydrogels.

Conductive self-healing hydrogels can be studied using same methods as previously presented, but additionally their electrical conductivity can be measured using a conductivity measurement test and LED bulb test in connection with a gel block fusion test, as also shown in Fig. 9. For example, Yuan et al. [19] studied the conductivity of self-healable polyacrylic acid (PAAc)/2-hydroxypropyltrimethyl ammonium chloride chitosan (HACC) physical hydrogels with different HACC content (n = 2, 3, 4 or 5). Fig. 9 (a) shows that these gels are conductive and can power a LED light, even after self-healing. The conductivity was

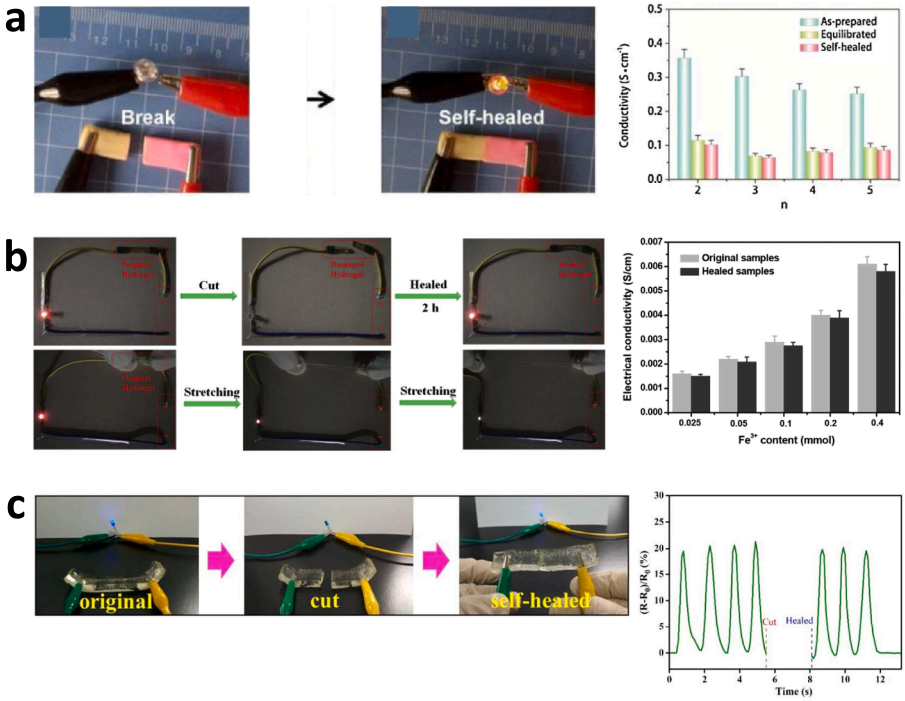

Fig. 9. Conductivity and LED bulb test of (a) PAAc/HACC physical hydrogels [19], (b) PEG/PAMAA DN hydrogel [67], and (c) TA@CNC ionic gels [20]. Reprinted with permission from Yuan et al. (2018) [19] Copyright ©2018 Elsevier, Liu, Oderinde et al. (2018) [67] Copyright ©2018 Elsevier, and Shao et al. (2018) [20] Copyright ©2018 ACS Publications.

also measured using a four-point probe method. The conductivity values of as-prepared, equilibrated (in water bath + dialysis) and self-healed gels were measured. Equilibrated gels had lower conductivity compared with as-prepared samples due to removal of NaCl ions during the dialysis. Self-healed gel had about the same conductivity as the equilibrated gel indicating gel's good self-healability of conductivity. It was also considered that the conductivity values (0.064 to 0.357 S/cm) of self-healed gel are sufficient for the transmission of bioelectrical signals and for the electrical stimulation of cell differentiation and proliferation in human body. [19] Liu, Oderinde et al. [67] studied the conductivity of self-healable polyethylene glycol (PEG)/poly(acrylamide-co-acrylic acid) (PAMAA) DN hydrogel. The conductivity was measured with a four-point method. Fig. 9 (b) shows that the electrical conductivity of self-healed gel (2 h of healing, 0.0029 S/cm) nearly reaches the conductivity of original sample. In addition, the LED test showed an initial high brightness indicating good conductivity that also recovered after self-healing. There was also a luminance variation seen with the increased strains. [67] Shao et al. [20] used also a LED bulb test to prove the electrical self-healing property of their tannic acid-coated cellulose nanocrystals (TA@CNC) ionic gels. Fig. 9 (c) shows that after cutting the gel pieces were immediately put back together and healed, the electric circuit was restored leading to lightening of the LED bulb. Additionally, they studied the time evolution of the electrical self-healing process using real-time relative resistance change measurements. Almost 100 % recovery of the relative resistance change of healed sample was seen within 3 s, meaning that these gels have good electrical restoration performance. [20] All the examples given here are also collected in Supplementary Table S3 with more detailed information.

8. Challenges in the characterization of self-healing hydrogels

As previously shown, there are many methods already used to characterize self-healing hydrogels. However, the lack of proper standardized characterization methods makes the comparison of different self-healing hydrogel materials impossible because the amount and use of analysis methods and parameters varies between different studies. The lack of standardization can also be considered as a lack of reliability of the method, because standardization ensures that the method is repeatable and reproducible. [24]

When considering the choice and standardization of characterization methods, there are a few things to keep in mind like Tang et al. [24] have nicely shown. Shortly, the quality of results given by different methods can vary. The results can be either quantitative or qualitative, but since qualitative results usually are based on observation, determination and detection without the actual measurement (e.g. 2D or 3D images) they are better for basic evaluation, whereas quantitative results (e.g. mechanical values) are preferable because something is actually measured resulting in figures and numbers. Second, the interpretation of results can be simple based on immediate results without the need for further analysis or it can be more complex based on raw data which needs further analysis and interpretation by a qualified analyst. The simplicity of the characterization method is also important for a good test. Many times specialized training is needed despite the detailed instructions. The safety of the procedure should not be forgotten either. The characterization method suitable for *in situ* applications is also very interesting. Methods like mechanical compression and tensile tests are destructive methods causing irreversible damage to the sample, whereas, for example, microscopic methods are considered as partially-destructive methods since small samples of the material are extracted. Non-destructive methods do not cause any damage to the structure and would be favorable. [24]

All the methods used for the characterization of self-healing hydrogels have limitations. Limitations common for all test methods are related to disturbing factors. First, the sample validity affects to the validity and accuracy of the method where sampling is needed. Second limitation is the instrumental errors caused by native manufacturing and design problems. Third limitation is the operational errors that are related to the errors caused by the operator and to the operation of the test instrument. [24] Naturally, different characterization methods themselves also have their specific limitations and drawbacks.

Characterization of hydrogels is not without problems. Due to their low solid polymer content and high water content the characterization of composition and structure, and in this case the presence of reversible interactions, can be challenging. Significant water molecules' signals can interfere the FTIR and NMR spectra by dominating the polymer chemical bonds. [10] In NMR, the data quality can be affected by the low solid content [10], also due to NMR's high detection limit, high concentration of end groups are needed [11]. Similarly in FTIR, the small signals caused by low volume fraction bonds can be difficult to resolve, as well as if there is small amount of some minor components. In addition to water, impurities and chemical bond interference may affect to FTIR spectra. [10,24] Additionally, in NMR, the solid samples like hydrogels can also be measured using solid-state NMR [11,33]. However, when at solid state, the molecule motions are restricted revealing such types of orientation-dependent internuclear and nuclear interactions that cannot be seen with liquid-state NMR. Even though they give some information about local electronic and geometric structure, the resolution is lost, the sensitivity is reduced and it is difficult to detect individual atomic sites since the line is broadened. However, there are some line-narrowing techniques that can be used to solve this problem, for example, combination of NMR with magic angle spinning (MAS), for example, CP/MAS ^{13}C NMR. [33] In case of Raman, low solid concentration causes lost of sensitivity, but also some chemical species' established information on Raman spectra may lack [10,24].

Water affects also for the scattering methods. Water background subtraction statically reduces the hydrogel structure morphology signals. Another problem of scattering methods is the scale disparity of nanoscale polymer chains and microscale mesh size, which is why not only one method can be used to measure both dimensions. Thus, a combination of different scattering methods is often needed to cover the whole scale. In addition, there is also a necessity of extensive modeling of the scattering curves that needs some pre-knowledgement of the hydrogel structure. Further, these methods provide no image. [10]

Despite the successful use of rheology to study the reversibility of the interactions, it also has some drawbacks, like the destructiveness of method for the sample which cannot recover after the measurement, as well as the use of different sized plates and geometries leading to different results despite using the same measuring parameters [10].

The self-healability of hydrogels have been observed using electron, AFM and optical microscopy methods that together cover structure information from atomic to microscopic scales. However, the combination of results may be misleading. The drawback of electron microscopy methods concerns preparation of the sample, which can be time-consuming and complex. Also, due to the high water content of the hydrogels, their direct analysis can be difficult. For electron microscopy due to need of the gas-free vacuum environment in the sample chamber, the sample needs to be water free (for example air- or freeze-dried). The drying process can cause artefacts into the structure and therefore give false information about the structure. When using cryo-SEM or -TEM, the rapid cooling of the sample into liquid nitrogen or ethane temperatures can minimize the changes, but still can have similar affect. In cryo-TEM, the imaging of bulky hydrogels is also difficult because cryo-cooled samples need to be very thin for electron beam transmission. Further, high energy electron beam is known to be able to damage the sample, whereas the image quality can be poor when using low electron energy. It is also not possible to measure the samples directly using these methods. The samples need to be made conducting, which polymers in hydrogels are usually not. Also, for TEM, the samples need to be very thin (100 nm). Further, with high resolution microscopy the structure information given by the measurement is localized into a limited area not statistically representative of the sample. [10,31] The disadvantage

of AFM is the tip which can apparently flatten the nanostructures of soft matter so that they appear shallower and wider than they really are [31]. Also, the image quality and magnification are dependent on the fraction of polymer chains and the cantilever probes. Further challenge with AFM is the sizing to get a good baseline caused by the network's many layers of polymer chains between the cantilever tip and the bottom. [10] Optical microscopy has poor resolution at nanoscale. The samples need to be transparent and the resolution is limited because of the wavelength of light itself and thin section thickness. Also, the sample can dry during the measurement which may give false information. [10,24] In addition to microscopic methods, the observation of self-healability has also been done by eye and taking digital photos which are very raw methods. Overall, the drawback of all these methods is that the results are only qualitative. Other methods, such as surface profilometry (contact or optical profilometry) measuring also the crack's width and depth, might be better options, although their drawbacks are slowness of the contact method and lower accuracy of the optical method. In case the crack is not only on the surface, techniques, such as X-ray computer tomography (CT) or micro-X-ray CT (μCT) as non-destructive methods offer a possibility to create a 3D-image or video of the material showing also the damage. However, the visualization of hydrogels due to their low polymer content can be difficult using these methods. [11]

The HE of hydrogels have been studied using mechanical testing methods, such as tensile and compression testing. The downside of these methods is that they usually require a large amount of sample [38] and the size of the sample may affect to the results [24]. Also, the comparative analysis of healing abilities may be difficult due to the different measuring parameters, environments, equipment etc. used, even though these are quantitative methods [9]. In addition, the drawbacks of compression testing are the sample bulging under the compressive loading, as well as the difficulty to apply evenly distributed pressure to sample [38]. The downsides of tensile testing are the limited specific geometries used, grips' possible misalignment and limited long-term monitoring of mechanical property changes [38].

In case of injectable/3D-bioprintable self-healing hydrogels, the injectability has been tested using simple injection test using a syringe and different sized needles. However, this method is very raw and does not give any quantitative information. For conductive self-healing hydrogels, the conductivity test and a LED bulb test may be performed. The drawback of 4-point probe measurement is however its better suitability for dry samples which wet, electrolyte swollen hydrogels are not. Two-point probe electrical resistivity measurements would therefore be more suitable for hydrogels. [68]

9. Future aspects of self-healing hydrogels and their characterization

The field of self-healing hydrogels has grown in recent years. Despite the great progress already made, there are still some challenges that need to be solved before fully ideal self-healing hydrogels can emerge. As already mentioned in the previous chapter, the characterization of the self-healing hydrogels needs some standardization in order to allow better comparability of the results between different studies, but it also gives reliability for the characterization methods and results given by them. In this way, the self-healing process can be learned better, also if the characterization methods are further developed.

As Taylor et al. [9] nicely show in their review, within the literature, there are number of different non-standardized methods used to characterize self-healing hydrogels, although there are different parameters within the techniques used to quantify the self-healing making the comparison of results impossible [9,4]. These methods analyze, for example, the chemical composition, molecule sizes, mechanical properties, and monitor microscopically or using other imaging techniques the structural reorganizations [11]. For the standardization, we already have the basic methods, but they are used very inconsistently [4]. Overall, the mechanical characterization and morphological/topological observation methods need some development. From a morphological point of view, multi-scale (atomic to macroscopic scale) characterizaton is needed. The healing process can be learned better based on the details given by the atomic and molecular scale observations. It would be also important to be able to translate the qualitative and visual information into quantitative and numerical information. [7] Quantitative methods with *in situ* evaluation and non-destructiveness is especially needed [1]. For the mechanical characterization, nowadays only the short-term healing has been studied through HE calculations, but also the long-term changes should be studied. Further development is also needed in order to demonstrate numerically the healing kinetics. [7] The methods that allow to measure the healing process or outcome at different conditions should be desired. This way the material could be tailored so that the reversible reactions would occur at conditions suitable for the application, but also in a reasonable time frame. For the analysis methods, these things impose some requirements, such as it should be able to monitor the time dependence of reaction relatively fast and allow the measurement at the actual reaction conditions with no changes during the measurement. [11] In addition, the self-healing ability should be studied in conjunction with the mechanical robustness, since as a property they are in opposition [9]. As a conclusion, however, in reality there seldom is just one suitable method, so a combination of complementary methods are needed [11]. Theoretical research is also needed in this field. Models or general correlations describing the healing process are needed in order to select proper interactions and functional groups for more ideal self-healing material. [4] When all these pieces of information are combined a more detailed information of the dynamic bonding process should be achieved [11].

Previous chapters have already shown some advantages and disadvantages of the characterization methods. According to that information, the suitable techniques should be selected carefully in order to be able to study the selected problem. The less number of techniques, the better. It is important to use different techniques that give information about the processes both microscopically and macroscopically from several points of view. In addition, the measurements should be made over a wide range of conditions, such as temperature or pH. Sample preparation should not be complicated and *in situ* monitoring should be possible. [11]Of course, according to the application, more extensive study of the material is needed, for example, the biodegradation, swelling, structure analysis (for example, porosity and mesh size) and *in vitro* cytotoxicity tests are needed in order to design suitable material for the application. For ideal self-healing hydrogel, a combination of better mechanical properties and higher HEs are needed, together with good biocompatibility. [7] Currently, the mechanical properties of self-healing hydrogels are still rather poor. Therefore more tough self-healing hydrogels are needed. However, efforts making higher strength self-healing hydrogels have been made, for example, by using double network or nanocomposite hydrogels, although they are still far from purely tough MPa level gels. [1] Multi-responsive hydrogels are also desired. [7] This means that self-healability is combined with other function(s), such as magnetism, electrical conductivity etc. into one system [1].

There is also a problem when comparing the advantage of self-healing hydrogel with non-healing hydrogel, since no corresponding control can be set. This is difficult for the biomedical application, if we do not know if the self-healability gives superior results and how much the self-healing ability contributes to tissue repair etc. For ideal self-healing hydrogel for biomedical application, it should have improved biocompatibility and biodegradability, and should heal autonomously without external stimulus. Further, the structure (e.g. porosity) and mechanical properties (e.g. elasticity, stiffness) should meet the ones of target tissue. Hydrogel should also be multi-functional with synergetic effect. The *in vivo* tracking of hydrogels should also be further studied, for example with the help of fluorescence or electric current. Last, the complicated bio-functionality should be fulfilled. [7]

It was said above, that autonomously self-healing hydrogels are desired. They do not need any external stimulus for the structure and function recovery. It is known that triggering with external stimuli, like heat, pH or light, consumes energy, but it also makes the realization of self-healing process more complicated. Triggers like temperature change can irreversibly damage cells, and light cannot be used for opaque samples or for implanted samples. Therefore, their applications are limited. [1]

According to current methods, we could already suggest a combination of methods to be used as a "standard" protocol when characterizing self-healing hydrogels and presenting the results in the literature. In accordance with Fig. 1, first, the presence of reversible interactions should be confirmed using suitable spectroscopic and/or X-ray techniques. The methods chosen should be justified and suitable for the healing method of the hydrogel, so there is no need to specify the methods at this point. Second, the reversibility of the interactions should be determined using strain dependent oscillatory shear measurement, i.e. alternate step strain measurement. This test should, however, be performed more consistently between different studies, although also here some of the measuring parameters, such as temperature or alternate strain values, are dependent on the healing method used. The larger strain value(s) should always be chosen based on the amplitude sweep measurement performed prior testing. Additional information about the material can be achieved if multiple higher strain values are used in addition to one value. Also, the number of loading/unloading cycles should be reasonable, for example, at least three cycles should be performed. Third, self-healing ability of the hydrogel should be tested using a gel block fusion test. An optical photo alone is not enough to present the self-healing process, so also some micro scale imaging method is needed, for example, optical microscopy or AFM. SEM is a questionable method since it requires sample drying. Staining of sample pieces with different dyes gives more information about the process since it reveals also the diffusion properties (which could be quantified quantitatively). Also, some mechanical stretching or bending tests using hands or tweezers should be done to show that the pieces are truly connected. Fourth and last method, involves mechanical testing that can also reveal the HE of the hydrogel. Whether to use tensile or compression testing at this point is again dependent on the healing method used and application. More softer and sensitive hydrogels should be measured using compression testing, whereas tensile testing is more suitable for tough and strong hydrogels that can withstand stretching better. As already shown in previous chapters, HE of hydrogels can be determined based on many different parameters given by the mechanical measurements, such as compressive load at breaking point, fracture strength at breaking point, elongation at break, or tensile strength at break, just to name a few. The HEs based on different parameters are not, however, comparable with each other since they do not represent the same situation so this causes a problem. The difficulty also lies in the different measuring parameters used, such as different strain rates used at compression test and extension rates at tensile test. Also, the size and shape of the sample have been shown to affect to the results, so more consistent use of those parameters between different studies would be advisable. However, we do not comment in this review what specific parameters should be used.

Unfortunately totally ideally standardized methods cannot be developed, for example, due to the different healing methods (e.g. different healing environment) used between different studies, which makes the setting of measuring parameters, such as temperature, difficult. Also, the testing equipment are different even if same parameters are measured, and different users are affecting, for example, to the sample preparation or setting the sample to the device. Despite these problems, more consistent use of methods and presenting of measuring parameters and results in the literature are needed in order to be able to compare different self-healing hydrogels with each other.

10. Conclusions

Self-healing hydrogels have become one of the most attractive artificial biomaterials in the past decades due to their self-healing nature i.e. their ability to repair their initial properties and structure in response to damage. Many types of self-healing hydrogels have already been studied but still some optimization needs to be done. Properties, like biocompatibility, toughness and multi-functionality are needed. Also, the applications should be explored. When designing ideal self-healing hydrogels it requires understanding of their properties but also about the actual healing process. There are currently different characterization methods that have been used to study the properties of self-healing hydrogels and the healing process. The lack of standardization of the characterization methods has, however, made the comparison of different hydrogels difficult. A group of characterization methods and further how to present the measuring parameters and results more consistently in the literature can be suggested in order to help the comparison of hydrogels with each other. However, the setting of measuring parameters due to different healing methods (e.g. different healing environment), or the effect of different testing equipment and user, used between different studies should be taken into account, making the total standardization of methods basically impossible. Anyway, the group of characterization methods should include methods suitable to determine the presence of reversible interactions, to study the reversibility of these interactions, to investigate the self-healability of hydrogels and to determine the healing efficiencies of hydrogels, not forgetting time dependence and dynamics of self-healing. Ideally an additional theoretical study of the healing mechanism would give more information and value for the research. Quantitative studies over qualitative ones should also be preferred.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work is supported by Academy of Finland (Center of Excellence in Body-on-Chip Research, CoEBoC decision number: 336663 (1.5.2020–31.12.2022)). The authors would like to thank Vilma Lampinen for making a good background work in her bachelor thesis for the subject.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eurpolymj.2022.111641.

References

[1] Z. Wei, J.H. Yang, J. Zhou, F. Xu, M. Zrínyi, P.H. Dussault, Y. Osada, Y.M. Chen, Self-healing gels based on constitutional dynamic chemistry and their potential applications, Chem. Soc. Rev. 43 (23) (2014) 8114–8131.
[2] Z. Wang, G. An, Y. Zhu, X. Liu, Y. Chen, H. Wu, Y. Wang, X. Shi, C. Mao, 3D-printable self-healing and mechanically reinforced hydrogels with host–guest non-covalent interactions integrated into covalently linked networks, Mater. Horizons 6 (4) (2019) 733–742.
[3] H. Yu, Y. Liu, H. Yang, K. Peng, X. Zhang, An injectable self-healing hydrogel based on chain-extended PEO-PPO-PEO multiblock copolymer, Macromol. Rapid Commun. 37 (21) (2016) 1723–1728.
[4] B. Gyarmati, B.Á. Szilágyi, A. Szilágyi, Reversible interactions in self-healing and shape memory hydrogels, Eur. Polymer J. 93 (2017) 642–669.
[5] C. Sun, H. Jia, K. Lei, D. Zhu, Y. Gao, Z. Zheng, X. Wang, Self-healing hydrogels with stimuli responsiveness based on acylhydrazone bonds, Polymer 160 (2019) 246–253.
[6] J.A. Yoon, J. Kamada, K. Koynov, J. Mohin, R. Nicolaÿ, Y. Zhang, A.C. Balazs, T. Kowalewski, K. Matyjaszewski, Self-healing polymer films based on

thiol–disulfide exchange reactions and self-healing kinetics measured using atomic force microscopy, Macromolecules 45 (1) (2012) 142–149.
[7] Q. Li, C. Liu, J. Wen, Y. Wu, Y. Shan, J. Liao, The design, mechanism and biomedical application of self-healing hydrogels, Chin. Chem. Lett. 28 (9) (2017) 1857–1874.
[8] Z. Deng, H. Wang, P.X. Ma, B. Guo, Self-healing conductive hydrogels: preparation, properties and applications, Nanoscale 12 (3) (2020) 1224–1246.
[9] D.L. Taylor, M. in het Panhuis, Self-healing hydrogels, Adv. Mater. 28 (41) (2016) 9060–9093.
[10] V.S. Raghuwanshi, G. Garnier, Characterisation of hydrogels: Linking the nano to the microscale, Advances in colloid and interface science 102044 (2019).
[11] J. Brandt, K.K. Oehlenschlaeger, F.G. Schmidt, C. Barner-Kowollik, A. Lederer, State-of-the-art analytical methods for assessing dynamic bonding soft matter materials, Adv. Mater. 26 (33) (2014) 5758–5785.
[12] A. Phadke, C. Zhang, B. Arman, C.-C. Hsu, R.A. Mashelkar, A.K. Lele, M.J. Tauber, G. Arya, S. Varghese, Rapid self-healing hydrogels, Proceedings of the National Academy of Sciences 109 (12) (2012) 4383–4388.
[13] G. Xiao, Y. Wang, H. Zhang, L. Chen, S. Fu, Facile strategy to construct a self-healing and biocompatible cellulose nanocomposite hydrogel via reversible acylhydrazone, Carbohydrate polymers 218 (2019) 68–77.
[14] G. Li, J. Wu, B. Wang, S. Yan, K. Zhang, J. Ding, J. Yin, Self-healing supramolecular self-assembled hydrogels based on poly (L-glutamic acid), Biomacromolecules 16 (11) (2015) 3508–3518.
[15] M. Khamrai, S.L. Banerjee, S. Paul, S. Samanta, P.P. Kundu, Curcumin entrapped gelatin/ionically modified bacterial cellulose based self-healable hydrogel film: An eco-friendly sustainable synthesis method of wound healing patch, International journal of biological macromolecules 122 (2019) 940–953.
[16] Z. Wei, J.H. Yang, Z.Q. Liu, F. Xu, J.X. Zhou, M. Zrínyi, Y. Osada, Y.M. Chen, Novel biocompatible polysaccharide-based self-healing hydrogel, Adv. Funct. Mater. 25 (9) (2015) 1352–1359.
[17] Z. Wei, J. Zhao, Y.M. Chen, P. Zhang, Q. Zhang, Self-healing polysaccharide-based hydrogels as injectable carriers for neural stem cells, Scientific reports 6 (2016) 37841.
[18] G. Zhang, Y. Chen, Y. Deng, T. Ngai, C. Wang, Dynamic supramolecular hydrogels: regulating hydrogel properties through self-complementary quadruple hydrogen bonds and thermo-switch, ACS Macro Letters 6 (7) (2017) 641–646.
[19] N. Yuan, L. Xu, B. Xu, J. Zhao, J. Rong, Chitosan derivative-based self-healable hydrogels with enhanced mechanical properties by high-density dynamic ionic interactions, Carbohydrate polymers 193 (2018) 259–267.
[20] C. Shao, M. Wang, L. Meng, H. Chang, B. Wang, F. Xu, J. Yang, P. Wan, Mussel-inspired cellulose nanocomposite tough hydrogels with synergistic self-healing, adhesive, and strain-sensitive properties, Chem. Mater. 30 (9) (2018) 3110–3121.
[21] P.R. Griffiths, J.A. De Haseth, Fourier transform infrared spectrometry, Vol. 171, John Wiley & Sons, 2007.
[22] Y.E. Shapiro, Structure and dynamics of hydrogels and organogels: An NMR spectroscopy approach, Prog. Polym. Sci. 36 (9) (2011) 1184–1253.
[23] A. Orlando, F. Franceschini, C. Muscas, S. Pidkova, M. Bartoli, M. Rovere, A. Tagliaferro, A comprehensive review on raman spectroscopy applications, Chemosensors 9 (9) (2021) 262.
[24] W. Tang, O. Kardani, H. Cui, Robust evaluation of self-healing efficiency in cementitious materials–a review, Constr. Build. Mater. 81 (2015) 233–247.
[25] Y. Wang, Z. Wang, K. Wu, J. Wu, G. Meng, Z. Liu, X. Guo, Synthesis of cellulose-based double-network hydrogels demonstrating high strength, self-healing, and antibacterial properties, Carbohydrate polymers 168 (2017) 112–120.
[26] F. Ding, S. Wu, S. Wang, Y. Xiong, Y. Li, B. Li, H. Deng, Y. Du, L. Xiao, X. Shi, A dynamic and self-crosslinked polysaccharide hydrogel with autonomous self-healing ability, Soft Matter 11 (20) (2015) 3971–3976.
[27] H. Liu, C. Li, B. Wang, X. Sui, L. Wang, X. Yan, H. Xu, L. Zhang, Y. Zhong, Z. Mao, Self-healing and injectable polysaccharide hydrogels with tunable mechanical properties, Cellulose 25 (1) (2018) 559–571.
[28] C. Qian, T. Zhang, J. Gravesande, C. Baysah, X. Song, J. Xing, Injectable and self-healing polysaccharide-based hydrogel for pH-responsive drug release, International journal of biological macromolecules 123 (2019) 140–148.
[29] Y. Wang, J. Niu, J. Hou, Z. Wang, J. Wu, G. Meng, Z. Liu, X. Guo, A novel design strategy for triple-network structure hydrogels with high-strength, tough and self-healing properties, Polymer 135 (2018) 16–24.
[30] S. Zhu, J. Wang, H. Yan, Y. Wang, Y. Zhao, B. Feng, K. Duan, J. Weng, An injectable supramolecular self-healing bio-hydrogel with high stretchability, extensibility and ductility, and a high swelling ratio, Journal of Materials Chemistry B 5 (34) (2017) 7021–7034.
[31] G. Yu, X. Yan, C. Han, F. Huang, Characterization of supramolecular gels, Chem. Soc. Rev. 42 (16) (2013) 6697–6722.
[32] Y.-J. Lin, W.-T. Chuang, S.-H. Hsu, Gelation mechanism and structural dynamics of chitosan self-healing hydrogels by in situ SAXS and coherent x-ray scattering, ACS Macro Letters 8 (11) (2019) 1449–1455.
[33] M.E.H. El Nokab, P.C. van der Wel, Use of solid-state NMR spectroscopy for investigating polysaccharide-based hydrogels: A review, Carbohydr. Polym. 240 (2020) 116276.
[34] L. Zedler, M.D. Hager, U.S. Schubert, M.J. Harrington, M. Schmitt, J. Popp, B. Dietzek, Monitoring the chemistry of self-healing by vibrational spectroscopy–current state and perspectives, Mater. Today 17 (2) (2014) 57–69.
[35] J. Patel, P. Parsania, Characterization, testing, and reinforcing materials of biodegradable composites, in: Biodegradable and biocompatible polymer composites, Woodhead Publishing United Kingdom, 2018, pp. 55–79.
[36] D.T. Chen, Q. Wen, P.A. Janmey, J.C. Crocker, A.G. Yodh, Rheology of soft materials, Annual Review of Condensed Matter Physics 1 (1) (2010) 301–322.
[37] T.G. Mezger, The rheology handbook: for users of rotational and oscillatory rheometers, Vincentz Network GmbH & Co KG, 2006.
[38] A. Vedadghavami, F. Minooei, M.H. Mohammadi, S. Khetani, A.R. Kolahchi, S. Mashayekhan, A. Sanati-Nezhad, Manufacturing of hydrogel biomaterials with controlled mechanical properties for tissue engineering applications, Acta biomaterialia 62 (2017) 42–63.
[39] R. Pugliese, F. Gelain, Characterization of elastic, thermo-responsive, self-healable supramolecular hydrogel made of self-assembly peptides and guar gum, Materials & Design 186 (2020) 108370.
[40] I. Tunn, M.J. Harrington, K.G. Blank, Bioinspired histidine–Zn2+ coordination for tuning the mechanical properties of self-healing coiled coil cross-linked hydrogels, Biomimetics 4 (1) (2019) 25.
[41] S. Sharma, A. Kumar, R. Kumar, N.K. Rana, B. Koch, et al., Development of a novel chitosan based biocompatible and self-healing hydrogel for controlled release of hydrophilic drug, International journal of biological macromolecules 116 (2018) 37–44.
[42] J. Qu, X. Zhao, Y. Liang, T. Zhang, P.X. Ma, B. Guo, Antibacterial adhesive injectable hydrogels with rapid self-healing, extensibility and compressibility as wound dressing for joints skin wound healing, Biomaterials 183 (2018) 185–199.
[43] S. Maity, A. Chatterjee, N. Chakraborty, J. Ganguly, A dynamic sugar based bio-inspired, self-healing hydrogel exhibiting ESIPT, New J. Chem. 42 (8) (2018) 5946–5954.
[44] I. Hussain, S.M. Sayed, S. Liu, O. Oderinde, M. Kang, F. Yao, G. Fu, Enhancing the mechanical properties and self-healing efficiency of hydroxyethyl cellulose-based conductive hydrogels via supramolecular interactions, Eur. Polymer J. 105 (2018) 85–94.
[45] S. Liu, M. Kang, K. Li, F. Yao, O. Oderinde, G. Fu, L. Xu, Polysaccharide-templated preparation of mechanically-tough, conductive and self-healing hydrogels, Chem. Eng. J. 334 (2018) 2222–2230.
[46] X. Jiang, X. Yang, B. Yang, L. Zhang, A. Lu, Highly self-healable and injectable cellulose hydrogels via rapid hydrazone linkage for drug delivery and 3D cell culture, Carbohydr. Polym. 273 (2021) 118547.
[47] X. Yang, G. Liu, L. Peng, J. Guo, L. Tao, J. Yuan, C. Chang, Y. Wei, L. Zhang, Highly efficient self-healable and dual responsive cellulose-based hydrogels for controlled release and 3D cell culture, Adv. Funct. Mater. 27 (40) (2017) 1703174.
[48] C. Cheng, X. Zhang, Y. Meng, Z. Zhang, J. Chen, Q. Zhang, Multiresponsive and biocompatible self-healing hydrogel: its facile synthesis in water, characterization and properties, Soft Matter 13 (16) (2017) 3003–3012.
[49] X. Han, X. Meng, Z. Wu, Z. Wu, X. Qi, Dynamic imine bond cross-linked self-healing thermosensitive hydrogels for sustained anticancer therapy via intratumoral injection, Materials Science and Engineering: C 93 (2018) 1064–1072.
[50] T. Cai, S. Huo, T. Wang, W. Sun, Z. Tong, Self-healable tough supramolecular hydrogels crosslinked by poly-cyclodextrin through host-guest interaction, Carbohydrate polymers 193 (2018) 54–61.
[51] L. Teng, Y. Chen, M. Jin, Y. Jia, Y. Wang, L. Ren, Weak hydrogen bonds lead to self-healable and bioadhesive hybrid polymeric hydrogels with mineralization-active functions, Biomacromolecules 19 (6) (2018) 1939–1949.
[52] Z. Wei, J.H. Yang, X.J. Du, F. Xu, M. Zrinyi, Y. Osada, F. Li, Y.M. Chen, Dextran-based self-healing hydrogels formed by reversible diels–alder reaction under physiological conditions, Macromolecular rapid communications 34 (18) (2013) 1464–1470.
[53] M. Hai, Q. Zhang, Z. Li, M. Cheng, A.J. Kuehne, F. Shi, Visualizing polymer diffusion in hydrogel self-healing, Supramolecular Materials 1 (2022) 100009.
[54] M. Mohamadhoseini, Z. Mohamadnia, Alginate-based self-healing hydrogels assembled by dual cross-linking strategy: Fabrication and evaluation of mechanical properties, Int. J. Biol. Macromol. 191 (2021) 139–151.
[55] W.D. Callister, D.G. Rethwisch, Materials science and engineering: an introduction, Vol. 7, Wiley, New York, 2007.
[56] J. Karvinen, J.T. Koivisto, I. Jönkkäri, M. Kellomäki, The production of injectable hydrazone crosslinked gellan gum-hyaluronan-hydrogels with tunable mechanical and physical properties, Journal of the mechanical behavior of biomedical materials 71 (2017) 383–391.
[57] C. Bilici, V. Can, U. Nöchel, M. Behl, A. Lendlein, O. Okay, Melt-processable shape-memory hydrogels with self-healing ability of high mechanical strength, Macromolecules 49 (19) (2016) 7442–7449.
[58] W.J. Zheng, J. Gao, Z. Wei, J. Zhou, Y.M. Chen, Facile fabrication of self-healing carboxymethyl cellulose hydrogels, Eur. Polymer J. 72 (2015) 514–522.
[59] W. Huang, Y. Wang, Y. Chen, Y. Zhao, Q. Zhang, X. Zheng, L. Chen, L. Zhang, Strong and rapidly self-healing hydrogels: Potential hemostatic materials, Advanced healthcare materials 5 (21) (2016) 2813–2822.
[60] W. Li, S. Lu, M. Zhao, X. Lin, M. Zhang, H. Xiao, K. Liu, L. Huang, L. Chen, X. Ouyang, et al., Self-healing cellulose nanocrystals-containing gels via reshuffling of thiuram disulfide bonds, Polymers 10 (12) (2018) 1392.
[61] L. Zeng, M. Song, J. Gu, Z. Xu, B. Xue, Y. Li, Y. Cao, A highly stretchable, tough, fast self-healing hydrogel based on peptide–metal ion coordination, Biomimetics 4 (2) (2019) 36.
[62] M. Yang, L. Wang, Y. Cheng, K. Ma, X. Wei, P. Jia, Y. Gong, Y. Zhang, J. Yang, J. Zhao, Light-and pH-responsive self-healing hydrogel, Journal of materials science 54 (13) (2019) 9983–9994.
[63] S.J. Bidarra, C.C. Barrias, P.L. Granja, Injectable alginate hydrogels for cell delivery in tissue engineering, Acta biomaterialia 10 (4) (2014) 1646–1662.
[64] S. Lü, X. Bai, H. Liu, P. Ning, Z. Wang, C. Gao, B. Ni, M. Liu, An injectable and self-healing hydrogel with covalent cross-linking in vivo for cranial bone repair, Journal of Materials Chemistry B 5 (20) (2017) 3739–3748.

[65] R. Zhang, Y. Tao, Q. Xu, N. Liu, P. Chen, Y. Zhou, Z. Bai, Rheological and ion-conductive properties of injectable and self-healing hydrogels based on xanthan gum and silk fibroin, Int. J. Biol. Macromol. 144 (2020) 473–482.
[66] J.M. Townsend, E.C. Beck, S.H. Gehrke, C.J. Berkland, M.S. Detamore, Flow behavior prior to crosslinking: The need for precursor rheology for placement of hydrogels in medical applications and for 3D bioprinting, Prog. Polym. Sci. 91 (2019) 126–140.
[67] S. Liu, O. Oderinde, I. Hussain, F. Yao, G. Fu, Dual ionic cross-linked double network hydrogel with self-healing, conductive, and force sensitive properties, Polymer 144 (2018) 111–120.
[68] T. Distler, A.R. Boccaccini, 3D printing of electrically conductive hydrogels for tissue engineering and biosensors–a review, Acta Biomater. 101 (2020) 1–13.