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Influence of dialkyne structure on the properties of new click-gels based on hyaluronic acid

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This paper is dedicated to Professor Vittorio Crescenzi who first worked on click-gels of hyaluronan.

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ABSTRACT

Hydrogels have been widely used in tissue engineering as a support for tissue formation and/or to deliver drug locally. A novel procedure for the *in situ* rapid chemical gelation of aqueous solutions of hyaluronan (HA) was employed. HA was functionalised with an arm bearing a terminal azido group (HAAA). When HAAA was mixed with a series of dialkyne reagents of different length, a 1,3-dipolar cycloaddition ("click-chemistry") reaction took place in the presence of catalytic amount of Cu(I) resulting in fast gelation at room temperature. The resulting gels were characterised in terms of degree of cross-linking by ^{1}H HR-MAS NMR. The kinetic of gelation and the determination of elastic moduli as well as the degree of swelling and the controlled release of a model drug, were studied as a function of chemical nature of the dialkyne group, catalyst concentration, HAAA concentration and temperature. All these variables allowed the swelling ratio and the extent of release of a drug, doxorubicin, entrapped within the gel, to be modulated. In all cases the kinetic of release reached the stationary state within 150 h. The height of the plateau was dependent on the overall (chemical and topological) degree of cross-linking.

1. Introduction

In situ gel forming systems have been widely investigated as vehicles for sustained drug delivery and tissue engineering applications. Fabrication of hydrogels *in situ* is an attractive topic for a variety of biomedical applications because it allows one to obtain complex shapes that adhere and match to tissue structures. The physical properties of natural and synthetic hydrogels (high water content and tissue-like elastic properties) are similar to those of the natural extracellular matrix, making these biomaterials appealing for numerous tissue engineering applications (Sakiyama-Elbert et al., 2001; Drury and Mooney, 2003). Furthermore, such hydrogels can be employed as localized drug delivery depots because of their good biocompatibility with hydrophilic, macromolecular drugs such as proteins or oligonucleotides and the ease of regulating drug release kinetic by controlling swelling, cross-link density, and degradation (Chowdhury and Hubbell, 1996; An and Hubbell, 2000; Lu et al., 2000). Another major advantage of polymerisation *in situ* is that liquid hydrogels precursors can be directly delivered to the specific site and cross-linked to form hydrogels *in situ* in a minimally invasive way, for example using laparoscopic devices (Hill-Wast et al., 1994a,b). This process also gives one spatial and temporal control over the conversion of a liquid to a gel, so that complex shapes can be obtained. Polymerization *in vivo* applications, however, are difficult since biological systems require a narrow range of temperature and pH values, as well as the absence of toxic materials such as most monomers and organic solvents. Polysaccharides are potential candidates as scaffold for tissue engineering and as drug carriers, since they are biodegradable and abundant in nature. Among them, hyaluronic acid (HA) based biomaterials and their functional derivatives have a great potential in anticancer drug delivery (Peer and Margalit, 2004; Hyung et al., 2008; Rosenthal et al., 2005) and tissue engineering applications. HA, a linear non-sulfated glycosaminoglycan of copolymer of β-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine is present ubiquitously in the extracelluar matrix (ECM) of virtually all mammalian connective tissues (Fraser et al., 1997) it is a major component of the synovial fluid of joints, vitreous fluid of the eye, and the scaffolding within cartilage and the umbilical cord. HA has been shown to play an important role in lubrication, cell differentiation and cell growth

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(Lee et al., 1994; Trochon et al., 1996). Cellular interactions with HA occur through cell surface receptors (CD44, RHAMM, ICAM-l) and influence processes such as morphogenesis, wound repair, inflammation, and metastasis (Yang et al., 1993; Peach et al., 1993; Entwistle et al., 1996; Toole, 1997).

In order to be able to undergo cross-linking, chemical modification of the native HA is a necessary prerequisite to obtain HA-based functional materials. In general, the most popular way of accomplishing chemical gelation *in vivo* is the photo cross-linking of vynilic derivatives of both synthetic polymers and biopolymers (Mann et al., 2001; Kristi et al., 2002; Nguyen and West, 2002; Park et al., 2003). A possible alternative way is offered by the dipolar cycloaddition between an azido group and an alkyno group, named Huisgen reaction, a type of click-chemistry process (Huisgen, 1989; Kolb et al., 2001; Rostovtsev et al., 2002; Tornøe et al., 2002; Li et al., 2004; Wu et al., 2004; Lee et al., 2006; Malkoch et al., 2006; Ossipov and Hilborn, 2006; Hasegawa et al., 2006), which presents numerous advantages. It occurs at physiological pH and temperature, ensuring a complete biocompatibility during cell encapsulation or incorporation of biologically active molecules. Furthermore, the use of Cu(II) catalyst accelerates the process by factors up to 10^7 thus preserving the inertness of both azides and alkynes toward the vast majority of functional groups that are typical of the biological environment (Rostovter et al., 2001; Wang et al., 2003; Dıaz et al., 2006). This route has been previously exploited by our group to synthesise hydrogels of HA (Crescenzi et al., 2007). With the aim of expanding the range of properties of click HA hydrogels, in the present work we have explored the influence of different dialkyne bridging units on the degree of cross-linking, on the kinetic of gelation and on the rheological properties of the resulting gels.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA) sodium salt, M_η = 200 kDa, and doxorubicin hydrochloride were provided by Fidia Advanced Biopolymers (FAB) srl and Fidia Farmaceutici SpA, Abano Terme, Padua, Italy respectively.

11-Azido-3,6,9-trioxaundecan-1-amine (technical, ≥90%) was purchased by Fluka, propargylamine (98.0%), 1,4-diethynylbenzene (96%), 1,6-heptadiyne (97%) and 1,8-nonadiyne (98%) were purchased by Aldrich. All other chemicals were reagent grade and used without further purification.

2.2. Synthesis of click-gels

The HA-azido derivative (HAAA) was prepared as previously reported (Crescenzi et al., 2007). Specifically, 1 g of HA was dissolved in 40 mL of MES [2-(*N*-morpholino)-ethanesulfonic acid] buffer (50 mM, pH 4); 1.44 g of EDC·HCl [*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodimide hydrochloride], 0.86 g of NHS (*N*-hydroxysuccinimide) and 2.75 mL of 11-azido-3,6,9-trioxaundecan-1-amine were added and the reaction was performed at room temperature under stirring for 24 h. The solution was dialyzed (cut-off = 12 kDa) against aqueous NaCl saturated solution for 1 day and then against distilled water for 5 days. Finally it was freeze-dried to recover the HAAA derivative.

The new click-gels were obtained using the azido derivative of HA, a dialkyne as cross-linker agent, and the system $CuSO_4$/ascorbic acid as catalyst. Briefly, in a typical reaction, 125 mg of HAAA were dissolved in 2 mL of distilled water and an adequate amount of the dialkyne dissolved in 250 μL of DMSO was added, specifically, 3.75 mg of 1,4-diethynylbenzene (D0), 3.37 μL of 1,6-heptadiyne (D1) or 4.37 μL of 1,8-nonadiyne (D2). Then, 12.5 mg of $CuSO_4{\cdot}5H_2O$ dissolved in 125 μL of water and 12.5 mg of ascorbic acid (Asc.) dissolved in 125 μL of water were added. The solution was stirred for few seconds until the formation of the gel. The dialkyne based click-gels were designated with the acronyms HAAA-D0 (Scheme 1a), HAAA-D1 (Scheme 1b) and HAAA-D2 (Scheme 1c), respectively.

The hydrogels were then dialyzed against EDTA 10 mM solution for 24 h and, then against distilled water until the nominal conductivity of water was reached.

2.3. Rheological measurements

Rheological measurements on the gelling solutions were performed on a Bohlin CS stress-controlled rheometer using a coaxial cylinder measuring system (C14).

Preliminary stress-sweep experiments on the completely formed gels were carried out to select a strain value in the range of linear viscoelasticity (target strain = 0.5 Pa). Storage and loss moduli, G' and G'', were measured as a function of the applied frequency in the 0.01–10 Hz range.

HAAA was dissolved in 1180 μL of distilled water. Dialkyne (D0, D1 or D2) solubilised in 20 μL of DMSO, 10 μL of $CuSO_4$ solution (0.5% w/v) and 10 μL of ascorbic acid solution (0.5% w/v) were added in sequence to the HAAA water solution. Then the mixture was vigorously stirred and quickly transferred into the rheometer at the fixed temperature.

2.4. UV–vis spectroscopy

Absorbance spectra were collected using an HP 8452A diode array spectrophotometer at 37 °C using 0.1, 0.5, and 1 cm quartz cells.

2.5. Equilibrium swelling of click-gels

The swelling capacity of the hydrogels, S_w, is defined as the ratio between the weight of swollen gels (W_s) after extensive dialysis against distilled water and the weight of the dry networks (W_d):

$$S_w = \frac{W_s}{W_d}$$

Samples of the cross-linked derivatives HAAA-D0, HAAA-D1 and HAAA-D2 were swollen in distilled water at 25 °C until constant weight.

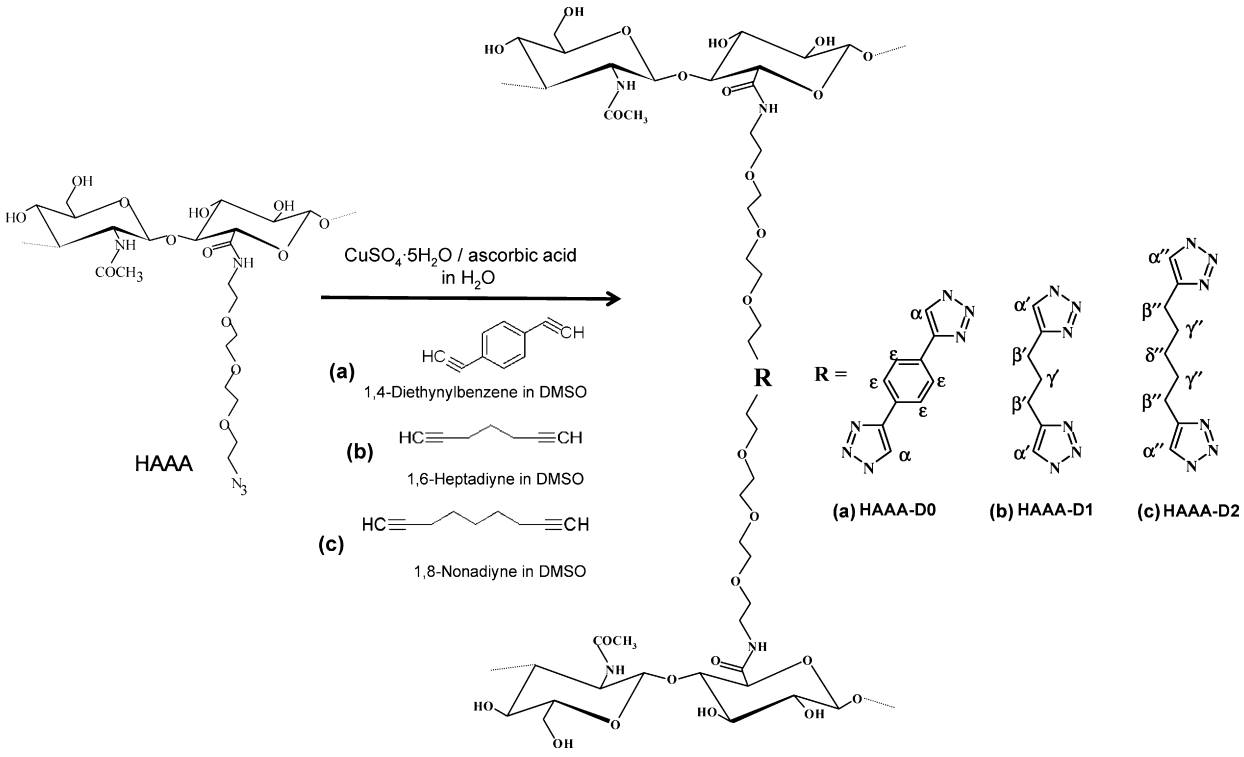
2.6. NMR spectroscopy

About 4 mg of HAAA were solubilized in 700 μL of D_2O. ^1H and ^{13}C NMR experiments were performed at 27 °C on a Bruker AVANCE AQS 600 spectrometer operating at 600.13 and 150.95 MHz respectively, and equipped with a Bruker multinuclear, z-gradient probehead. In all ^1H spectra, a soft pre-saturation of the HOD residual signal was applied (Guerènon et al., 1991).

^1H and ^{13}C assignments were obtained using ^1H–^1H COSY and ^1H–^{13}C HSQC experiments with a z-gradient coherence selection. All 2D experiments were carried out using 1024 data points in the f2 dimension and 512 data points in the f1 dimension. The HSQC experiment was performed using a coupling constant of 150 Hz and processed in the phase sensitive mode (TPPI) with 512 × 512 data points.

^1H and ^{13}C chemical shifts were reported in ppm with respect to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as an internal standard.

^1H HR-MAS experiments were performed on a Bruker AVANCE 600 spectrometer operating at 600.13 MHz, using a Bruker HR-MAS probe with an external lock. Samples were loaded in 4 mm ZrO_2



Scheme 1. Schematic representation of the HAAA-D0 (a), HAAA-D1 (b) and HAAA-D2 (c) click-gels formation.

cylindrical rotors and hydrated with a phosphate-buffered D_2O solution (100 mM, pD = 7). The spin-rate was 4 kHz.

^{1}H HR-MAS NMR spectra were recorded using 32K data points and collecting 128 scans. In all spectra a soft pre-saturation of the HOD residual signal was applied. The $\pi/2$ pulse width was 4 μs, and the recycle delay was 2 s. Before applying the Fourier transformation, data were zero filled to 32K points and apodized using an exponential line broadening of 1 Hz.

2.7. Synthesis of HAAA-D click-gels in doxorubicin aqueous solution

Doxorubicin hydrochloride, a powerful anti-tumour drug, was physically entrapped in the hydrogels during the cross-linking reaction. Doxorubicin and HAAA were dissolved in 160 μL of distilled water. Dialkyne (D0, D1 or D2) solubilized in 20 μL of DMSO, and in sequence 10 μL of $CuSO_4$ solution (0.5% w/v) and 10 μL of ascorbic acid solution (0.5% w/v) were added to the doxorubicin/HAAA/water solution, and the mixture was vigorously stirred until the formation of dark red gels.

2.8. Doxorubicin release from click-gels

The doxorubicin-containing click-gels were placed in 10 mL of distilled water at 37 °C, and the release of the drug was monitored by UV–Vis spectrophotometry, measuring the increase of absorbance *vs* time at λ = 486 nm. The doxorubicin concentration in the solution was extrapolated from the calibration curve obtained with doxorubicin standard solutions from 0.01 to 0.2 mM.

3. Results and discussion

3.1. NMR in solution

The HSQC map of HAAA derivative is reported in Fig. 1. Besides the cross peaks due to the HA moiety, other cross peaks due to the trioxaundecan-azido chain are also observed. In particular, methylenes 8 and 1 show well distinguishable cross peaks centred at 3.506 and 52.29 ppm, and 3.300 and 41.61 ppm, see also Table 1. 2D COSY allowed the assignment of methylenes 2 and 7 through the cross peaks with methylenes 1 and 8, respectively (data not shown). All resonances of methylenes 3, 4, 5 and 6 fully overlap giving rise to a cross peak centred at 3.713 and 71.58 ppm in the HSQC map, see Fig. 1 and Table 1.

3.2. ^{1}H HR-MAS analysis

^{1}H HR-MAS spectra of HAAA-D0, HAAA-D1 and HAAA-D2 hydrogels are reported in Fig. 2a, b, and c, respectively. Beside the signals

Table 1
^{1}H and ^{13}C NMR chemical shifts of HAAA derivative.

HAAA	^{1}H (ppm)	^{13}C (ppm)
1a	4.556	102.75
2a	3.839	56.28
3a	3.725	84.49
4a	3.529	70.64
5a	3.483	77.20
6a	3.912	62.61
6a′	3.578	62.61
8a	2.005	24.40
1b	4.499	104.90
2b	3.350	74.39
3b	3.600	75.65
4b	3.766	82.37
5b	3.829	76.99
1	3.300	41.61
2	3.590	71.79
3	3.713	7.58
4	3.713	7.58
5	3.713	7.58
6	3.713	7.58
7	3.629	70.97
8	3.506	52.29

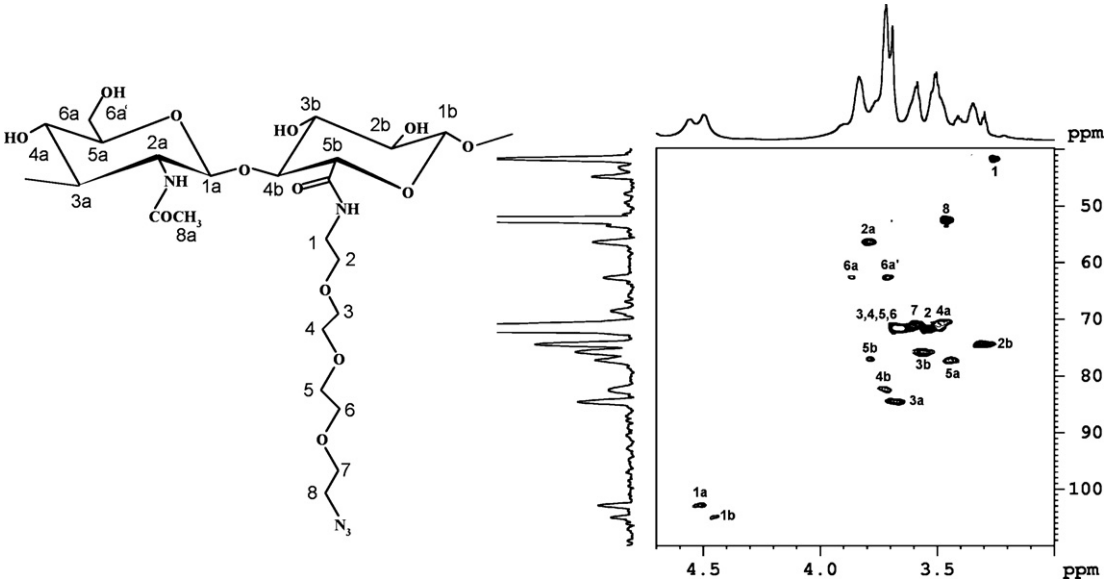


Fig. 1. ^{1}H–^{13}C HSQC map of HAAA in D_2O at 27 °C, along with the assignment of the resonances. ^{1}H and ^{13}C spectra are reported as projections in the f2 dimension and f1 dimension, respectively. The scheme of HAAA derivative along with the labelling used for the assignment is also reported.

of HA moiety, in the spectrum of HAAA-D1 hydrogel, see Fig. 2b, the resonances due to the α′ protons of the triazolic rings and to the resonances of β′ methylenes are clearly observed at 7.83 and 2.73 ppm, respectively. It is worth noting that the integral of the methine groups α′ and the integral of methylene groups β′ are in the correct ratio, i.e. 2:4, respectively. The resonance of methylene γ′ is overlapped with the resonance of the HA acetyl group. Also the resonances due to protons of the chemical bridge between HA chains are completely hidden by the intense resonances of the HA moiety between 3 and 4 ppm. A semi-quantitative evaluation of the hydrogel cross-linking degree was obtained by integrating the resonances of the triazolic rings protons at 7.83 ppm with respect to the resonance of OCH_3 group of HA at 2.00 ppm. Note that the integral of methylene γ′, $I(\gamma')$, must be subtracted from the integral of OCH_3, $I(A)$. We assume that $I(\gamma') = I(\alpha')$, where $I(\alpha')$ is the integral of the two protons due to the two triazolic rings formed during the cross-linking reaction. Accordingly, the cross-linking degree (CLD) turns out to be:

$$\mathrm{CLD(HAAA\text{-}D1)} = \frac{I(\alpha')/2}{[I(A) - I(\gamma')]/3} \times 100 = 22\%$$

In the spectrum of HAAA-D2 hydrogel, see Fig. 2c, protons α″ of the triazolic rings are observed at 7.78 ppm, methylenes β″ at 2.67 ppm, methylenes γ″ at 1.65 ppm and, finally, methylene δ″ at 1.30 ppm. The integral of the methine groups α″, the integrals of methylene groups β′, γ″ and δ″ are in the correct ratio, i.e. 2:4:4:2, respectively.

The degree of cross-linking can be calculated by integrating the resonance of OCH_3 of HA with respect to the resonance of protons α″, $I(\alpha'')$:

$$\mathrm{CLD(HAAA\text{-}D2)} = \frac{I(\alpha'')/2}{I(A)/3} \times 100 = 28\%$$

The spectrum of HAAA-D0 hydrogel, see Fig. 2a, clearly shows the α proton resonances of the triazolic rings at 8.34 ppm and the protons ε of the benzenic ring at 7.80 ppm. Note that these resonances are very broad with respect to the resonances of the HA moiety, possibly due to the stiffness of the planar system formed by the three aromatic rings. The different mobility of these protons and consequently their different line-broadening impairs a quantitative evaluation of the degree of cross-linking.

3.3. Rheology and swelling data

The kinetic of gelation as a function of different compositional and physical parameters was monitored by following the time evolution of the storage modulus (G'). In the first instance, the influence of the different cross-linking units on the kinetic of gelation and the final mechanical properties of the gels were determined at 30 °C, all the other physical variables being constant.

Fig. 3 shows that the storage modulus rises faster in the case of HAAA-D2 gel compared to HAAA-D1 and HAAA-D0 gels and that the resulting plateau moduli, G'_p (Table 2), follow the same order. This result is in agreement with the cross-linking densities determined by NMR for HAAA-D1 and HAAA-D2 gels. Evidently, the cross-linking units which lead to more flexible bridges, still leave in the incipient network enough freedom to allow further cross-linking to progress. Even though no quantitative data regarding the degree of cross-linking is available, in the case of HAAA-D0 gel, the comparison of the profiles reported in Fig. 3 and the values of the elastic gels moduli reported in Table 2 support the hypothesis that this gel is characterised by the lowest degree of cross-linking.

Table 2
Influence on the gels plateau elastic moduli G'_p and swelling values (S_w) of the dialkyne cross-linking units of Scheme 1 ([HAAA] = 5% (w/v), [$CuSO_4$] = 0.5% (w/v), [Asc.] = 0.5% (w/v)).

	G'_p (Pa)	S_w
HAAA-D0	2230	324
HAAA-D1	4640	250
HAAA-D2	5500	169
[HAAA] (%, w/v)		
2.0	1520	551
5.0	4640	250
10.0	6700	161
[$CuSO_4$] (%, w/v)		
0.2	1490	560
0.5	1520	551
1.0	1560	543

Influence of the concentration of HAAA in HAAA-D1 hydrogel ([$CuSO_4 \cdot 5H_2O$] = 0.5% (w/v), [Asc.] = 0.5% (w/v)) and catalyst concentration [$CuSO_4 \cdot 5H_2O$] ([Asc.] = 0.5% (w/v), [HAAA] = 2% (w/v)) on G'_p and S_w of the click-gels. T = 30 °C.

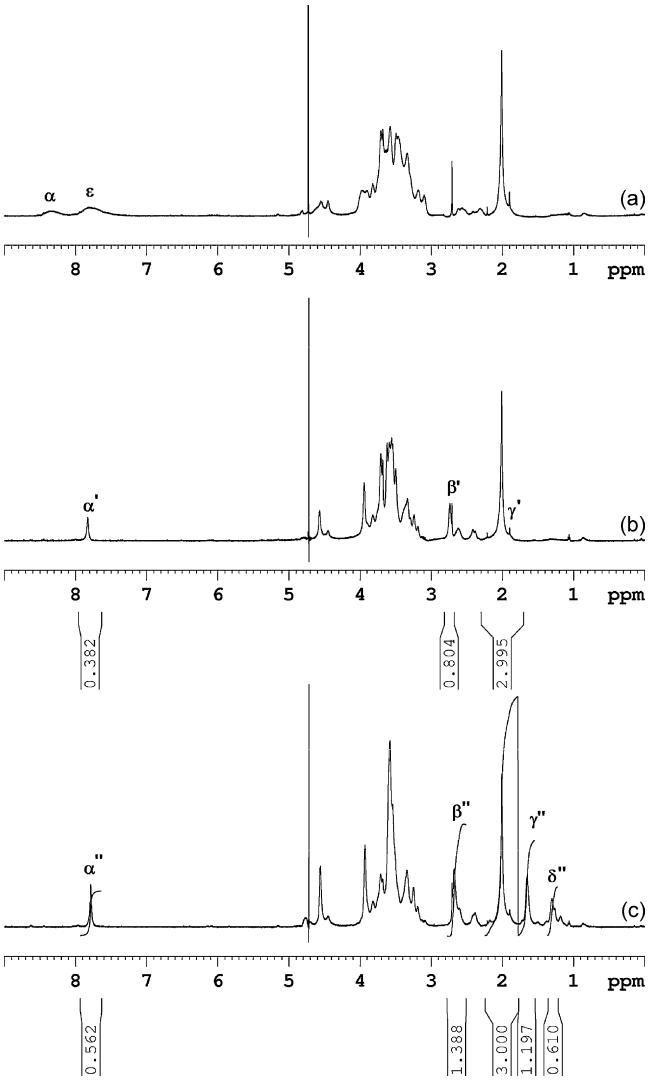


Fig. 2. 600.13 MHz ^{1}H HR-MAS spectra of HAAA-D0 (a), HAAA-D1 (b) and HAAA-D2 (c) click-gels hydrated with phosphate-buffered D_2O solution (100 mM, pD = 7) at T = 27 °C. The labels are used in accordance to Scheme 1a–c.

The degree of equilibrium swelling (S_w) of a polymeric hydrogel is known to be inversely proportional to hydrogel mechanical strength (Anseth et al., 1996; Kavanagh and Ross-Murphy, 1998). The swelling of polymer chains reaches the equilibrium value at a point where the swelling (osmotic) force is balanced by the elastic restoring (entropic) force acting in the opposite direction. From the results of Table 2, the degree of swelling is shown to be an inverse function of the bridging unit length. These results confirm that HAAA-D0 has the lowest degree of cross-linking among the three kinds of click-gels.

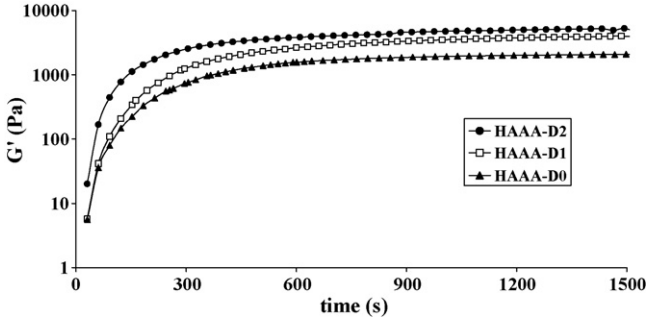


Fig. 3. Evolution of the storage modulus (G') at 30 °C of gelling systems made of [HAAA] = 5% (w/v) and one of the alkyne bridging units of Scheme 1; [$CuSO_4$] = 0.5% (w/v); [Asc.] = 0.5% (w/v).

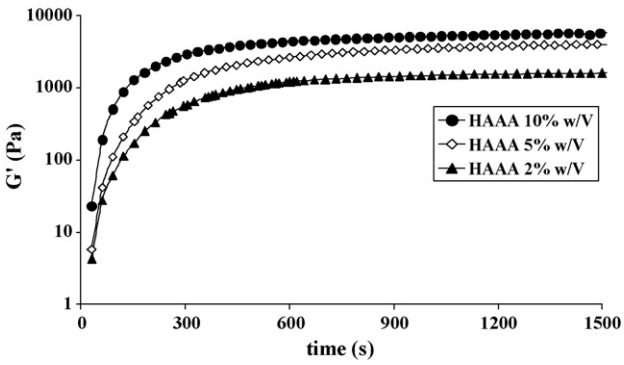


Fig. 4. Evolution of the storage modulus (G') at 30 °C of HAAA-D1 hydrogels obtained with different polymer concentration ([HAAA] = 2, 5, 10% (w/v); [$CuSO_4$] = 0.5% (w/v); [Asc.] = 0.5% (w/v)).

Further, the influence of compositional parameters, such as HAAA and catalyst ($CuSO_4$) concentrations, as well as a physical parameter, such as temperature, on the kinetic of gelation, on the storage modulus G' and on the swelling value S_w was investigated.

The effect of [HAAA] on G' evolution of the HAAA-D1 gel is displayed in Fig. 4 and the G'_p values are reported in Table 2. Both the gel strength and the kinetic of gelation increase with [HAAA]. Since the degree of coils interpenetration (chain entanglement) increases with polymer concentration, when chemical cross-linking starts, the structure of the solution is locked-in giving rise to a gel with an overall more constrained network structure. The gel elastic modulus is sensitive to the density of junctions (physical and chemical) among polymeric chains, as a consequence there is a direct proportionality between G' and [HAAA].

For the same reason swelling ratios which are a measure of networks elasticity follow an inverse relationship with [HAAA] (Table 2).

As expected [$CuSO_4$] influences only the kinetic of gelation and not the G'_p value of HAAA-D1 gel (Fig. 5 and Table 2). As it can be noted, the main effect is obtained in the range of [$CuSO_4$] = 0.2–0.5% (w/v). Beyond 0.5% (w/v) the increment in terms of reaction rate is not particularly significant. This is an important information since in biomedical applications in which gelation *in situ* is involved, a fast gelation kinetic is required but at the same time the level of copper should be kept to very low level in order to avoid toxicity effects.

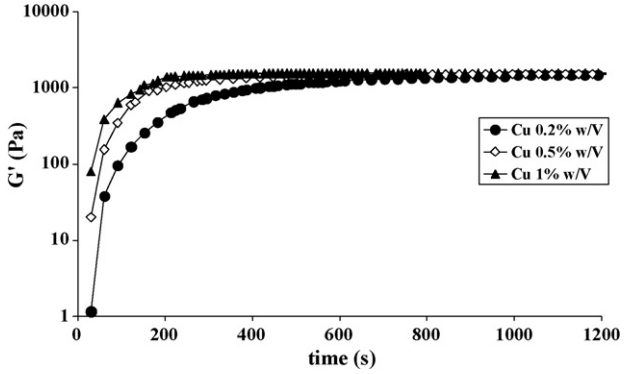


Fig. 5. Evolution of the storage modulus (G') at 30 °C of HAAA-D1 hydrogels obtained with different concentrations of $CuSO_4$ ([HAAA] = 2% (w/v); [$CuSO_4$] = 0.2, 0.5, 1% (w/v); [Asc.] = 0.5% (w/v)).

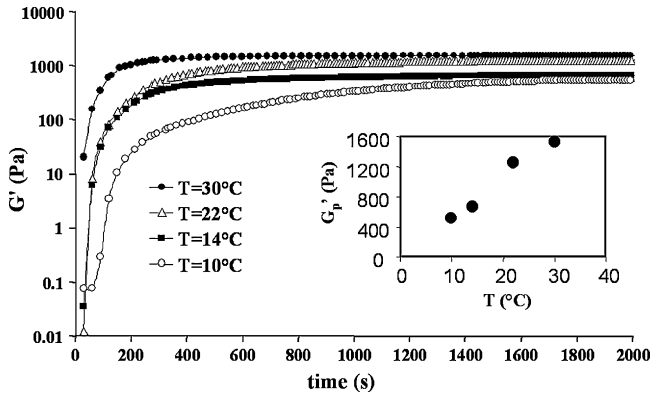


Fig. 6. Evolution of the storage modulus (G') of HAAA-D1 hydrogels ([HAAA] = 2% (w/v); [$CuSO_4$] = 0.5% (w/v); [Asc.] = 0.5% (w/v)) at different temperature (T = 10, 14, 22, 30 °C). In the insert the temperature dependence of the plateau elastic modulus (G'_p) is shown.

In line with rheological results (G'_p constant), the catalyst concentration does not have any influence on the swelling ratio (Table 2).

The effect of temperature increase resolves in a corresponding increase in the kinetic of gelation and also in the plateau value of G'_p of HAAA-D1 gel (Fig. 6 and the insert therein shown). It is evident that enhancing the thermal agitation of the macromolecular chains favours the frequency of contacts among dangling side chains resulting in an increase in cross-linking density.

3.4. Doxorubicin release from click-gels

Since the new click-gels presented in this work may be formed *in situ*, they can be used as devices for a post-surgical oncology treatment to fill the gap left in a human organ or tissue after a surgical treatment of a solid cancer and, at the same time, to release anti-tumoral drugs. To mimic this process, doxorubicin, a potent anticancer agent, was entrapped into a click-gel during its formation (Crescenzi et al., 2007), subsequently monitoring the drug release in physiological conditions.

Generally, all three types of click-gels exhibited a stepwise kinetic of release (Fig. 7). Within the first 4 h, the mean cumulative release was approximately 35% for HAAA-D0, 12% HAAA-D1 and 10% for HAAA-D2. After about 50 h, the kinetic of drug release reached a pseudo stationary state. The percentage of doxorubicin

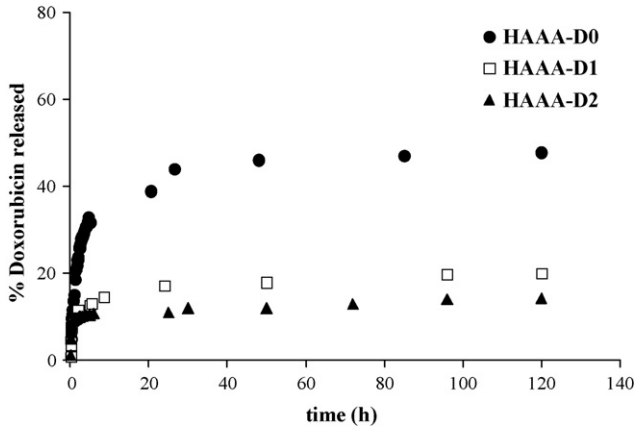


Fig. 7. Doxorubicin release in distilled water from click-gels obtained by using the three different dialkyne units of Scheme 1. [Doxo] = 0.4 mM, ([HAAA] = 5% (w/v); [$CuSO_4$] = 0.5% (w/v); [Asc.] = 0.5% (w/v); T = 37 °C. The error associated with the experimental points lies within 10%.

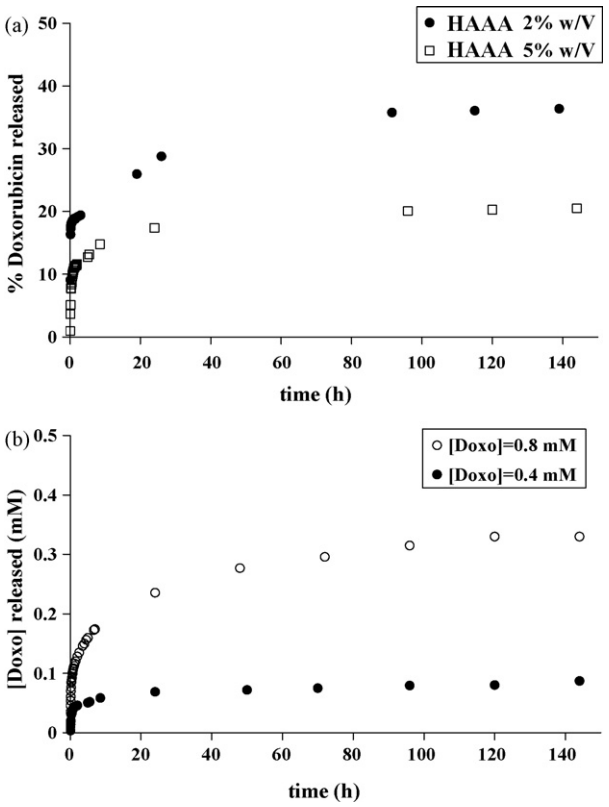


Fig. 8. Doxorubin release in distilled water from HAAA-D1 click-gels upon variation of (a) [HAAA] or (b) [Doxo] at T = 37 °C. The error associated with the experimental points lies within 10%.

released from the three gels follow the order: HAAA-D0 > HAAA-D1 > HAAA-D2. The higher release rate of HAAA-D0 is most likely due to the larger mesh size characterising this network which swells to a higher extent (Table 2) and is thus more permeable to doxorubicin. This behaviour is in turn a consequence of the cross-linking density which presumably is the lowest among the three kinds of click-gels. In general, the results indicate a slow drug release: in fact, after 10 days approximately 47% of doxorubicin was released from the gel HAAA-D0 and approximately 20 and 14% from HAAA-D1 and HAAA-D2, respectively. This is probably due to two combined effects: the ionic interactions between amino groups of the drug molecules and the carboxylate groups of HA and the ϑ–ϑ stacking interactions between the heteroaromatic 1,4-triazole ring and the aromatic moiety of doxorubicin. We can, anyway, suppose that *in vivo* gel degradation by hyaluronidase could accelerate the kinetic of release of the loaded drug. In *in vivo* applications it is to be expected that the permanence of the gel characterised by the higher degree of cross-linking (namely, HAAA-D2 and HAAA-D1) will be longer, thus providing a longer-term release of doxorubicin. This outcome is a combination of a slower kinetic of release (Fig. 7), and a higher resistance towards degradation operated by hyaluronidase (Vermonden et al., 2008). Thus, after the initial burst, the more heavily cross-linked gels should guarantee a prolonged release of doxorubicin.

Measurements were also carried out on two HAAA-D1 hydrogels obtained using different initial concentrations of HAAA (2 and 5% w/v, respectively) and the same concentration of doxorubicin ([Doxo] = 0.4 mM), or using HAAA-D1 gels at the same concentration (5% w/v) and different amounts of doxorubicin ([Doxo] = 0.4 or 0.8 mM), see Fig. 8a and b.

As expected, the reduction of the initial HAAA concentration gives rise to a gel characterised by a weaker network structure

as evidenced by rheological measurements (Table 2). As a consequence, both the kinetic of release and the cumulative amount of released drug follow an inverse relationship with [HAAA]. Analogously, loading the gel HAAA-D1 with higher amounts of drug lead to an enhancement in both the rate and the amount of drug released. In conclusion, it is possible to modulate the anticancer release, in the target tissue for loco-regional applications, simply by varying the polymer or drug concentration in the hydrogel.

4. Conclusion

New HA-based hydrogels were obtained by means of a "click-chemistry reaction", i.e. the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide derivative of HA and a series of three dialkynes of different length. Gels were characterised by ^{1}H HR-MAS NMR which allowed the degree of cross-linking (CLD) to be established, showing an inverse relationship between the CLD and the flexibility of the bridging unit: longer spacer arm gives rise to higher cross-linking densities. Both the kinetic of gelation and the determination of the elastic moduli by rheology as well as the swelling ratios were in agreement with such a finding.

These click-gels are highly tuneable and can be employed to identify the design parameters that optimise the delivery *in situ* of a specific drug, determining the rate and extent of the release.

We are currently studying the use of injectable gel based on hyaluronic click-gels in the repairment of infartuated hearth using stem cells and in this context the evaluation of gel cytotoxicity is being pursued.

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