Novel Hydrogels via Click Chemistry: Synthesis and Potential Biomedical Applications

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A novel procedure for the in situ rapid chemical gelation of aqueous solutions of hyaluronan has been employed. In brief, water-soluble polysaccharide derivatives bearing side chains endowed with either azide or alkyne terminal functionality have been prepared. When the latter two types of derivatives are mixed together in aqueous solution they give rise to a 1,3-dipolar cycloaddition reaction resulting in fast gelation (in the presence of catalytic amounts of Cu(I)) at room temperature. Gel formation has been characterized rheologically and could also be followed qualitatively by means of IR spectroscopy. The resulting gels have been studied in terms of swelling properties and, in particular, NMR spectral features. Carrying out the gelation process in aqueous solutions of benzidamine and doxorubicin, respectively, the polysaccharide networks acted as drug reservoirs. The doxorubicin release resulted in well controllable acting upon the gels degree of cross-linking. Finally, formation of the click-gels using aqueous suspensions of *Saccharomices cerevisiae* yeast cells allowed the obtainment of scaffolds inside which cells were homogeneously distributed and smoothly adhered to the inner pores surfaces, according to SEM analysis. After 24 h about 60% of the entrapped cells exhibited proliferating activity. Click-gels prepared as detailed herein do have a number of positive features that make them, in perspective, materials of choice for drug release and tissue engineering manipulations.

Introduction

Scaffolds, including hydrogel networks, for tissue engineering applications are typically prefabricated, employing a number of different technologies,[1−3] and then seeded with cells by placing them in contact with aqueous suspensions of cells that slowly diffuse inside and attach to the inner walls of scaffolds' cavities. While cells can easily migrate into the outermost portions of the scaffolds, the cell distributions may not be uniform throughout the scaffold due to random motility and limitations in the diffusion of nutrients.

One solution consists of having the capability to simultaneously add cells to the scaffold during the fabrication process in order to have better control over the cellular distribution provided that the fabrication process does not involve heat or toxic chemicals that would jeopardize cells survival.

In this direction we report here on a novel procedure for the fabrication of hydrogels suitable for tissue engineering applications and which appears to comply with the above stringent requirements based on biocompatible polymeric derivatives of hyaluronan.[4−9] In brief, these new derivatives bear azido and alkyne groups linked by short arms to the polysaccharide main chains. Once the two types of derivatives are mixed in aqueous media they give rise to dipolar cycloaddition, Huisgen reaction,[10−22] a type of click-chemistry process. The latter, as is well known, is defined as a particular chemical reaction characterized by important criteria of simple experimental conditions, rapidity, high yields, stereospecificity, simple product recovery, absence of toxic byproducts, and high thermodynamic driving force (more than 20 kcal/mol).

Such cycloadditions can be carried out in aqueous cells suspensions without harm for the cells inasmuch as azido and alkyne groups can react exclusively with each other irrespective of different functional groups present in the mixture.

Likewise, if controlled drug delivery is being pursued, the latter procedure allows for a fast and homogeneous entrapment of drugs and biomolecules, including biomacromolecules, inside the hydrogels during their formation.

The synthesis and structural characterization of the novel gels, named here "click-gels",[23] as well as preliminary data on their use as cells scaffolds and drug release materials are herein described.

Materials and Methods

Materials. Hyaluronic acid sodium salt (HA), $M_n = 200$ kDa, was provided by Fidia Advanced Biopolymers (FAB) srl, Abano Terme, Padua, Italy. Doxorubicin hydrochloride and benzidamine hydrochloride were supplied by Fidia Farmaceutici SpA., Abano Terme, Padua, Italy. *Saccharomyces cerevisiae* cells were kindly supplied by Prof. C. Palleschi (Department of Developmental and Cell Biology, University of Rome Sapienza, Rome, Italy).

11-Azido-3,6,9-trioxaundecan-1-amine (technical, ≥90%) was a Fluka (Milan, Italy) product, and propargylamine (98.0%) was an Aldrich (Milan, Italy) sample.

All other chemicals were reagent grade and used without further purification.

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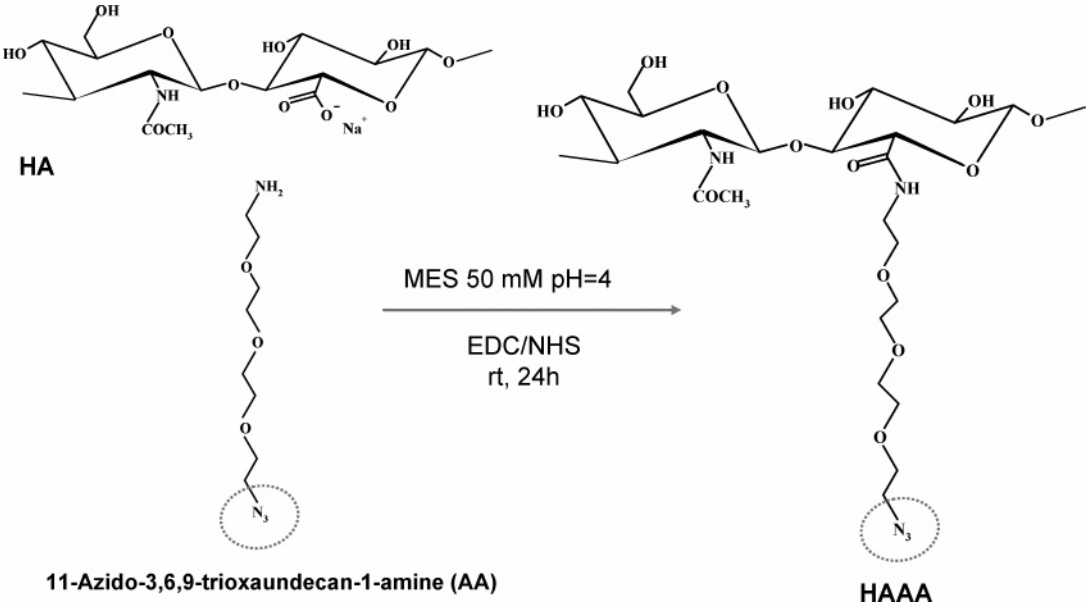
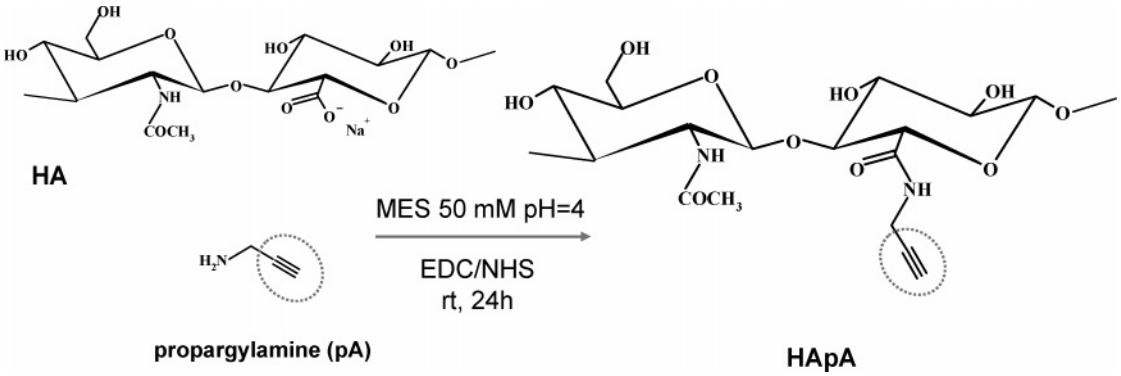
Scheme 1. Schematic Representation of the Reaction between HA and AA



Scheme 2. Schematic Representation of the Reaction between HA and pA



Methods. *Synthesis of Hydrosoluble HA Derivatives Bearing Azide Groups (HAAA).* A 200 mg amount of HA was dissolved in 8 mL of MES [2-(*N*-morpholino)-ethanesulfonic acid buffer (50 mM, pH = 4). In two synthesis, 478 or 143 mg of EDC·HCl [*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride)], 287 or 86 mg of NHS (*N*-hydroxysuccinimide), and 770 or 330 μL of 11-azido-3,6,9-trioxaundecan-1-amine, respectively, were added to the HA solution. In both cases an excess of reagents with respect to HA has been employed in view of the limited yield of amidation processes in aqueous media.[24−25] The reaction was performed at room temperature under stirring for 24 h. The solutions were dialyzed (cutoff = 12 kDa) against aqueous saturated NaCl for 1 day and then against distilled water for 5 days. Finally, the solutions were freeze-dried to recover the HAAA derivatives (see Scheme 1): the latter are indicated as HAAA-1 and HAAA-2.

Synthesis of HA−Propargylamide (HApA) Hydrosoluble Derivatives. HA (≈200 mg) was dissolved in 8 mL of MES [2-(*N*-morpholino)-ethanesulfonic acid buffer (50 mM, pH = 4). In two synthesis, 478 or 143 mg of EDC·HCl [*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride)], 287 or 86 mg of NHS (*N*-hydroxysuccinimide), and 243 or 104 μL of propargylamine, respectively, were added to the HA solution. In both cases an excess of reagents with respect to HA has been employed. The reaction was performed at room temperature under stirring for 24 h. The solutions were dialyzed (cutoff = 12 kDa) against a saturated NaCl solution for 1 day and then against distilled water for 5 days. Finally, the HApA derivatives (see Scheme 2) were recovered by freeze-drying. The latter samples are indicated as HApA-1 and HApA-2.

Synthesis of HA-Based Click-Gels. A 60 mg amount of HAAA-1 and 60 mg of HApA-1 were dissolved in 2.4 mL of distilled water. Then, 1 mL of CuCl aqueous solution (1% w/v) was added, and the mixture was vigorously stirred. After a few minutes a stable gel was formed (click-gel: Scheme 3). The gel was left at rest overnight and then dialyzed against EDTA solution (10 mM) for 12 h and finally against distilled water until constant weight. The same procedure was followed starting from HAAA-2 and HApA-2 samples.

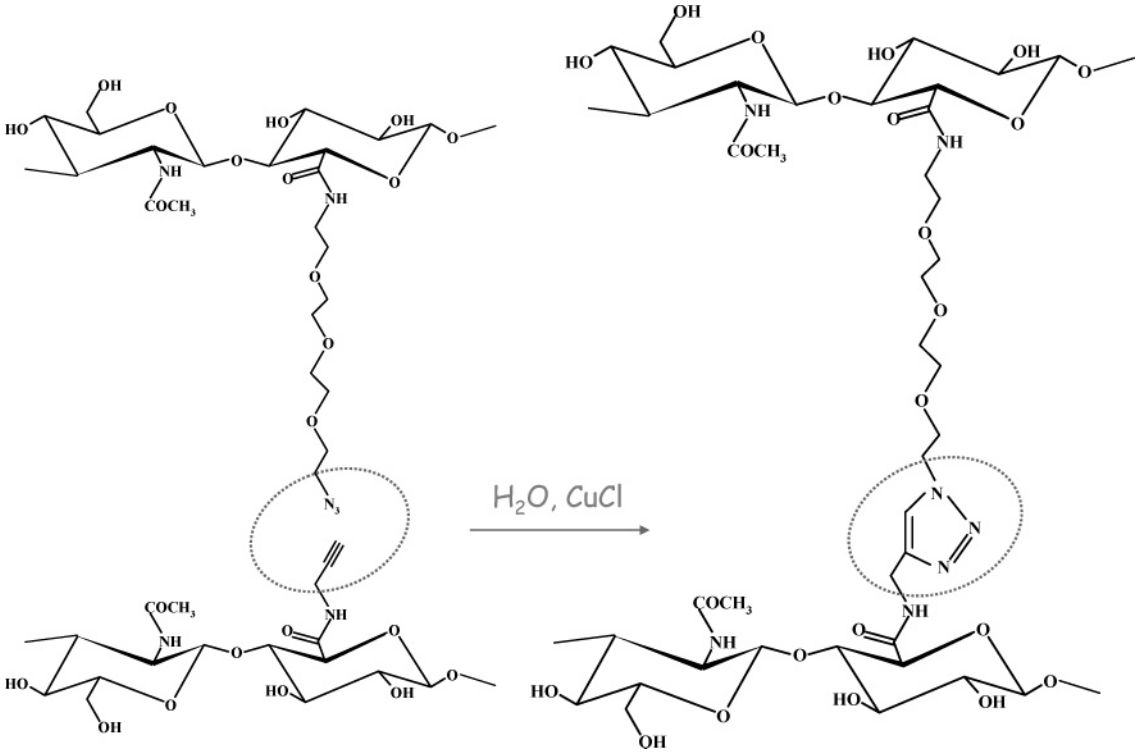
Click-gels having two different stoichiometric cross-linking degrees were thus obtained. The latter are indicated as CG-1 and CG-2.

Rheological Measurements. The kinetics of formation of the click-gels CG-1 and CG-2 was followed by rheological measurements performed on a Bohlin CS Rheometer using a coaxial cylinders geometry (C14) in oscillation mode. A 0.8 mL amount of CuCl aqueous solution (1% w/v) was added to the solution of HAAA-1 (50 mg) and HApA-1 (50 mg) derivatives in 2 mL of distilled water; then the final solution was quickly transferred into the rheometer at 30 °C. An identical procedure was followed using the HAAA-2 and HApA-2 samples.

The gels were subjected to oscillation at a frequency of 0.1 Hz; a frequency sweep was performed from 0.01 to 1 Hz.

Infrared Spectroscopy. IR spectra were recorded in ATR mode with a Shimadzu 8300 FT-IR spectrometer equipped with an ATR Golden Gate accessory (Specac Inc.) collecting 32 scans in the 500−4000 cm^{-1}

Scheme 3. Schematic Representation of the Formation of HA-Based Click-Gels



range with a resolution of 4 cm^{-1}. Spectra were normalized with respect to the HA−glycosidic stretching peak at 1151 cm^{-1}.

UV−Vis Spectroscopy. Absorbance spectra were collected with a HP 8452A diode array spectrophotometer at 37 °C using 0.1, 0.5, and 1 cm quartz cells.

Swelling Measurements. 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): $S_w = W_s/W_d$.

Samples of the cross-linked derivatives CG-1 and CG-2 were swollen in distilled water at 25 °C until constant weight.

^{1}H HR-MAS NMR. ^{1}H 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. The samples (CG-1 and CG-2) were loaded in 4 mm ZrO_2 cylindrical rotors and hydrated with phosphate-buffered D_2O solution 100 mM, pD = 7, and spun at a spinning rate of 4 kHz.

^{1}H HR-MAS NMR spectra were recorded accumulating 128 scans with 32K data points and using a spectral width of 10 ppm. In all spectra a soft presaturation of the HOD residual signal was applied. A 4 μs 90° excitation pulse was used with a relaxation delay of 2 s. Prior to Fourier transformation the data were zero filled to 32K points and apodized using an exponential line broadening of 1 Hz.

Synthesis of HA-Based Click-Gels in Benzidamine Aqueous Solution. A 6.9 mg amount of benzidamine hydrochloride was dissolved in 200 μL of distilled water. A 5 mg amount of HAAA-1 and 50 mg of HApA-1 were dissolved in this solution. Then, 83 μL of CuCl aqueous solution 1% w/v was added, and the solution was vigorously stirred. After a few minutes the gel CG-1 was formed. The gel was left at rest before benzidamine release measurements.

An identical procedure was followed using the HAAA-2 and HApA-2 samples obtaining the benzidamine-containing click-gel CG-2.

Synthesis of HA-Based Click-Gels in Doxorubicin Aqueous Solution. A 5 mg amount of HAAA-1 and 5 mg of HApA-1 were dissolved in 200 μL of an aqueous solution containing 2.9 mg of doxorubicin hydrochloride until complete solubilization. Then, 83 μL of aqueous 1% w/v CuCl was added, and the mixture was vigorously stirred. After a few minutes a dark red gel CG-1 was formed, and it has been used for doxorubicin release measurements.

An identical procedure was followed using the HAAA-2 and HApA-2 samples, obtaining the doxorubicin-containing click-gel CG-2.

Benzidamine Release from HA-Based Click-Gels. The click-gels containing benzidamine were placed in 10 mL of distilled water at 37 °C, and benzidamine release was monitored by UV−vis spectrophotometry, measuring the increase of absorbance vs time at $\lambda = 308$ nm. The benzidamine concentration was extrapolated from the calibration line obtained with benzidamine solutions from 0.1 to 2.0 mM (see Supporting Information).

Doxorubicin Release from HA-Based Click-Gels. The click-gels containing doxorubicin were placed in 10 mL of distilled water at 37 °C, and doxorubicin release was monitored by UV−vis spectrophotometry, measuring the increase of absorbance vs time at $\lambda = 486$ 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 (see Supporting Information).

Synthesis of HA-Based Click-Gels as "in Situ" Scaffolds for S. cerevisiae Cells. There were 5×10^8 *S. cerevisiae* cells suspended in 2 mL of YPD (2% polypeptone + 1% yeast extract + 2% d-glucose) medium. A 50 mg amount of HAAA-2 and 50 mg of HApA-2 were dissolved in this suspension until complete solubilization. Then, 0.5 mL of CuCl aqueous solution (1% w/v) was added, and the mixture was vigorously stirred. After a few minutes, the gel was formed. The product was left at 28 °C for 48 h.

SEM Analysis. The internal structure and cells behavior inside click-gels were studied using scanning electron microscopy (SEM) (LEO 1450VP) operating at 10 or 20 kV. Samples were fractured and mounted on aluminum stubs using adhesive carbon disks and then sputtered with a thin layer of gold (~10 nm) in argon atmosphere using a SEM coating unit 953, Agar Scientific (England), to ensure conductivity.

Results and Discussion

^{1}H-HR-MAS Analysis. ^{1}H-HR-MAS spectra of the two click-gels are shown in Figure 1 a (CG-1) and b (CG-2). Note

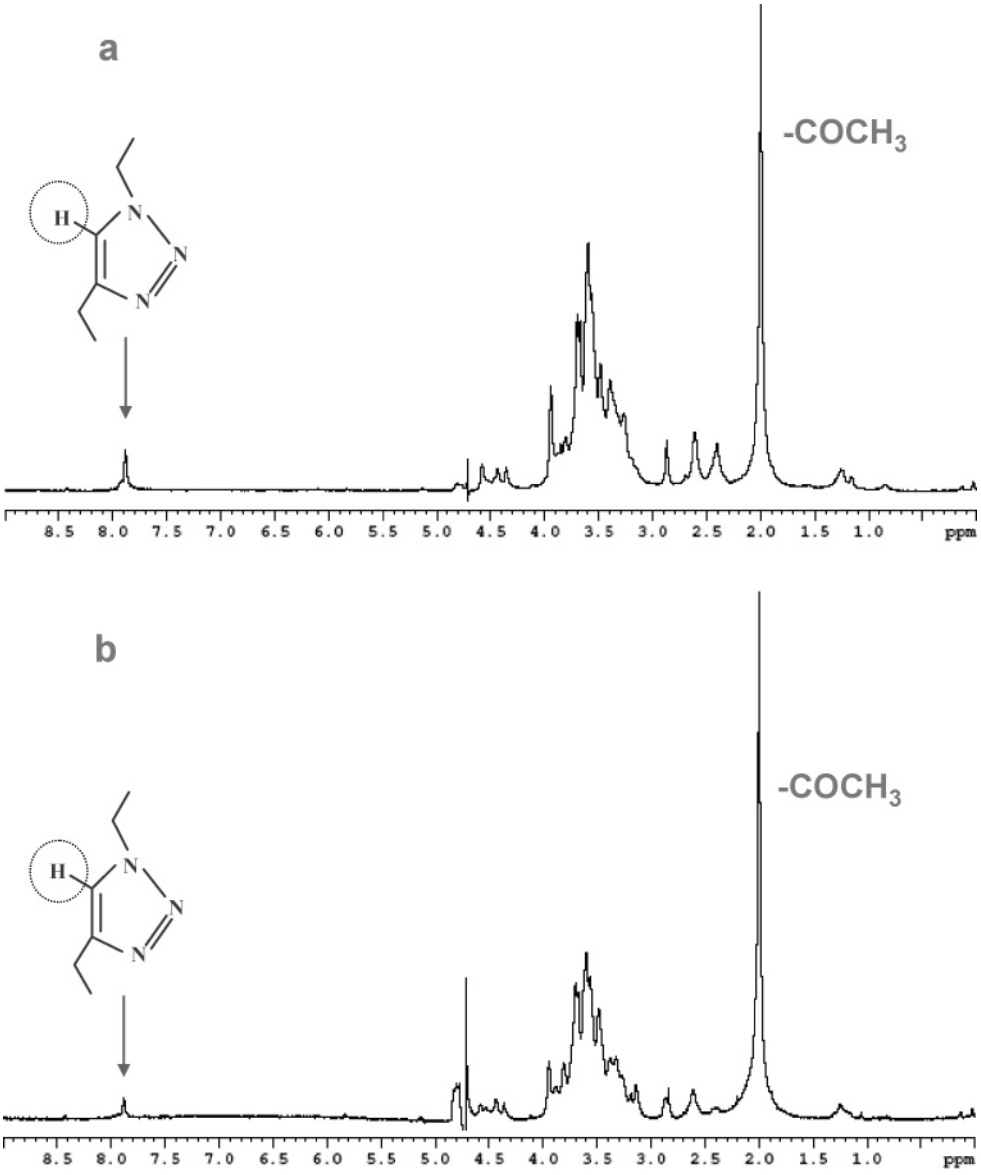


Figure 1. ^{1}H HR-MAS NMR (600.13 MHz) spectra of click-gel CG-1 (a) and click-gel CG-2 (b). The resonances used for the semiquantitative analysis are indicated, i.e., the triazole ring proton and acetyl protons.

that the signals of the HA moiety are clearly identified as well as the proton of the triazole ring formed during the cross-linking reaction. Other signals due to protons belonging to the chemical bridges between HA polymeric chains are also present.

It is possible to obtain a semiquantitative estimate of the degree of cross-linking of the two gels by integrating the resonance at 2.00 ppm due to the acetyl protons of HA, I(A), with respect to the resonance at 7.88 ppm due to the triazole ring proton,[20] I(T), Scheme 4. The cross-linking degree (Cld) of the click-gel CG-1 is obtained according to the relationship

$$\mathrm{Cld} = \frac{I(\mathrm{T})}{\frac{I(\mathrm{A})}{3}} \times 100 = 21\%$$

Analogously, we obtain for the cross-linking degree of CG-2

$$\mathrm{Cld} = \frac{I(\mathrm{T})}{\frac{I(\mathrm{A})}{3}} \times 100 = 8\%$$

Table 1. Gel Points, Elastic Modulus, and Swelling Values for HA-Based Click-Gels

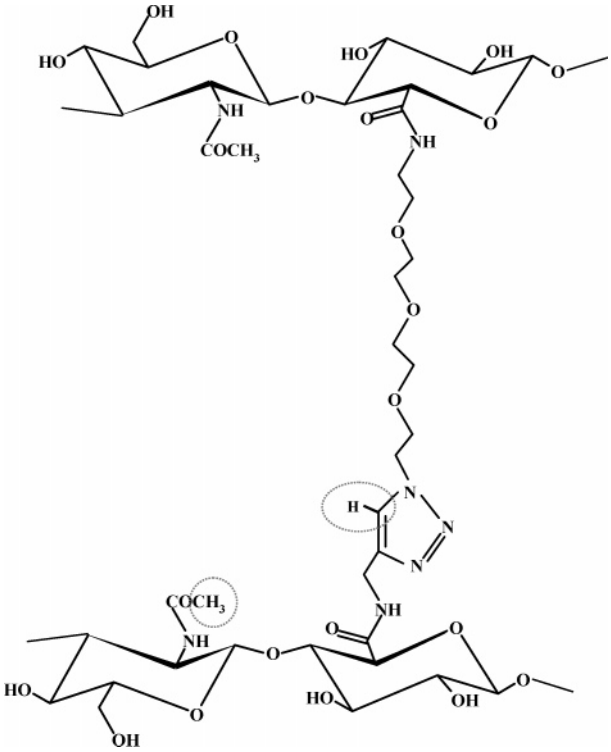
	gel CG-1	gel CG-2
gel point (s)	130	160
G' (Pa)	14 900	6900
swelling	108	167

Rheology Data. The aim of the rheological measurements was to characterize the course of the Cu(I)-catalyzed cross-linking process of the HA-based click-gels in terms of "gel points", i.e., the time at which $G' = G''$, and of the plateau G' values (Figure 2). The latter are the values of the elasticity modulus for each given gel when the cross-linking reaction has been completed.

The results indicate that for the two gels considered the reticulation process is characterized by a relatively short time (130 and 160 s, see Table 1), and the increasing G' values give evidence for an increase in the product's elasticity. Results obtained are reported in Table 1 along with swelling data.

Results of frequency-sweep and stress-sweep experiments show also that the G' values can be considered reliable ones.

Scheme 4. Structure of HA-Based Click-Gels[a]



[a] Protons used for the semiquantitative evaluation of cross-linking degree are evidenced.

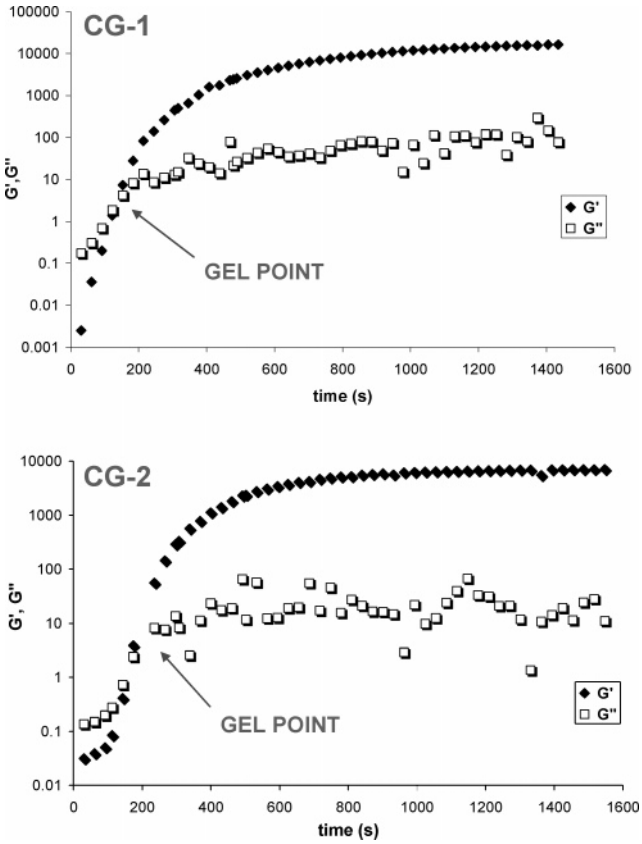


Figure 2. Sol−gel transition for HA-based click-gels.

In fact, the plots indicate the absolute independence of G' and stress with respect to the applied frequencies near the working frequency used in oscillation experiments (0.1 Hz). Moreover, for any given frequency we verify the theoretical relationships

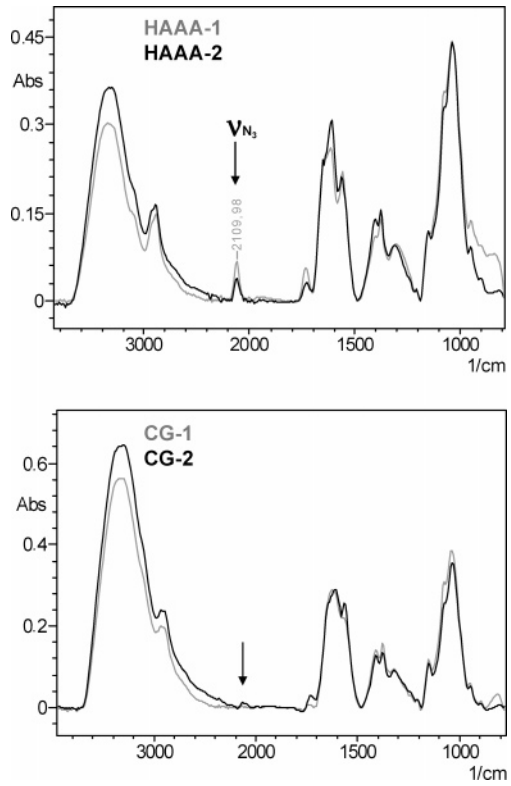


Figure 3. FTIR spectra of HAAA-1 and HAAA-2 derivatives (top) and CG-1 and CG-2 (bottom). The arrows indicate the HAAA derivative azide stretching peak that disappears in the gels.

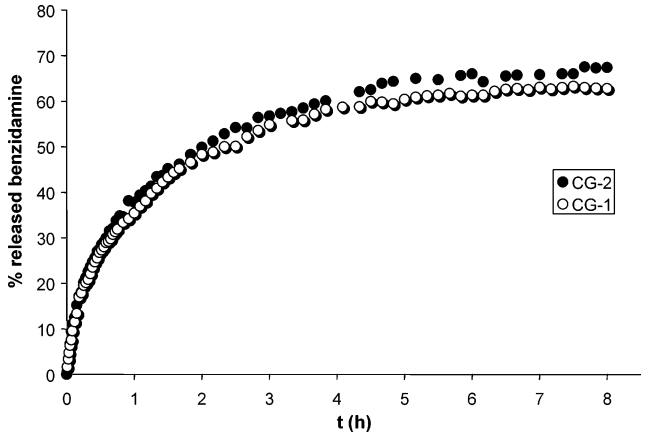


Figure 4. Kinetics of benzidamine releases from HA-based click-gels CG-1 and CG-2 at $T = 37$ °C.

between G' and strain (constant function) and between stress and strain (linear function); see Supporting Information.

FTIR Analysis. The gelation process of HA-based click-gels was followed by FTIR measurements, observing the disappearance of the azide stretching peak at about 2110 cm^{-1} of HAAA-1 and HAAA-2 derivatives after reaction with HApA-1 and HApA-2 derivatives, respectively, with formation of click-gels (see Figure 3).

Benzidamine Entrapment and Release from Click-Gels. The benzidamine releases from click-gels CG-1 and CG-2 was monitored for 8 h, when about 70% of total loaded benzidamine was released in solution. The benzidamine release is thus found to be fast and about quantitative (see Figure 4).

Doxorubicin Entrapment and Release from Click-Gels. By virtue of their formation in situ, the new HA-based click-gels can be useful devices for an Innovative Post-Surgical

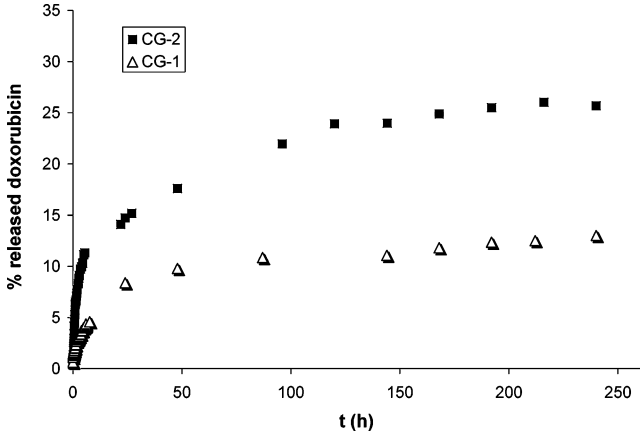


Figure 5. Kinetics of doxorubicin releases from HA-based click-gels CG-1 and CG-2 at $T = 37$ °C.

Oncology Treatment, i.e., to fill the gap left in a human organ or tissue after a surgical treatment of a solid cancer.

To mimic this process doxorubicin, a potent anticancer agent,[25] was successfully entrapped into a click-gel during its formation (as reported in detail in the Experimental Section). Drug release was monitored spectrophotometrically in physiological conditions (Figure 5).

The results indicate a slow drug release: in fact, after 10 days approximately 14% of doxorubicin was released from the gel CG-1 and approximately 25% from CG-2. 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 $\pi-\pi$ stacking interactions between the heteroaromatic 1,4-triazole ring and the aromatic moiety of doxorubicin.

This preliminary analysis will be extended to other important antitumor agents, such as 5-fluorouracil and irinotecan.

Preliminary Study of Cells Behavior in Click-Gels-Based Scaffolds. The aim of this preliminary study was to verify the possibility of employing click-gels-based scaffolds for tissue engineering. We employed *S. cerevisiae* yeast as probes for survival and proliferating test of cells in click-gels. The physical entrapment of cells was carried out easily by directly forming the gel in an YPD cell suspension. The gel was stored 2 days at 28 °C and then mechanically broken, and the extracted cells were examined on YPD plates. After 24 h 80% of the cells exhibited proliferating activity.

SEM analysis of freeze-dried samples showed a porous structure of the scaffold, a homogeneous cell distribution inside the scaffold, and good cell adhesion on the gel internal surface; moreover, the cells showed proliferating activity inside the gel (Figure 6).

Concluding Remarks

New HA-based hydrogels have been obtained by means of a "click chemistry reaction", i.e., the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide derivative and an alkyne derivative of HA obtained by amidation in aqueous solution of HA using an amino-azide bifunctional linker and propargylamine, respectively.

HA-based click-gels with two different cross-linking degrees have been characterized by ^{1}H HR-MAS NMR, rheology, and swelling measurements.

The ability of such networks to act as controlled drug-release agents and new scaffolds of interest in tissue engineering by virtue of their formation in situ in drug solution or cell suspension was also investigated in a preliminary fashion.

The amount of Cu(I) used showed not to be toxic for the yeast cells, nor for red blood cells included in the same way in

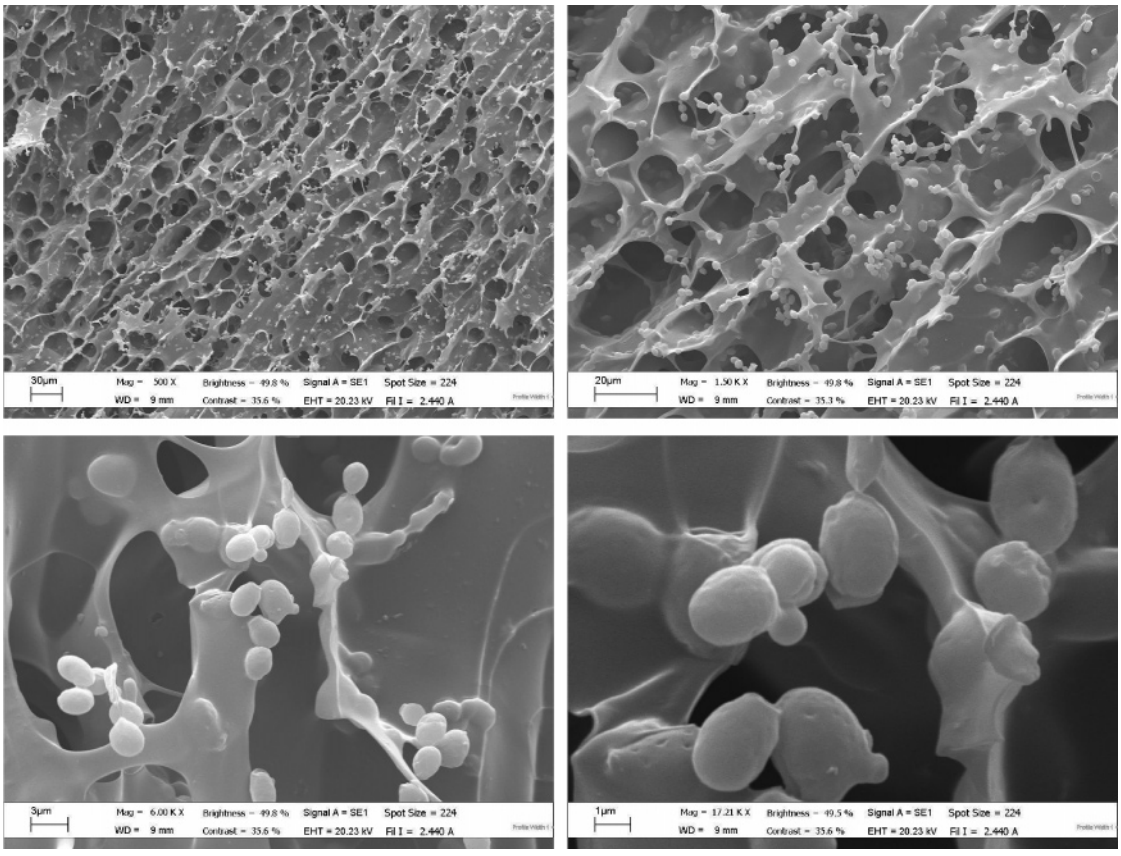


Figure 6. SEM images of HA-based click-gel CG-2 containing *S. cerevisiae* cells.

the HA-based click-gels (data not shown), and allowed a facile rheological characterization of the gelation process. However, for different types of cells it will be necessary to lower the Cu(I) concentration:[22] we found that reducing the catalyst concentration four times allows for gel formation in a few minutes. Experiments are under way using cardiac stem cells.

In conclusion, it appears that HA-based click-gels can be useful materials for controlled drug release of therapeutically relevant biomolecules as well as for cells scaffolding in tissue engineering.

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Supporting Information Available. UV−vis calibration curves of benzidamine and doxorubicin solutions in distilled water and frequency-sweep and stress-sweep experiments performed on gel CG-1. This material is available free of charge via the Internet at http://pubs.acs.org.

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