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Isosteric Substitution of 4*H*-1,2,4-Triazole by 1*H*-1,2,3-Triazole in Isophthalic Derivative Enabled Hydrogel Formation for Controlled Drug Delivery

Marleen Häring,^a Julio Rodríguez-López,^b Santiago Grijalvo,^{c,d} Markus Tautz,^a Ramón Eritja,^{c,d} Víctor S. Martín^b and David Díaz Díaz^{*a,d}

^aInstitut für Organische Chemie, Universität Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany. ^bInstituto Universitario de Bio-Organica “Antonio González” (CIBICAN), “Síntesis Orgánica Sostenible, Unidad Asociada al CSIC”, Departamento de Química Orgánica, Universidad de La Laguna, Francisco Sánchez 2, 38206 La Laguna, Tenerife, Spain. ^cBiomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, 08034 Barcelona, Spain. ^dInstitute of Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

ABSTRACT: In this work we demonstrated that the simple substitution of the 1,2,4-triazole moiety in 5-(4*H*-1,2,4-triazol-4-yl)isophthalic acid (**5-TIA**) by the 1*H*-1,2,3-triazol-5-yl unit

enables the preparation of a hydrogelator (**click-TIA**). In sharp contrast to **5-TIA**, its isostere **click-TIA** undergoes self-assembly in water upon sonication, leading to the formation of stable supramolecular viscoelastic hydrogels with a critical gelation concentration of 6 g/L. Hydrogels made of **click-TIA** as well as hybrid hydrogels made of the mixture **click-TIA** + **5-TIA** (molar ratio 1:0.2) were used to compare different properties of the materials (i.e., rheological properties, thermal properties, mechanical stability, morphology). In terms of toxicity, nor **click-TIA** neither **5-TIA** showed cytotoxic effects on cellular viability of HeLa cells up to 2.3×10^{-3} g/L when compared to untreated cells incubated with DMSO. Furthermore, the hydrogels were used for the encapsulation and *in vitro* controlled release of oxytetracycline that followed first-order kinetics. For the hydrogel made of **click-TIA**, a maximum drug release of ca. 60% was reached after ca. 8 h within a pH range between 6.5 and 10. However, the release rate was reduced to approximately half of its value at pH values between 1.2 and 5.0, whereas the use of hybrid hydrogels made of **click-TIA** + **5-TIA** allowed to reduce the original rate at $\text{pH} \leq 6.5$.

KEYWORDS: *Supramolecular gels, hydrogels, drug delivery, isosteres, click chemistry*

INTRODUCTION

Supramolecular gels have gained great attention due to their potential applications in diverse fields including, among others, biomedicine,¹⁻⁴ sensors,^{5,6} catalysis,⁷ environmental remediation,⁸⁻¹⁰ and materials synthesis.¹¹⁻¹³ The use of gel materials as carriers for drug delivery has been also an area of major expansion during the last decade.¹⁴⁻¹⁶ In contrast to swellable chemical gels based on covalent bonds (e.g., cross-linked polymers unable to redissolve), physical or supramolecular gels are made by self-assembly of either low-molecular-weight (LMW)

molecules or polymers -so-called gelators- via non-covalent interactions (e.g., hydrogen-bonding, van der Waals, π - π stacking, charge-transfer, coordination interactions, etc).¹⁷⁻²² The solid-like appearance of these materials and their rheological properties are the result of the immobilization of the liquid phase (major component) in the interstices of an entangled self-assembled solid matrix usually by capillary forces.²³⁻²⁸

A few years ago, we demonstrated that the isosteric replacement, a well-established paradigm in medicinal chemistry,^{29,30} can be successfully transplanted to the synthesis of new supramolecular gels with tailored functionality.³¹ Isosteres³² have been defined by Burger³³ as compounds or groups that possess near-equal molecular shapes and volumes, approximately the same distribution of electrons, and which exhibit similar physical properties. As a proof-of-concept, we compared the gelation ability of *N*-stearoyl-L-glutamic acid (**C₁₈-Glu**) to that of its isostere namely **click-Glu**, where the 1,4-disubstituted 1,2,3-triazole unit replaced the amide moiety in **C₁₈-Glu**. The results showed that **click-Glu** displayed superior features with respect to CGC, *gel-to-sol* transition temperature (T_{gel}) and mechanical stabilities in polar protic solvents, whereas **C₁₈-Glu** exhibited improved properties in non-polar solvents. Furthermore, co-assembly of both isosteres was proved to be a useful approach for fine-tuning the release of the antibiotic vancomycin. In addition, we should not underestimate the fact that triazole nucleus is one of the most well-known and important heterocycles, which constitute a core structural component in a large variety of natural and medicinal products. Indeed, the broad and potent activity of triazoles and their derivatives has established them as pharmacologically significant scaffolds.³⁴

More recently, we also reported the use of 5-(4*H*-1,2,4-triazol-4-yl)isophthalic acid (**5-TIA**) for the preparation of unprecedented 3D porous, crystalline metal-organic framework (MOF) and metallo-organogels using Ca^{2+} as metal ion (Figure 1).³⁷ In good agreement with the

delicate balance existing between gelation and crystallization,³⁵ the only presence of water in the solvent system was found to favor the formation of crystalline Ca-MOF (space group $C m c a$) (Figure 1A), whereas pure organic solvent induces gelation (Figure 1B). Interestingly, the xerogel obtained by freeze-drying the corresponding metallo-organogel showed 20% higher CO₂-uptake than the crystalline MOF at 1 atm and 298 K. More recently, Gao and co-workers described the remote stabilization propagated into copper paddlewheel-based molecular building blocks to afford isorecticular rht-MOFs with remarkably enhanced water/chemical stabilities.³⁶ In that work, the use of 5-(1*H*-1,2,3-triazol-5-yl)isophthalic acid (**click-TIA**), which can be considered as an isostere of **5-TIA**, yielded rht-MOF-tri (space group $Fm3m$) (Figure 1C). Motivated by our previous results obtained from the isosteric substitution of *N*-stearoyl-L-glutamic acid,³¹ we decided to explore in this work the intrinsic gelation ability of **click-TIA**. We were delighted to observe that simple ultrasound treatment of **click-TIA** in aqueous media afforded a stable hydrogel, which could be suitable as drug delivery carrier (Figure 1D). Thus, the use of the isosteric replacement approach discussed in this contribution would expand considerably the potential applications of triazolyl-containing isophthalic acids as versatile building blocks for materials synthesis.

Figure 1. Relevant uses of **5-TIA** and **click-TIA** as building blocks for the preparation of MOFs (A, C) and gel-based materials (B, D). The present work is focused on the ultrasound-induced hydrogel formation using **click-TIA** (D). Hydrogen atoms in the 3D structures are omitted for clarity.

EXPERIMENTAL SECTION

Materials

Unless otherwise noted, all reagents were purchased from commercial suppliers (Sigma-Aldrich, TCI Europe) and used as received without further purification. All solvents for synthesis were dried by distillation. Phosphate-buffered saline (PBS) solutions were prepared with Na_2PO_4 , KH_2PO_3 , NaCl and KCl , and the pH values were adjusted by using diluted HCl or NaOH solutions. Deionized water was used to prepare the buffer solutions and to prepare the hydrogels. Oxytetracycline hydrochloride ($\text{OXT} \cdot \text{HCl}$) was purchased from Sigma Aldrich ($\geq 95\%$, CAS

2058-46-0).

Synthesis and characterization of compounds

General remarks: Nuclear magnetic resonance (NMR) spectra, ^1H -NMR (400 or 500 MHz) and ^{13}C -NMR (75 or 125 MHz), were recorded using Bruker Avance spectrometers at room temperature (RT). Chemical shifts for ^1H -NMR and ^{13}C -NMR were reported as δ , parts per million (ppm), relative to the signal of the residual solvent (for ^1H -NMR: $\text{CHCl}_3 = 7.26$ ppm, DMSO = 2.50 ppm; for ^{13}C -NMR: $\text{CHCl}_3 = 77$ ppm, DMSO = 39.5 ppm). Coupling constants (J) are given in Hertz (Hz). The following notations indicate the multiplicity of the signals: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet. Multiplicity in ^{13}C -NMR spectra was assigned from DEPT135 and DEPT90 experiments and was expressed by the abbreviations s (C), d (CH), t (CH_2) and q (CH_3). Estimated error of reported values: 0.01 ppm (δ , ^1H -NMR), 0.1 ppm (δ , ^{13}C -NMR), 0.1 Hz (J , coupling constant). All NMR spectra are provided in the Supporting Information. Melting points were determined on a Büchi B-540 model. Mass spectra were recorded with a Waters LCT Premier XE Micromass spectrometer by using electrospray ionization (ESI^+ -TOF). Flash chromatography was performed with silica gel (230–400 mesh) as the stationary phase. The solvent system used as mobile phase is indicated in each case. Thin-layer chromatography (TLC) analysis were carried out on silica gel 60 F₂₅₄ aluminum sheets and visualized using UV light (365 nm), a phosphomolybdic acid solution 10 wt.% in MeOH or a vanillin solution (6 g of vanillin, 450 mL of ethanol, 40 mL of AcOH and 30 mL of H_2SO_4). Chemical nomenclature was generated using ChemBioDraw Ultra 13.0.0.3015, and atoms of all the compounds were numbered according to the IUPAC name. Sonication treatments were done using a sonication bath USC200th VWR, 45 kHz, 60 W.

Preparation of 5-(1*H*-1,2,3-triazol-5-yl)isophthalic acid (click-TIA)

Compound **click-TIA** was synthesized in four steps as described below (Scheme 1).

Scheme 1. Synthetic route for the preparation of **click-TIA**.

Dimethyl 5-((trimethylsilyl)ethynyl)isophthalate (3): Pyridine (7.47 g, 7.64 mL, 94.5 mmol) was added to a solution of dimethyl 5-hydroxyisophthalate (**1**) (13.5 g, 63.0 mmol) in dry CH_2Cl_2 (420 mL) under argon atmosphere. Then, the mixture was cooled to $0\text{ }^\circ\text{C}$ and trifluoromethanesulfonic anhydride (19.75 g, 11.78 mL, 69.3 mmol) was added dropwise over a period of 30 min. The reaction mixture was stirred for 1 h at $0\text{ }^\circ\text{C}$, after which time an aqueous HCl solution (1 M, 150 mL) was added. The organic layer was washed with deionized water (200 mL) and brine (100 mL), dried over anhydrous MgSO_4 , filtered and concentrated under reduced pressure to yield a crude, which was used in the next step without further purification.

To a solution of the above crude in benzene (150 mL) under argon at RT were added, subsequently, 1,4-diazabicyclo[2.2.2]octane (DABCO) (7.78 g, 69.3 mmol), trimethylsilylacetylene (**2**) (6.95 g, 10.0 mL, 69.3 mmol), $\text{PdCl}_2(\text{Ph}_3)_2$ (880 mg, 1.26 mmol) and CuI (120 mg, 0.63 mmol). The reaction mixture was stirred overnight at RT, after which time the

solvent was removed under vacuum. The resulting solid was re-dissolved in CH₂Cl₂ (200 mL) and washed with aqueous HCl solution (1M, 100 mL) and brine (100 mL). The combined organic layers were dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The obtained residue was purified by column chromatography in silica gel using 5% EtOAc/hexane as eluent, affording compound **3** as a white solid (17.38 g, 59.85 mmol, 95% overall yield): ¹H-NMR (500 MHz, CDCl₃) δ (ppm) 0.25 (s, 9H), 3.93 (s, 6H), 8.27 (d, *J* = 1.7 Hz, 2H), 8.58 (dd, *J* = 1,7 Hz, 1H); ¹³C-NMR (125 MHz, CDCl₃) δ (ppm) –0.1 (q), 55.6 (q), 96.8 (s), 102.9 (s), 124.4 (s), 130.4 (d), 131.0 (s), 137.0 (d), 165.7 (s); mp 103–104 °C; HRMS (ESI): *m/z*: calcd for C₁₅H₁₈O₄NaSi [M+Na⁺]: 313.0872, found: 313.0873.

Dimethyl 5-ethynylisophthalate (4): To a solution of compound **3** (17.0 g, 58.5 mmol) in THF (300 mL) at 0 °C was added water (10.5 mL, 585 mmol) and TBAF (64.35 mL of 1 M solution in THF, 64.35 mmol). The mixture was stirred for 5 min and then, the reaction was quenched with saturated aqueous NH₄Cl (40 mL) and extracted with CH₂Cl₂ (2 × 100 mL). The combined organic layers were dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure. The resulting crude was purified by column chromatography with silica gel using 10% EtOAc/hexane as eluent, affording compound **4** (12.1 g, 55.45 mmol, 95% yield) as a white solid: ¹H-NMR (500 MHz, CDCl₃) δ (ppm) 3.17 (s, 1H), 3.95 (s, 6H), 8.31 (d, *J* = 1.7 Hz, 2H), 8.63 (dd, *J* = 1,6 Hz, 1H); ¹³C-NMR (125 MHz, CDCl₃) δ (ppm) 52.7 (q), 79.3 (d), 81.7 (s), 123.4 (s), 130.8 (d), 131.2 (s), 137.2 (d), 165.6 (s); mp 127–128 °C; HRMS (ESI): *m/z*: calcd for C₁₂H₁₀O₄Na [M+Na⁺]: 241.0477, found: 241.0472.

Dimethyl 5-(1*H*-1,2,3-triazol-5-yl)isophthalate (6): CuI (525 mg, 2.75 mmol) and azidotrimethylsilane (**5**) (10.0 g, 11.5 mL, 82.5 mmol) were added to a solution of compound **4** (12.0 g, 55.0 mmol) in a mixture DMF:MeOH 9:1 (99 mL:11 mL). The reaction mixture was

heated to 100 °C overnight. After this time, the solution was filtered through a pad of Celite and the solvent removed under reduced pressure. The resulting residue was dissolved in CH₂Cl₂ (200 mL), washed with deionized water (100 mL), dried over anhydrous MgSO₄, filtered and concentrated under reduced pressure to yield **6** (14.3 g, 54.74 mmol, > 99% yield) as a brown solid, which was used in the next step without further purification: ¹H-NMR (500 MHz, DMSO-*d*₆) δ (ppm) 3.90 (s, 6H), 8.34 (dd, *J* = 1.6 Hz, 1.5 Hz, 1H), 8.58 (d, *J* = 1.6 Hz, 2H), 8.61 (bs, 1H); ¹³C-NMR (125 MHz, DMSO-*d*₆) δ (ppm) 52.5 (q), 128.6 (d), 129.9 (d), 130.9 (q), 165.1 (s); mp 213–215 °C; HRMS (ESI): *m/z*: calcd for C₁₂H₁₀N₃O₄ [M-H]: 260.0671, found: 260.0661.

5-(1*H*-1,2,3-Triazol-5-yl)isophthalic acid (click-TIA): Compound **6** (14.0 g, 53.5 mmol) was dissolved in 200 mL of NaOH aqueous solution at 15% w/w. The mixture was stirred at RT for 2 h and then, the reaction was acidified with concentrated HCl. So-formed white precipitate was filtered and washed with cold water and acetone to afford compound **click-TIA** (12.4 g, 53.18 mmol, > 99% yield) as a pale white solid: ¹H-NMR (500 MHz, DMSO-*d*₆) δ (ppm) 8.43 (s, 1H), 8.63 (s, 3H), 13.44 (bs, 2H), 15.26 (bs, 1H); ¹³C-NMR (125 MHz, DMSO-*d*₆) δ (ppm) 129.3 (d), 130.0 (d), 132.2 (s), 166.5 (s); mp 271–272 °C; HRMS (ESI): *m/z*: calcd for C₁₀H₆N₃O₄ [M-H]: 232.0358, found: 232.0357.

Preparation of 5-(4*H*-1,2,4-triazol-4-yl)isophthalic acid (5-TIA)

Compound **5-TIA** was synthesized following the previously reported procedure^{37,38} with slight modifications as described below (Scheme 2).

Scheme 2. Synthetic route for the preparation of **5-TIA**.

(*E*)-*N'*-((*E*)-(Dimethylamino)methylene)-*N,N*-dimethylformohydrazoneamide

dihydrochloride (9): SOCl_2 (11.9 g, 7.26 mL, 100 mmol) was added dropwise to dimethylformamide (DMF, **8**) (50 mL) at $0\text{ }^\circ\text{C}$ and stirred at RT. Then, hydrazine monohydrate (**7**) (2.50 g, 2.58 mL, 50.0 mmol) was added slowly at $0\text{ }^\circ\text{C}$ leading to a white suspension. The mixture was allowed to warm to RT and stirred overnight. After filtration, the obtained solid was washed with DMF ($2 \times 10\text{ mL}$) and Et_2O ($2 \times 10\text{ mL}$). The residue was dried under reduced pressure affording compound **9** (5.80 g, 27.0 mmol, 54% yield) as a white solid: $^1\text{H-NMR}$ (300 MHz, D_2O) δ (ppm) 8.24 (s, 2H), 3.18–2.99 (m, 12H); $^{13}\text{C-NMR}$ (75 MHz, D_2O) δ (ppm) 158.5, 43.9; FT-IR (solid, cm^{-1}) $\nu = 3473, 2945, 2571, 2079, 1945, 1703, 1609, 1479, 1398, 1297, 1092, 753$; mp $> 200\text{ }^\circ\text{C}$; MS (ESI): $m/z = 143\text{ [M+H]}^+ (-2\text{HCl})$ (100%), 144 (6.4%).

5-(4*H*-1,2,4-Triazol-4-yl)isophthalic acid (5-TIA): Compound **9** (1.20 g, 5.58 mmol) was added to a solution of 5-aminoisophthalic acid (**10**) (842.3 mg, 4.65 mmol) in xylene (mixture of isomers, 15 mL) and the mixture was stirred at $135\text{ }^\circ\text{C}$ for 7 h. After this time, the solvent was decanted and the precipitate was washed with EtOAc (20 mL) and EtOH (20 mL). The residue was dried under reduced pressure and recrystallized from propan-2-ol, affording compound **5-TIA** (236 mg, 1.01 mmol, 22% yield) as a white solid. $^1\text{H-NMR}$ (400 MHz, $\text{DMSO-}d_6$) δ (ppm) 9.32 (s, 2H), 8.48 (m, 1H), 8.42 (m, 2H). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ (ppm) 165.6, 141.4,

134.6, 128.8, 125.7. FT-IR (solid, cm^{-1}) $\nu = 3123, 2948, 1703, 1561, 1446, 1286, 1259, 1144, 1080, 890, 764, 697$; MS (ESI): $m/z = 234.0 [M+H]^+, 467.1 [2M+H]^+$.

Procedure for the preparation of the hydrogels

Typically, a weighted amount of the corresponding gelator (i.e., **Click-TIA** or a hybrid made of **Click-TIA** + **5-TIA**) and an appropriate amount of water were placed into a screw-capped glass vial (4 cm length $\times$ 1 cm diameter). The mixture was quickly stirred with a spatula and then sonicated for ca. 2–3 min until everything was homogeneous. The resulting solution was rest at RT until gel was formed. The material was preliminary classified as gel if it did not exhibit gravitational flow upon turning the vial upside-down at RT. The gel state was further confirmed by oscillatory rheological measurements.

Characterization of gel-based scaffolds

Critical gelation concentration (CGC), defined as the minimum gelator concentration necessary for gelation, was estimated by weighting different amounts of **click-TIA** to reach concentrations ranging from 2 to 25 g/L and applying the sonication protocol as described above. The maximum waiting time to define the gel- or non-gel state of the material was ca. 12 h. Herein, CGC was established with respect to the absence of gravitational flow.

Thermal gel destruction (T_d) values were determined using a custom-made set-up where the sealed vial was placed into a mold of an alumina block and heated up at ca. 2 °C/5 min using an electric heating plate equipped with a temperature control couple (Figure S1).³⁹ Herein, T_d was defined as the temperature at which the gel material was no longer stable upon inversion of the vial. Each measurement was made at least by duplicate and the average values were reported. Due to potential variations depending on factors such as cooling rate, aging time, thermal

history, and degree of hysteresis,⁴⁰ the values obtained with this apparatus have been previously confirmed by differential scanning calorimetry (DSC) measurements.³⁹

Field Emission Scanning Electron Microscopy (FESEM) images of the bulk xerogels were obtained with a Zeiss Merlin, Field Emission Scanning Electron Microscope operated at an accelerating voltage of 10 kV. For visualization, samples were prepared by the freeze-drying (FD) method as following: A 4 mL glass vial containing the corresponding gel (total volume = 1 mL) was frozen in liquid nitrogen and the solvent was immediately evaporated under reduced pressure (0.6 mmHg) overnight at RT. The obtained fibrous solid was placed on top of a tin plate and shielded with Pt (40 mA during 30–60 s; film thickness = 5–10 nm). Images were taken at the University of Zaragoza (Servicio General de Apoyo a la Investigación-SAI).

Fourier transform infrared (FT-IR) absorption spectra were recorded at RT using a Cary 630 FTIR spectrometer (Agilent Technologies) equipped with a single reflection ATR (attenuated total reflection) accessory. For each sample, 32 scans were performed between 4000 and 600 cm^{-1} with a spectral resolution of 8 cm^{-1} .

Powder X-ray diffraction (PXRD) experiments were performed on a STOE STADI P powder diffractometer (Start Fragment Transmission mode, flat samples, Dectris Mythen 1k microstrip solid-state detector, Debye-Scherrer geometry) with $\text{CuK}\alpha 1$ radiation ($\lambda = 1.540598 \text{ \AA}$) operated at 40 kV and 40 mA. The experiments were measured in the range $2^\circ \leq 2\theta \leq 90^\circ$. Conditions: Start Fragment 0.0151° , time 60 s/step. Lattice spacings were calculated from Bragg's law.

Dynamic oscillatory rheological measurements of the gels (total volume = 2 mL) were performed with an AR 2000 Advanced rheometer (TA instruments) equipped with a Julabo C cooling system. A 500 μm gap setting and a torque setting of $5 \times 10^{-4} \text{ N/m}$ at 25 $^\circ\text{C}$ were used

for the measurements in a plain-plate geometry (40 mm, stainless steel). Evolution of the storage (G') and loss (G'') moduli was studied as following: Dynamic strain sweep (DSS) measurements were first performed between 0.1% and 100% strain at 1 Hz frequency to establish the strain value at which reasonable torque values were given (ca. 10 times of the transducer resolution limit). Subsequently, dynamic frequency sweep (DFS) measurements (from 0.1 to 10 Hz at 0.1% strain) and time sweep measurements (DTS) within the linear viscoelastic regime (0.1% strain and 1 Hz frequency) were carried out.

Cell viability studies

The metabolic activity of the cells was measured after 18 h using the MTT test (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). HeLa cells (8000 cell/well) were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37 °C for 24 h. Several working concentrations of **Click-TIA** or **5-TIA** were previously prepared in DMSO in order to get a final **Click-TIA** or **5-TIA** concentration of 2.3×10^{-4} , 9.3×10^{-4} , 1.9×10^{-3} , 2.3×10^{-3} , 9.3×10^{-3} and 3.7×10^{-2} g /L, respectively. Cell viability studies were performed using 0.5 % DMSO (v/v) at all sample concentrations. HeLa cells were incubated with the corresponding concentration of **Click-TIA** or **5-TIA** for 24 h at 37 °C (5% CO₂). Subsequently, DMEM was removed and cells were washed with PBS (200 µL). Fresh DMEM (200 µL) was added and cells were incubated for 12 h at 37 °C. Finally, 25 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a concentration of 5 g/L in PBS were added directly in each well and cells were incubated for 4 additional hours at 37 °C. The liquid was removed and formazan crystals were dissolved in DMSO (200 µL). After 15 min incubation, absorbance was measured at 560 nm wavelength on Promega Glomax Multidetecion System instrument. Results were expressed as a relative percentage to untreated cells (control).

Procedure for the preparation of drug-loaded hydrogels and *in vitro* drug release experiments

A weighted amount of the corresponding gelator, and 1 mL of an aqueous stock solution of oxytetracycline hydrochloride (OXT·HCl) (0.8 g/L) were placed in a screw-capped glass vial and sonicated until the gel was formed (ca. 3–5 min). Obtained hydrogel materials equilibrated at RT for 16 h. After this time, the gels were overlaid with PBS buffer (1 mL, pH 1–10)⁴¹ corresponding to time = 0 of the release studies. At selected points of time, aliquots (100 μ L) were removed from the supernatant and diluted with PBS to reach 1 mL of total volume. Then fresh PBS buffer (100 μ L) was added over the gel to maintain infinite sink conditions. Drug concentrations were determined by UV-vis spectroscopy (Ocean Optics DH-2000-BAL UV-VIS-NIR spectrophotometer) after proper calibration using the maximum absorbance of OXT at $\lambda = 353$ nm. Each experiment was performed at least in triplicate. Experiments with different gelator concentrations and compositions, as described in the text, were carried out in a similar manner. The data obtained from the *in vitro* release experiments were fitted according to four drug release mathematical models, namely first-order release,⁴² Higuchi,⁴³ Korsmeyer-Peppas⁴⁴ and Weibull.⁴⁵ Note: It is known that OXT undergoes degradation at high temperatures (i.e., ≥ 43 °C), which is also accelerated by light.⁴⁶ However, we have previously observed that OXT starts degrading in PBS gel media even at 37 °C after 48 h of incubation.⁴⁷ Therefore, we limited the *in vitro* release studies in this work to 24 h and the OXT-loaded gels were maintained in dark to avoid photodecomposition.

RESULTS AND DISCUSSION

Synthesis of TIA derivatives

Click-TIA was synthesized in four steps starting from commercially available dimethyl 5-hydroxyisophthalate (**1**, Scheme 1). The alkyne unit was nearly quantitatively introduced into the aromatic ring by a classical Sonogashira cross-coupling reaction using trimethylsilylacetylene **2** and the corresponding aromatic trifluoromethanesulfonate derivative of **1**. Deprotection of trimethylsilyl (TMS) group of the alkyne with tetra-*n*-butylammonium fluoride (TBAF), and subsequent copper(I)-catalyzed azide-alkyne cycloaddition, the most famous click reaction,⁴⁸ using azidotrimethylsilane (**5**) as azide source afforded the expected 5-substituted-1*H*-1,2,3-triazole derivative **6** in excellent overall yield. Finally, alkaline hydrolysis of the ester groups yielded the desired **click-TIA** in nearly quantitative yield.

On the other hand, compound **5-TIA** was obtained as a white solid in two steps following the procedure described in the experimental section. The process involved the formation of a *N,N*-dimethylformohydrazonamide derivative intermediate **9** (Scheme 2) from the reaction between hydrazine and DMF, followed by cyclization reaction in the presence of 5-aminoisophthalic acid in hot xylene.

Hydrogelation ability of click-TIA

In our previous contribution³⁷ we reported the gelation ability of **5-TIA** in some organic solvents (i.e., DMF, DMSO, DMA, quinoline, DEF) when combined with Ca(OAc)₂. Interestingly, the replacement of the 4*H*-1,2,4-triazole moiety by the isostere 1*H*-1,2,3-triazole (**click-TIA**) allowed us to obtain stable hydrogel upon simple sonication of the mixture for ca. 2–3 min at RT. However, the critical gelation concentration (CGC) for **click-TIA** was estimated in 6 g/L (0.026 mM). This value indicates the immobilization of ca. 2150 molecules of water per molecule of gelator, which is a common ratio among many known LMW gelators.⁴⁹ The gelation time at concentrations $6 \leq c \text{ (g/L)} < 18$ varied between 30 and 60 min, whereas at $c > 18 \text{ g/L}$,

hydrogels were formed immediately. In previous reports, sonication-induced gelation has been associated to the solvent cavitation.³⁹ Due to the low water solubility of the gelator, it forms aggregated particles on water upon mixing at room temperature (entropic or hydrophobic effect). We believe that the mechanical energy provided by ultrasound may help to destroy the initial assemblies and facilitate a new supramolecular organization through the entire sample leading to the formation of the gel network.

Characterization of hydrogels made of click-TIA

The visual appearance of so-formed hydrogels was white opaque (Figure 2, inset), suggesting the formation of aggregates larger than the wavelength of visible light (ca. 380–780 nm), which was confirmed by electron microscopy imaging (see below). Moreover, the hydrogels did not show gravitational flow upon turning the vials upside-down and the gel nature was later confirmed by oscillatory rheological measurements (see below).

These gels were observed to remain stable for at least one month under steady neutral or acidic conditions, but they also responded to several stimuli (Figure S2). However, the addition of aqueous NaOH solution (1 M) caused the dissolution of the hydrogels within minutes leading to homogeneous clear solutions. In addition, irreversible *gel-to-sol* transition was induced by the addition of DMSO on top of the hydrogel, whereas overlying other solvents such as EtOAc, EtOH, *i*-PrOH and *n*-hexane did not cause gel disruption overnight. Moreover, a good thermal stability up to 65 °C was confirmed with a custom-made apparatus (see Experimental Section) for the hydrogel prepared at the CGC (6 g/L). However, the bulk gel material did not show the typical $gel \leftrightarrow sol$ thermoreversibility associated to most physical (or supramolecular) gels upon a heating-cooling cycle.¹⁷⁻²² For this reason, and to avoid confusion, we defined the above temperature as destruction temperature (T_d) instead of the classical *gel-to-sol* transition

temperature (T_{gel}) provided in most publications. Thus, T_d represents here the temperature when the entire bulk gel was no longer stable after inversion of the vial regardless the fate of the phase (e.g., precipitate, solution). Although further studies are currently in progress to understand the underlying mechanism responsible for this unusual behavior, preliminary results suggest an irreversible thermal transition to a stable crystalline phase. Interestingly, although no thermoreversibility (i.e., *gel-to-sol* and subsequent *sol-to-gel* transition) was achieved with these materials upon a classical heating-cooling cycle, reversible transitions were achieved by heating hydrogels made of **click-TIA** at concentrations below 12 g/L (i.e., *gel-to-sol* phase transition) and subsequent sonication of the obtained isotropic solutions for ca. 2–3 min (i.e., *sol-to-gel* phase transition) (Figure S2). It is worth mentioning that such heating-sonication protocol induces gelation almost instantaneously even at the CGC (6 g/L), whereas gel formation by only sonication usually takes ca. 3 h. In previous work we have highlighted the beneficial effect of combining heating and sonication on the gelation kinetics of supramolecular gels.⁵⁰ In general, the gel phase destroyed either by heating or vigorous mechanical agitation could be easily restored by application of ultrasound (Figure S2). As observed with other gels made of LMW compounds,¹⁷⁻²⁸ the transition temperature gradually increased with the gelator concentration (Figure 2). Astonishing, T_d reached a value of 104 °C, slightly above the boiling point of water, when the concentration of **click-TIA** was adjusted to 25 g/L. Concentrations above this value were not evaluated in this work. In a previous report, we demonstrated the use of a supramolecular co-assembly strategy for fine-tuning the properties of hydrogels for drug delivery applications.⁵¹ With this in mind, we checked that stable gels could also be made of mixtures **click-TIA:5-TIA** at different molar ratios being **click-TIA** always the major component (see below). The incorporation of **5-TIA** into the gel formulation (i.e., **click-TIA:5-TIA** = 1:0.2)

caused a decrease of the transition temperature in ca. 20 °C at low concentrations, whereas this difference was reduced to ca. 10 °C at higher concentrations, allowing to reach a T_d as high as 90 °C at 25 g/L.

Figure 2. Evolution of T_d with gelator concentration for the gels made of **click-TIA** and **click-TIA + 5-TIA** (molar ratio 1:0.2). Inset: Digital photograph of upside-down vial containing the hydrogel made of **click-TIA** at CGC ($c = 6$ g/L).

FT-IR spectroscopy was also performed for xerogels, obtained by freeze-drying the corresponding hydrogels, and as-synthesized solid gelators (Figure S3). The solid **click-TIA** and the corresponding xerogel showed not major differences in the characteristic infrared absorption bands, suggesting that the gelator could also be aggregated, at least to some extent, involving intuitive hydrogen bonding and π -stacking interactions in its solid state similarly to that in the gel phase. The following expected absorption regions were clearly identified: 3300–2500 cm^{-1} (O–H and aromatic C–H stretching, triazole ring); 1718–1670 cm^{-1} (C=O stretching of aromatic carboxylic acid); 1650–1530 cm^{-1} (aromatic C=C bending); 1468–1382 cm^{-1} (O–H bending;

C–N stretching); 1315–1190 cm^{-1} (O–H and C–H stretching); 1170–1110 cm^{-1} (C–O, C–N, N=N and C=C stretching); 1010–890 cm^{-1} (C=C, C–O and C–N stretching, O–H bending); 750–680 cm^{-1} (aromatic C–H bending). The broad region with overlapped frequencies between 3300 and 2500 cm^{-1} is typically observed in hydrogen bonding-forming aggregates. On the other hand, the hybrid xerogel made of **click-TIA** and **5-TIA**³⁷ displayed evident frequencies of both compounds (Figure S4). The potential similarities between the aggregation mode of **click-TIA** in the solid state and in the gel state were further confirmed by powder X-ray diffraction (PXRD). The spectrum of as synthesized solid **click-TIA** demonstrated a mesoscale ordering with peaks centered at $2\theta = 21.95^\circ, 25.70^\circ, 27.35^\circ, 27.52^\circ, 27.77^\circ, 31.69^\circ, 45.44^\circ, 56.45^\circ, 66.25^\circ, 75.28^\circ$ and 83.99° , corresponding to lattice spacings d of 4.05 Å, 3.46 Å, 3.26 Å, 3.24 Å, 3.21 Å, 2.82 Å, 1.99 Å, 1.63 Å, 1.41 Å, 1.26 Å and 1.15 Å, respectively (calculated from Bragg's law) (Figure S4). An almost identical crystalline pattern was obtained for the xerogel material obtained after freeze-drying the corresponding hydrogel, suggesting the preservation of the potential crystal packing of **click-TIA** in the gel phase, even in the presence of **5-TIA** (i.e., hybrid hydrogel) (Figure S4).

The weak and brittle nature of these gels was confirmed by oscillatory rheological experiments. Measurements were performed with the gels that were selected for subsequent drug release studies (see below): a) **click-TIA** ($c = 19 \text{ g/L}$), and b) hybrid **click-TIA:5-TIA** = 1:0.2 ($c = 19 \text{ g/L}$). In good agreement with viscoelastic network-like structures, the rheological data (Table 1 and Figure S5) showed that the elastic component or storage modulus (G') was one order of magnitude higher than the viscous component or loss modulus (G'') for both gel systems, maintaining also a weak frequency dependence ($G' \sim \omega^{0.05}$). The incorporation of a small amount of **5-TIA** into the hydrogel formulation reduced the storage modulus to ca. half its value compared to the pure **click-TIA** gel. Moreover, the $\tan \delta$ (G''/G') value was slightly lower

for the hybrid gel, whereas the critical stress or yield point (γ) was significantly higher for the **click-TIA** gel. These results indicate that the **click-TIA** gel has the greater mechanical stability and damping coefficient, thus being more efficient in effectively accomplishing energy absorption and dispersal.⁵²

Table 1. Rheological properties of gel systems used for drug release studies. Frequency was adjusted to 1 Hz (see Experimental Section).

Hydrogel	Concentration (g/L)	G' (Pa)	G'' (Pa)	$\tan \delta$	γ (%)
click-TIA	19	7927	686	0.087 ± 0.01	85
click-TIA:5-TIA (molar ratio 1:0.2)	19	3986	302	0.076 ± 0.01	60

Field Emission Scanning Electron Microscopy (FESEM) of the above-mentioned model gels were recorded in order to gain insights about the microstructure of these materials (Figures S7-S10). Samples obtained from the hydrogel made of **click-TIA** showed clustered structures with elongated aggregates in the form of leaves of micrometer diameters, resembling some flowering plants like *Kleinia neriifolia* (i.e., endemic plant of the Canary Islands known as Verode) (Figure 3A). The incorporation of oxytetracycline hydrochloride (OXT·HCl), the drug selected for release studies (see below), in these hydrogels did not cause significant changes in the morphology observed for the pristine gels (Figure 3C), suggesting a good robustness of the gel network for the encapsulation of therapeutic molecules. In the case of the hybrid hydrogel made of **click-TIA** and **5-TIA** (molar ratio 1:0.2), the globular and leaves-like aggregates were still observed in some areas (Figure 3B), even after incorporation of OXT·HCl (Figure S9), albeit fibrillar features with high aspect ratio and extended laminar structures were mainly

observed in the bulk material (Figure 3D). Although we have recorded representative images of the bulk material, it should be stressed that significant changes in the microstructures could take place during the preparation of the samples and, hence, the interpretation of these images should always be done cautiously.

Figure 3. Representative FESEM images of xerogels prepared by freeze-drying the corresponding hydrogels derived from: A) **click-TIA** ($c = 19$ g/L); B) **click-TIA** + **5-TIA** (molar ratio 1:0.2; overall $c = 19$ g/L); C) **click-TIA** ($c = 19$ g/L) + OXT·HCl ($c = 0.8$ g/L); D) **click-TIA** + **5-TIA** (molar ratio 1:0.2; overall $c = 19$ g/L) + OXT·HCl ($c = 0.8$ g/L).

***In vitro* cytotoxicity evaluation of click-TIA and 5-TIA**

In order to determine the toxicity levels of **click-TIA** and **5-TIA**, we screened a broad range of concentrations (i.e., 2.3×10^{-4} , 9.3×10^{-4} , 1.9×10^{-3} , 2.3×10^{-3} , 9.3×10^{-3} and 3.7×10^{-2} g /L, respectively) by incubating the corresponding amount of **TIA** derivatives in the presence of HeLa cells for a period of 24 h. Under our experimental conditions, **click-TIA** and **5-TIA** did

not show any cytotoxic effect on cellular viability up to 1.9×10^{-3} g/L (90 and 95 %, respectively) when compared to untreated cells incubated with DMSO (0.5 %; *v/v*). However, the cellular viability profile of both compounds remarkably differed at high concentrations (2.3×10^{-3} , 9.3×10^{-3} and 3.7×10^{-2} g/L), as illustrated in Figure 4. While cell viability decreased with the increase of the **5-TIA** concentration (66, 61 and 50 % at 2.3×10^{-3} , 9.3×10^{-3} and 3.7×10^{-2} g/L, respectively), the cellular proliferation, in the case of **click-TIA** derivative, was less affected at the same concentrations (89, 83 and 65 %) and prolonging the incubation time. These results indicate the suitability of **click-TIA** to be used as part of hydrogel formulations for *in vitro* drug release applications.

Figure 4. Cellular viabilities of HeLa cells in the presence of **click-TIA** and **5-TIA** at specified concentrations according to the MTT colorimetric assay.

OXT encapsulation and release *in vitro*

The possibility of preparing hydrogels at different pH values using non-toxic **click-TIA** motivated us to evaluate their ability for the encapsulation and controlled release of OXT (used in the form of hydrochloride salt) as model water-soluble therapeutic cargo. The release of OXT from other hydrogel systems, mainly polymer gels, has been also reported.⁵⁴⁻⁵⁶ In addition, light absorption of OXT does not overlap with that of **click-TIA** (Figure S10), which makes it a good candidate for drug delivery studies with our gel formulations. OXT is a broad-spectrum antibiotic, isolated from the actinomycete *Streptomyces rimosus* and active against a wide variety of gram-positive and gram-negative bacteria.⁵⁷ Nowadays, it is mainly used to treat acne, flare-ups of chronic bronchitis, and infections caused by *Chlamydia* (e.g., trachoma, urethritis) and *Mycoplasma* organisms (e.g., pneumonia).

UV-vis spectra of OXT hydrochloride dissolved in PBS buffer (0.01 M) were recorded to build the calibration curve based on the maximum absorption peak of OXT at 353 nm (Figure S10). It is worth mentioning that the absorption spectrum of OXT stays nearly unchanged regardless the pH,⁵⁶ although tetracyclines usually show a complex acid-base behavior in aqueous solutions.^{58,59} In this work, for release experiments the content of OXT embedded in each hydrogel system corresponded to 0.8 g/L (100% loading efficiency, see Experimental Section). Moreover, no leaching of **click-TIA** was detected from the bulk gels during the equilibration period and release experiments. Figure 5 displays selected cumulative drug release profiles using the hydrogels made of **click-TIA** or the mixture **click-TIA:5-TIA** ($c = 19$ g/L) at different pH values (i.e., pH 1.2–10). In the case of the hydrogel made of **click-TIA**, a plateau drug release zone (ca. 60%) was reached in ca. 8 h (Figure 5A). Although this is similar to the release pattern observed from some polymeric gel matrices,⁵⁶ it should be noted that chemical

and physical gels do not necessarily share the same transport mechanisms, and hence comparison should be made cautiously. The estimated release kinetics from **click-TIA** gels was comparable for pH values between 6.5 and 10. However, the release rate was reduced to ca. half of its value at pH values between 1.2 and 6.5, achieving the same maximum drug concentration (ca. 60%) in ca. 17 h. Thus, the use of hybrid hydrogels made of **click-TIA** + **5-TIA** allowed to reduce the release rate at $\text{pH} \leq 6.5$ (Figure 5B). Similar kinetics and plateau concentrations to those obtained with the **click-TIA** gel were also observed for the hybrid hydrogel in the range of pH studied, except for pH 7.4, where the maximum released drug was ca. 33%. Nevertheless, the amount of **5-TIA** in the gel formulation (i.e., within the limits that allowed the formation of stable gels) seemed to play a specific effect on the release kinetics at pH 7.4. The release rate observed in the hybrid formed by **click-TIA** + **5-TIA** at molar ratios of 1:0.1 and 1:0.4 were similar, whereas that at a molar ratio of 1:0.2 was significantly slower (Figure S11). Thus, further experimentation is still necessary to understand the reason for this particular behavior of the hybrid gel. Furthermore, both the release rate and final drug concentration could be reduced in ca. 10% by simply increasing 1.3-fold the gelator concentration, which increased the entanglement and stability of the gel network (Figure S12).

The foregoing results suggest that the gel network may act as a pH-responsive drug reservoir, which undergoes expansion at alkaline pH due to deprotonation events weakening the supramolecular structure and allowing a faster drug release. Considering the pKa values of model unsubstituted 1,2,3-triazole (ca. 9.3) and isophthalic acid (ca. 3.46, 4.46), it seems reasonable that protonation/deprotonation events at the carboxylic acid groups play a significant role on the release process. Thus, pH-induced conformation changes of the supramolecular gel networks due to strong electrostatic interactions constitute a plausible scenario.⁶⁰ Moreover, the

evolution of zwitterionic OXT at pH 5.0 to anionic OXT at higher pH (e.g., at pH 8.9 mono-anionic OXT prevails⁵⁶) should also be considered to rationalize the faster release under basic conditions due to potential repulsive interactions between negatively charged drug and gelator molecules.

A first-order release model fitted very well all release data regardless the gel formulation and pH value, showing almost perfect linear regression with correlation coefficients > 0.99 in most cases (Figure 5 and Figure S14), indicating a dependence of the release rate with the drug concentration. Although the first-order was identified as the best-fitting model, other kinetic models such as Korsmeyer-Peppas and Weibull were found to be also good models (correlation coefficients > 0.98) for many of the examples. In contrast, the Higuchi model failed in describing the experimental data in several cases (Table S1). The release exponents obtained with the Korsmeyer-Peppas model were always < 0.45 , suggesting a quasi Fickian transport mechanism for the release of OXT from these supramolecular hydrogel systems. Overall, the observed release rates are comparable to those obtained with some polymer gel composite matrices previously used under similar conditions.⁵⁶

Figure 5. Representative *in vitro* release curves of OXT obtained at 37 °C and different pH values from gels made of A) **click-TIA** ($c = 19$ g/L) and B) **click-TIA:5-TIA** (molar ratio 1:0.2; overall $c = 19$ g/L). PBS was used as the release medium. Additional plots corresponding to other gel compositions at different pH values and fitting curves obtained from Higuchi, Korsemeyer-Peppas and Weibull models are given in the Supporting Information.

CONCLUSIONS

In conclusion, the replacement of 1,2,4-triazole moiety in 5-(4*H*-1,2,4-triazol-4-yl)isophthalic acid (**5-TIA**) by 1,2,3-triazole enabled the preparation of a new LMW hydrogelator (i.e., 5-(1*H*-1,2,3-triazol-5-yl)isophthalic acid; **click-TIA**). In sharp contrast to **5-TIA**, its isostere **click-TIA** undergoes self-assembly in water upon sonication, leading to the formation of stable supramolecular hydrogels with a critical gelation concentration of 6 g/L. The viscoelastic and brittle nature of the materials were demonstrated by oscillatory rheological measurements. Gels made of **click-TIA** as well as hybrid gels made of **click-TIA** + **5-TIA** (molar ratio 1:0.2) were used to compare different properties of the materials. Although no thermoreversibility was achieved with these materials upon a classical heating-cooling cycle, reversible transitions were

achieved by heating hydrogels made of **click-TIA** at concentrations below 12 g/L (i.e., *gel-to-sol* phase transition) and subsequent sonication of the corresponding isotropic solutions for ca. 2–3 min (i.e., *sol-to-gel* phase transition). The thermal stability of the hydrogels increased with the gelator concentration allowing gels that remained stable up to 104 °C when the concentration of **click-TIA** was adjusted to 25 g/L. In general, the incorporation of **5-TIA** into the gel formulation (i.e., **click-TIA:5-TIA** = 1:0.2) caused a decrease of the thermal stability, which could be overcome by increasing the gelator concentration. Gels made of only **click-TIA** showed greater mechanical stability than the hybrid gels, and morphologies consisting in clustered structures were observed by SEM of the corresponding xerogels. Finally, nor **click-TIA** neither **5-TIA** showed cytotoxic effects on cellular viability up to 2.3×10^{-3} (~ 86%) when compared to untreated cells incubated with DMSO (1%). Furthermore, the hydrogels allowed the encapsulation and *in vitro* controlled release of oxytetracycline that followed first-order kinetics. In the case of the hydrogel made of **click-TIA**, a maximum drug release of ca. 60% was reached after ca. 8 h within a pH range between 6.5 and 10. Interestingly, the release rate was reduced to ca. half of its value at pH values between 1.2 and 5.0, whereas the use of hybrid hydrogels made of **click-TIA** + **5-TIA** allowed to reduce the rate at $\text{pH} \leq 6.5$. Complementary research to fully understand the underlying mechanism of the pH-induced release with these hydrogels, as well as additional studies in the presence of release modifiers (e.g., ions) are currently underway.

ASSOCIATED CONTENT

Supporting Information. NMR and IR spectroscopy, additional pictures, electron microscopy images, rheology and drug release plots. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

* E-mail: David.Diaz@chemie.uni-regensburg.de; Fax: + 49 941 9434121.

Author Contributions

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Notes

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Isosteric substitution of 4*H*-1,2,4-triazole by 1*H*-1,2,3-triazole in a triazolyl-containing isophthalic acid molecular building block induced, upon sonication, the formation of supramolecular hydrogels with potential as carrier for controlled drug delivery.