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Bolaamphiphile-based supramolecular gels with drugs eliciting membrane effects

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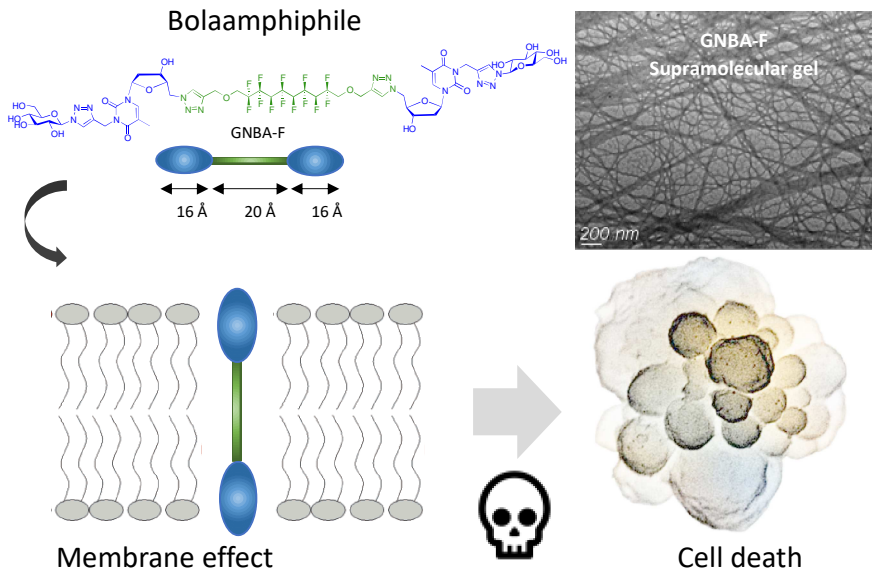
TOC



Abstract: Supramolecular chemistry has garnered important interest in recent years toward improving therapeutic efficacy via drug delivery approaches. Although self-assemblies have been deeply investigated, the design of novel drugs leveraging supramolecular chemistry is less known. In this contribution, we show that a Low Molecular Weight Gel (LMWG) can elicit cancer cell apoptosis. This biological effect results from the unique supramolecular properties of a bolaamphiphile-based gelator, which allow for strong interaction with the lipid membrane. This novel supramolecular-drug paradigm opens up new possibilities for therapeutic applications targeting membrane lipids.

Keywords: supramolecular; bolaamphiphiles; hydrogels; membrane; fluorocarbon

INTRODUCTION

Most drugs elicit their pharmaceutical activity thanks to interactions with cellular biomolecules such as peptides, proteins[1], sugars[2–4] or nucleic acids[5],[6]. In fact, depending on their concentration, the active pharmaceutical ingredients (API) of drugs induce a chemical and/or a physiological effect at their site of action[7]. Several recent reports highlighted the central role of membrane lipids in many biological functions and mechanisms[8,9]. Hence membrane lipids have emerged as an alternative therapeutic target[10]. It is anticipated that modification at the level of lipid membranes *via* insertion of an API may elicit a biological activity allowing changes in the cellular fate and the subsequent therapeutic effect. In this context, amphipathic peptides have been identified as attractive APIs[11] as they exert biological activities *via* different mechanisms[12,13] against bacteria[14], fungi[15], viruses[16] and cancer cells[17,18]. Likewise, amphiphilic compounds such as saponins can perturb biologic membranes leading to their perforation and/or lysis[19–21]. It was recently reported that several drugs, including anthracyclins and edelfosine[22], could also interplay with biological membranes[23]. In all these examples, the biological activities were explained by the intrinsic properties of the API. As a result, their effects on biological membranes have been mainly considered at the molecular level, which occult their own supramolecular properties and the possible impact of these assemblies on the final membrane activity. Except in a few recent cases,[24] less attention has been paid to the supramolecular behaviors of these membrane elicitors in the design of new drugs.

Recently, Low Molecular Weight Gels (LMWGs) have attracted great interest as supramolecular materials for biomedical applications[25–34],[35],[36],[37],[38]. Among the gelators reported in this field, the bolaamphiphiles (molecular structure with two hydrophilic heads linked by a hydrophobic segment (Fig. 1)) appear as promising materials as they feature physicochemical and biological properties allowing both *in vitro* and *in vivo* applications[39,40],[41],[42]. The purpose of our study was

to investigate the use of a novel bolaamphiphile, featuring a fluorinated hydrophobic segment, as a new supramolecular gelator. Fluorinated compounds display a unique combination of physico-chemical properties[43]. The very low polarization of F-alkyl chains prevents the instantaneous dipole formation with H-alkyl chains: fluorocarbons are therefore lipophobic. On the other hand, provided that more than four CF2 groups are involved, London cohesive forces between F-chains occur: fluorocarbons are then fluorophilic too [44]. Their thermal and chemical stability as well as their biological inertness may also originate from the larger bond dissociation energy of the C-F bond compared to C-H (485 vs 425 kJ/mol). With a Van der Waals radius of 1.47 Å for fluorine vs. 1.20 Å for hydrogen, the cross-section of a fluoroalkyl chain is significantly larger than that of its hydrogenated counterpart (28 vs. 18 Å2 respectively) [45],[44]. This bulkiness explains the experimentally well-known hydrophobicity of fluorocarbons [46] and the greater stiffness of perfluorinated chains [47]. It was previously demonstrated that bolaamphiphiles with rigid linking hydrophobic chains strongly prefer extended, membrane spanning conformations[48]. Hence, we postulated that the incorporation of a rigid highly fluorinated segment in the bolaamphiphile architecture would affect its supramolecular behavior and its interaction with biological membranes.

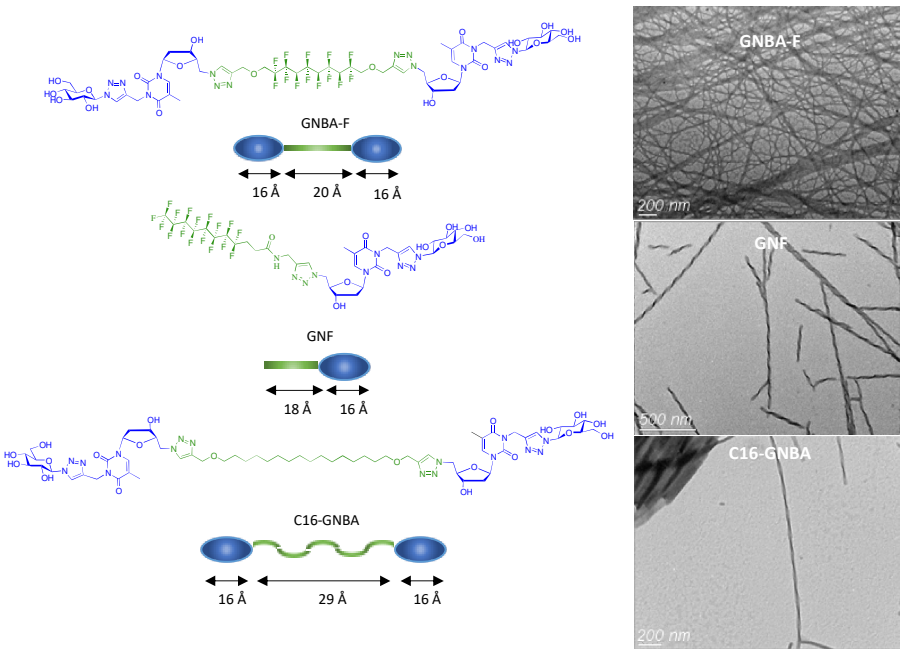


Figure 1. Left) Amphiphilic derivatives used in this study (chemical structures, schematic representation and size). Right) TEM images of the supramolecular networks of the corresponding hydrogels.

In this contribution, we report a new bolaamphiphile with N-thymine glycosylated polar groups linked to a highly fluorinated segment *via* either functions (Fig. 1). We showed that this fluorinated moiety impacts strongly on both the supramolecular properties and targeting of lipid membranes. Contrary to previous non-toxic Glycosyl-Nucleoside-Bolaamphiphiles (GNBA[39], Fig 1) and Glycosyl-Nucleoside-Fluorocarbon amphiphiles[49–51] (GNF, Fig 1), the novel GNBA-F features membrane spanning properties, which induce cancer cell apoptosis.

RESULTS AND DISCUSSIONS

The molecules selected in this contribution are depicted in Figure 1. Besides the already reported GNF and GNBA, we synthesized a new bola-amphiphile (C16-GNBA) comprising 16 methylene groups to serve as a second reference compound. C16-GNBA is expected to better match the greater hydrophobic character of the fluorinated compound (GNBA-F) as the latter exhibits eight CF2 groups among ten carbons overall. This ratio of 1.75 CH2 per CF2 group was determined after Krafft and Goldmann [52] who quoted that a highly stable Langmuir monolayer is easily obtained from C8F17COOH whereas 13 CH2 groups are needed for a similar result with a hydrocarbon acid. The bola-amphiphiles synthesis relied on a straightforward double click-chemistry strategy starting from the appropriate long chain diols (see SI for details) and the target compounds are obtained in reasonably high yields.

In the case of physical gels, the formation of a gel at the macroscopic level is driven by non-covalent intermolecular interactions occurring at the molecular level. Hydrogen bonds, π-π stacking interactions, hydrophobic effects, etc., promote the stabilization of LMWG supramolecular assemblies.

All the glycoconjugate amphiphiles studied were able to form gels (Fig SI1) stabilized by a supramolecular network at 11.55 mM (Fig 1 and Fig SI7). The visco-elastic properties of the gels were characterized by measuring the elastic (G') and viscous (G") moduli. For all hydrogels, G' consistently

exceeds G" throughout the frequency range tested (0.1-10 Hz), indicating the dominant elastic character (solid-like) as expected for a cross-linked network. The fluorinated compounds presented similar mechanical strength with G' moduli of 1680 ± 158 Pa and 1609 ± 175 Pa for GNBA-F and GNF respectively (Fig SI3). In comparison, the hydrocarbon homologues C12-GNBA[39] and C16-GNBA were characterized with G' 20-fold and 10-fold higher respectively (Fig SI3). The linear visco-elastic region (LVR) was determined up to 0.25 % and their breaking points characterized by a moduli inversion were found at 5 % for GNBA-F and 16 % for both GNF and C16-GNBA (Fig SI2). The thixotropic behavior was highlighted for the supramolecular hydrogels by the succession of two destructuration / restructuration cycles (Fig SI4). The point where the hydrogel goes from a gel-like state to a liquid-like state under temperature ramp is called Gel-Sol temperature ($T_{gel-sol}$). As presented in Fig. SI5, $T_{gel-sol}$ was higher than 37°C (~310 K) for each hydrogel, enabling potential biomedical applications. GNBA-F was characterized by a $T_{gel-sol}$ of 46°C (~319 K), which is similar to the hydrocarbon analog C12-GNBA 46°C (~319 K)[39]. The two other supramolecular gels required higher temperature for the gel-sol transition 61°C (~334 K) for the GNF and 77°C (~350 K) for the C16-GNBA (Fig SI5). On the macroscopic scale, rheology experiments, including visco-elasticity, Gel-Sol, thixotropy studies, showed that GNBA-F possess a similar behavior compared to GNF and hydrocarbon bolamphiphiles.

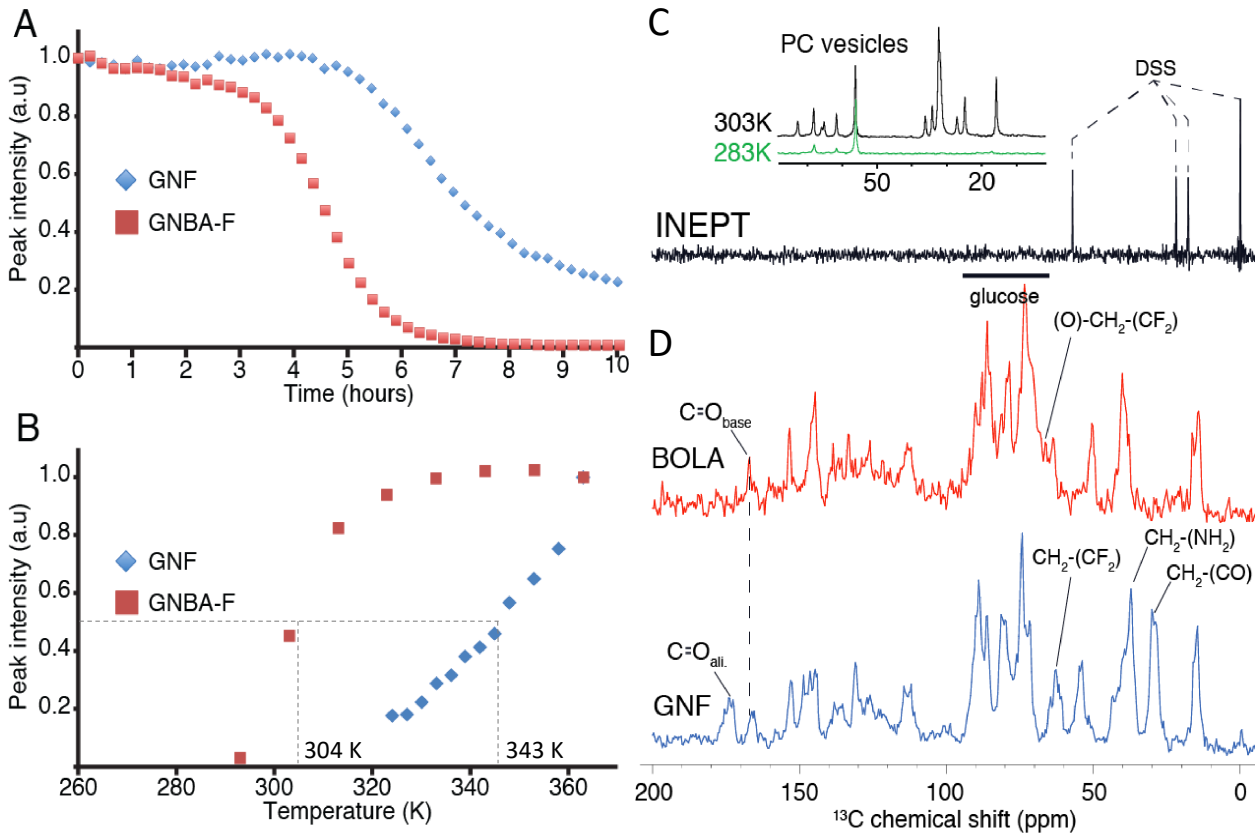


Figure 2. NMR studies of supramolecular assemblies. A) Liquid-to-gel transition of GNF (blue, [GNF] = 11.55 mM) and GNBA-F (red, [GNBA-F] = 11.55 mM) as observed by 1D ^{19}F liquid-state NMR (400 MHz, 310 K), over 10 hours. B) Liquid-to-gel phase transition monitored by 1D ^{19}F liquid-state NMR (400 MHz), as a function of temperature (363-283 K) for GNF (blue) and GNBA-F (red) initial samples were in a liquid phase with well-resolved NMR peaks and all final samples clearly under-went a phase transition to rigid gel C) ^{13}C INEPT spectra of DMPC liposomes were acquired at 37°C, 10 kHz MAS D) ^{13}C CP spectra of GNBA-F (red) and GNF (blue).

Next, we investigated at the molecular scale the liquid-to-gel phase transition using solution NMR spectroscopy (Fig. 2) by monitoring the NMR signal during the assembly process. In comparison to GNF, GNBA-F exhibited a faster transition to the gel phase as a function of time (inflection point ~4.5 hours for GNBA-F vs. ~7 hours for GNF) at the constant physiological temperature of 310 K (Fig. 2A), suggesting that the BOLA architecture increases the cooperativity of the GNBA-F molecules to self-assemble in the hydrogel form. Interestingly, when monitoring the phase transition as a function of temperature (Fig. 2B), GNBA-F undergoes a phase transition ~304 K, which was significantly lower

than that of GNF at ~343 K. This relatively low temperature of phase transition for GNBA-F reinforces its potential therapeutic application compared to its counterpart GNF because monomeric GNBA-F molecules may be more bioavailable at physiological temperatures for interactions with endogenous molecules, in contrast to GNF that would be predominantly found as supramolecular species. Furthermore, the phase transition observed for GNBA-F occured in a short temperature range (~15 K) compared to GNF (~39 K), supporting the cooperativity character of the GNBA-F self-assembly promoted by its particular BOLA shape.

As both GNBA-F and GNF molecules undergo a phase transition to form a hydrogel, we use magic-angle spinning (MAS) NMR spectroscopy performed in the solid-state (SSNMR) to probe the local conformation of the hydrogels. MAS frequency of 10 kHz was applied to improve the spectral resolution and observe isotropic chemical shift values. First, we performed ^{13}C-detected experiments using both cross-polarization (CP) and INEPT-based pulse sequences. CP polarization transfer relies on dipolar coupling and is used to probe the rigid components of the hydrogels, while INEPT-based polarization transfer enables the observation of the highly mobile part. The ^{13}C INEPT experiment (here exemplified on GNF, see Fig 2C) only displayed signals of the DSS (internal reference for chemical shift and temperature calibration) after 12k scans, indicating the absence of highly mobile moieties (typically faster than the microsecond) within the hydrogel assembly. In comparison, phosphatidylcholines (PC) vesicles led to signals in an INEPT experiment above and below the PC phase transition (i.e. 297K, inset Fig 2C), indicating that in the gel phase, GNF and GNBA-F lipid-like molecules adopted a much more compact organization compared to non-fluorinated natural lipids in membranes. The compactness of the molecule within the hydrogel organization was probed with ^{13}C CP experiments (Fig. 2D). We observed isolated signals encoding for the different parts including carbons close to the fluorinated acyl chain, carbohydrate and the nucleoside. A single set of resonances was observed for both GNBA-F (Fig 2D red) and GNF (Fig 2D blue). As NMR chemical shift is an

exquisite probe of local environment and is very sensitive to molecular polymorphism, the observation of an unique set of NMR resonances strongly suggests that a single molecular conformation predominates the hydrogel assembly.

Next, we conducted ^{19}F SSNMR under MAS condition using a Hahn-echo sequence to observe that the isotropic chemical shift of the -(CF_2)- hydrophobic tail was identical in both GNF and GNBA-F samples, additional resonances being observed (methyl CF_3 and CF_2-CH_2) for the GNF hydrogel (Fig SI9). Consistent with ^{13}C-detected SSNMR, ^{19}F data highlighted the same chemical shift distribution (except for few positions that are chemically different in the two molecules) of GNF and GNBA-F indicating that both molecules arranged with a similar local conformation in the gel phase.

To gain further insights into the cytotoxic properties of amphiphilic derivatives, we investigated *in vitro* their ability to interact with membrane models mimicking the properties of the cell barriers. Fluorocarbon amphiphile chemical properties were compared in the presence of lipid liposomes (DMPC) using ^{19}F SSNMR (Fig 3A and 3B). Samples were incubated at 37°C during one night before spectra acquisition, in the presence or absence of DMPC liposomes. For GNF (Fig 3A) and GNBA-F (Fig 3B), ^{19}F NMR spectra in water exhibited well-resolved peaks for the hydrophobic core, which reflected a high sample homogeneity.

In the presence of a lipid membrane, we detected ^{19}F chemical shift perturbations (Fig 3A-B, Fig SI12) indicating a change in the isotropic chemical shift due to the binding to the membrane. The chemical shift perturbation of the -(CF_2)- signal between GNBA-F in water and in contact with membranes is ~0.4 ppm (113 Hz) while we observed a perturbation of ~0.75 ppm (211Hz) for GNBA-F. Such ^{19}F chemical shift perturbations of fluorinated pharmaceuticals upon anchoring to the membrane have been already reported Bhattacharya and co. [53] as a method of choice to identify membrane-bound compounds. GNBA-F ^{19}F perturbations is significantly higher than GNF, reflecting an insertion of bolaamphiphiles in the core of the lipid bilayer because of their hydrophobic properties. GNF also

induces a small chemical shift pertubation, suggesting a less facilitated insertion in the DMPC bilayer. To confirm this hypothesis, we monitored the integrity of the lipid bilayer as a function of time upon the addition of GNBA-F or C16-GNBA (Fig 3C).

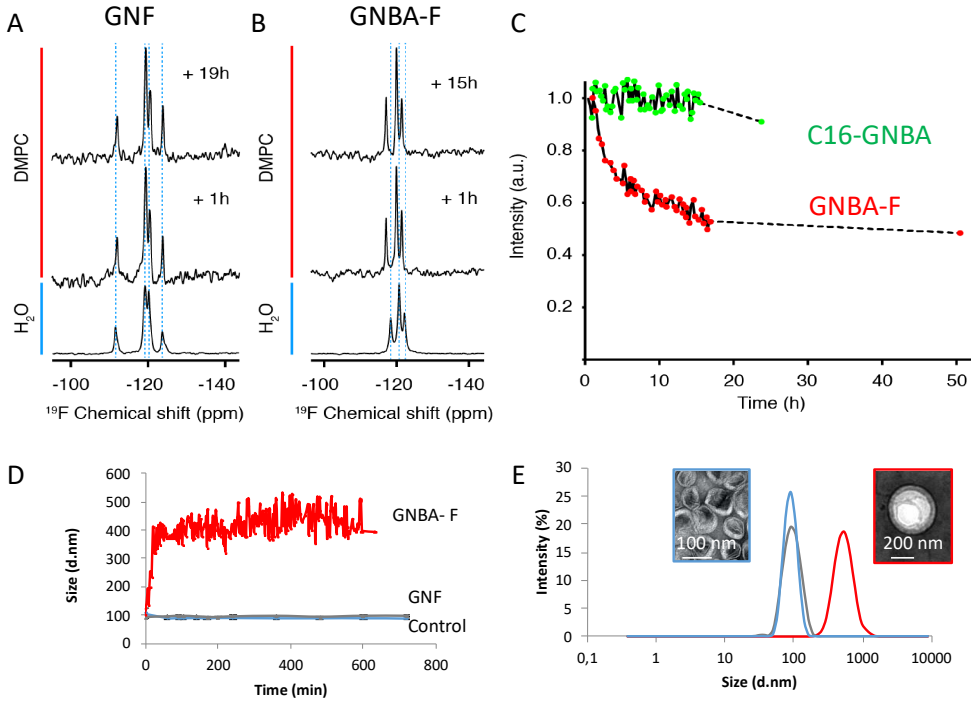


Figure 3. Effects of amphiphiles on a biomimetic membrane; NMR (A, B and C) and DLS (D and E) studies. Solid-state ^{19}F NMR spectrum of GNF (A) and GNBA-F (B) at 250 µM in DMPC liposome and aqueous solution. C). Intensity of solid-state NMR ^{13}C liposomes signal versus time after addition of C16-GNBA (green), GNBA-F (red) at 250 µM. Lipid spectra were recorded every 15 minutes. D) DMPC Liposome diameter versus time in the absence (control) or presence of amphiphiles (GNF or GNBA-F). E) Size of liposomes after incubation in the presence of GNF (blue) or GNBA-F (red), inset corresponding TEM images.

C16-GNBA was used as a control for a non-cytotoxic amphiphilic derivative with a hydrophobic character comparable to GNBA-F. SSNMR ^{13}C INEPT DMPC signal was detected and integrated every 30 minutes after addition of 250 µM C16-GNBA (green) or GNBA-F (red) for a total of 16 hours. Considering the accuracy of the intensity measurement, the lipid signals in the presence of C16-GNBA remained stable over the time (Fig. 3C, green curve) indicating that the flexible hydrocarbon bola-amphiphile C16-GNBA did not perturb the lipid bilayer. However, a different behavior was observed for the fluorocarbon GNBA-F, as after adding the compound, a decrease of the lipid signals was

immediately observed to reach a plateau around 50 % of the initial intensity (Fig 3C, red curve, Fig SI11). The NMR signal loss of DMPC lipids in the presence of GNBA-F correlated to a rapid disruption of the membrane to form bigger objects as observed by DLS (Fig 3D and E, red curve). Altogether, these results confirmed the ability of GNBA-F to destabilize the biological membranes after penetrating inside the hydrophobic core.

External matrix surrounding the cells impacted strongly on cellular fate[54],[39]. For example, in the case of cancer cells, the matrix stiffness were reported to be an important factor in tumorogenesis[55]. Our hypothesis is that since the matrix is in close contact with the cells, it may also exchange matrix molecules, which would elicit a biological activity. In this context, LMWGs appeared ideal materials since gelator molecules may be released from the supramolecular material. However, most of these materials featured equilibria strongly shifted in favor of the supramolecular structures with almost no single molecule in the bulk phase. Hence, in order to solve this paradoxical situation, and obtain a potential biological effect, the LMWGs must possess self-assembly properties allowing both the stabilization of the supramolecular network and strong interactions with the biological membranes. Thus, we hypothesized that a bolaamphiphile with a rigid fluorinated hydrophobic segment would encompass these properties. Liposomes are useful models to assess different biophysical parameters and are often used to investigate drug-membrane interactions[23]. Thus, in order to determine a membrane effect of our gelators, we investigated the impact of gelator molecules (GNF, GNBA-F, C12-GNBA and C16-GNBA) on the DMPC-based liposomes. As shown in figures 3D and 3E, the liposomes were strongly and quickly affected after incubation in the presence of GNBA-F, whereas their size remained unchanged with GNF or C16-GNBA, indicating that GNBA-F was interacting strongly with the DMPC biomimetic membrane. Such an exclusive membrane effect may be explained by the unique properties of the GNBA-F, including the BOLA shape, hydrophobic rigid segment and a similar size of the cell bilayers[56] (Fig 4C). Due to its rigid segment GNBA-F adopted an extended transmembrane

orientation.

The modulation of membrane biophysics *via* the insertion of an amphipathic molecule could open new perspectives in cancer therapies[57]. As a proof of concept, the cytotoxicity of the gelators was investigated for the Hela carcinoma cell line. Cell viability experiments (Fig 4B) showed that GNBA-F was effective in killing human cancer cells (IC50<200 µM), while GNF was non-toxic in similar conditions after several days of incubation. Also, this anticancer efficacy in the case of GNBA-F and the lack of toxicity for GNF were visualized by following the fate of HeLa cells in the presence of gelators (see Fig SI13). Also, the cytotoxic effect of GNBA-F at a concentration of 11.55 mM on HeLa cells was followed for 17h. The phase and fluorescence microscopies merged indicates a total cell death after 17h in the presence of GNBA-F, whereas in the same conditions HeLa cells are still alive in the absence of gel and/or in the presence of GNF (SI movie). These experiments showed the presence of cells death undergoing morphological changes typical of apoptosis[58] (Fig 4A) such as loss of cell volume and membrane blebbing[59].

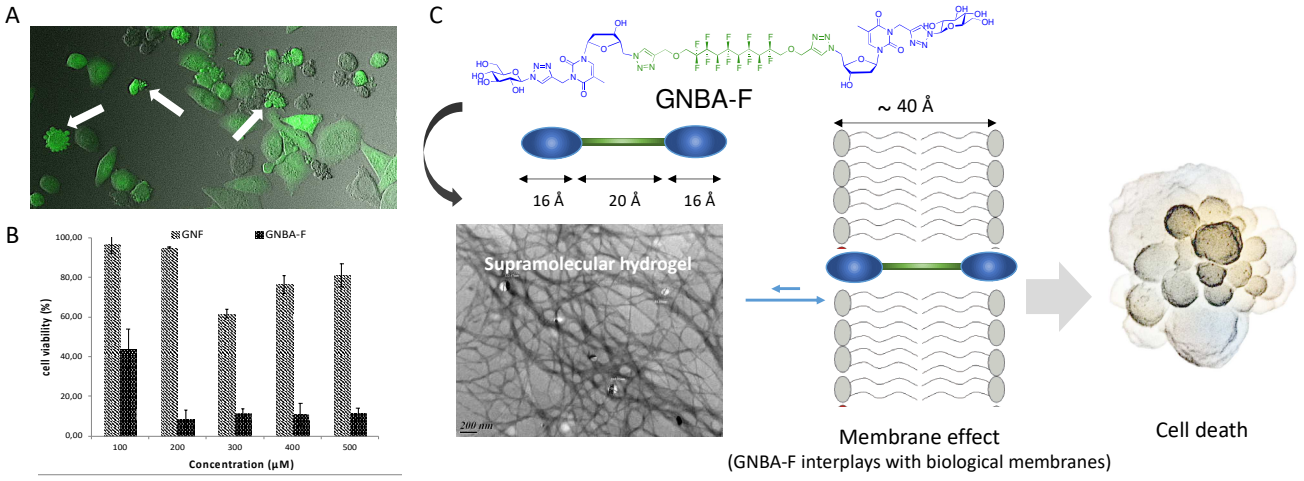


Figure 4. Cell viability studies and conceptual scheme. A) Fluorescence microscopy image showing the apoptotic cells (white arrows). B) Cancer Hela cells viability versus GNF or GNBA-F concentrations (for details see Fig. SI14). C) Conceptual scheme illustrating the membrane effect mechanism. GNBA-F self-assembled at 37°C to stabilize a supramolecular network (TEM image) in equilibrium with monomeric species, which interacted with biological membrane thanks to GNBA-F properties, including hydrophobic character, rigid highly fluorinated segment and a size similar to biological membrane thickness. The GNBA-F insertion perturbed biologic membranes leading to cancer cell death.

CONCLUSIONS

In this work we successfully synthesized a new fluorinated bola-amphiphile (GNBA-F), which exhibits eight CF_2 groups among ten carbons overall and two N-thymine glycosylated head groups. GNBA-F self-assemble through weak interactions to provide a network of nanofibers in aqueous media. The resulting hydrogel features thixotropic and thermal reversibility properties. Rheological studies revealed that the GNBA-F based hydrogel possesses a similar mechanical strength as its single chain fluorinated amphiphile analogue (GNF). Also, the GNBA-F gel features a gel-sol phase transition temperature 15 K lower ($T_{gel\text{-}sol}$ = 46°C ~ 319 K) than GNF ($T_{gel\text{-}sol}$ = 61°C ~ 334 K) and 39 K lower than its hydrocarbon analogue C16-GNBA ($T_{gel\text{-}sol}$ = 77°C ~ 350 K). Importantly, NMR investigations showed that monomeric and supramolecular species coexist in the case of the GNBA-F gel at physiological temperature (37°C ~ 310 K), whereas the GNF is predominantly involved in supramolecular assemblies. Such a behavior indicates that GNBA-F monomers can interact with biological membranes in a biological environment at 37°C. Hence, contrary to its analogues (hydrocarbon bolaamphiphile C16-GNBA and fluorocarbon single chain GNF), the novel GNBA-F was able to strongly interact with DMPC bilayers and promote the destabilization of liposomes. The molecular characteristics (rigid fluorocarbon, bolaamphiphile structure, molecular size) and the self-assembly behaviors of GNBA-F (presence of monomers at 37 °C, high cooperativity, low gel-sol temperature) allow its incorporation in biological membrane. As a result, contrary to the non-toxic hydrocarbon bolaamphiphiles and GNF, which do not perturb bilayers, the cell viability was drastically affected by the presence of GNBA-F in their membranes. Thanks to these properties this soft material exhibited cancer cell toxicity (HeLa), whereas the GNBA and GNF gels previously reported are not toxic. The consequential loss of membrane integrity would result in the alteration of membrane transport and/or stability,[60] inducing cancer cell apoptosis. To the best of our knowledge, GNBA-F

represents the first bolaamphiphile-based supramolecular gel eliciting anti-cancer activities *via* a membrane effect. Controlling supramolecular systems based on Low Molecular Weight Gel (LMWG) open new perspectives to impact on cellular fate and viability. This approach appears to be a promising therapeutic option to effectively struggle against diseases such as cancers.

EXPERIMENTAL

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing finan-cial interests or personal relationships that could have appearedto influence the work reported in this paper

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AUTHOR CONTRIBUTIONS

PB, LL and AL conceived the study, designed the experiments, critically interpreted data and wrote the manuscript. LL performed the synthesis and characterization of GNBA-F. AG realized rheology and microscopy studies. The physico-chemical characterizations and biological investigations were achieved by SB. JT, DM, EM, AG, BH completed the NMR studies and characterization. All authors analyzed and interpreted data and critically read the manuscript.

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