Interaction between a cationic bolaamphiphile and DNA: The route towards nanovectors for oligonucleotide antimicrobials

(Article begins on next page)

12 September 2024

Interaction between a cationic bolaamphiphile and DNA:

The route towards nanovectors for oligonucleotide antimicrobials

Marianna Mamusa [a], Claudio Resta [a], Francesco Barbero [b], Davide Carta c,d,Doroty Codoni [c,e], Kostas Hatzixanthis [c,f], Michael McArthur [c,g], Debora Berti [a,*]

a Department of Chemistry "Ugo Schiff" and CSGI, University of Florence. Via della Lastruccia 3, 50019 Sesto Fiorentino (FI), Italy

b Nanovector s.r.l. Via Livorno 60, 10144 Torino, Italy

c Procarta Biosystems Ltd. Innovation Centre, Norwich Research Park, Norwich NR4 7GJ, UK

d Biological Chemistry Department, John Innes Centre, Norwich NR4 7UH, UKeKuecept Ltd. 16/17 Station Close, Potters Bar EN6 1TL, UK

f School of Pharmacy, University of East Anglia, Norwich NR4 7TJ, UKgNorwich Medical School, University of East Anglia, Norwich NR4 7UQ, UK

ABSTRACT

Bacterial resistance to antimicrobials is a global threat that requires development of innovative therapeu-tics that circumvent its onset. The use of Transcription Factor Decoys (TFDs), DNA fragments that act byblocking essential transcription factors in microbes, represents a very promising approach. TFDs requireappropriate carriers to protect them from degradation in biological fluids and transfect them throughthe bacterial cell wall into the cytoplasm, their site of action. Here we report on a bolaform cationicsurfactant, [12-bis-THA]Cl2,with proven transfection activity in vivo. By studying the physical-chemicalproperties of its aqueous solutions with light scattering, cryo-TEM, ζ-potential, absorption and fluores-cence spectroscopies, we prove that the bolaamphiphiles associate into transient vesicles which convertinto one-dimensional elongated structures over time. These surfactant assemblies complex TFDs withextremely high efficiency, if compared to common cationic amphiphiles. At $Z+/-= 11$, the nanoplexesare stable and have a size of 120 nm, and they form independently of the original morphology of the[12-bis-THA]Cl2aggregate. DNA is compacted in the nanoplexes, as shown through CD spectroscopy andfluorescence, but is readily released in its native form if sodium taurocholate is added.

Introduction

The "golden age" of antimicrobial breakthrough lasted 60 yearsfrom the discovery of penicillin in 1928 and supplied the largemajority of the drugs in current use [1]. However, bacteria nat-urally develop resistance, and contributory factors include: thelimited number of therapeutic targets that antimicrobials act on;the greater need due to the demands of modern medicine and agingpopulations; their misuse and overuse; most importantly, the fail-ure to find new antimicrobials to restock the pipeline [2]. Hence,antimicrobial resistance now constitutes a serious global threat[3]. Several alternative approaches to traditional small moleculediscovery are being developed, such as

bacteriophage therapy [4],antibodies [5], peptidomimetics [6] and nucleic acid therapies [7]. Previous work has demonstrated the efficacy of oligonucleotideTranscription Factor Decoys (TFDs) in controlling gene expressionin both eukaryotic [8] and bacterial cells [9]. However, oligonu-cleotide delivery faces an important challenge: how to transport anenzymatically labile, negatively charged molecule across biologicalfluids and through the bacterial cell wall.The bacterial membrane is negatively charged due to the pres-ence of phospholipids such as phosphatidylglycerol, cardiolipin, orphosphatidylserine [10]. The structural integrity of the membranecan be disrupted by initial electrostatic interaction using cationiclipids or surfactants. These are commonly employed as vectors fornon-viral gene delivery for therapeutic purposes, as they are effec-tive in the condensation and transfection of genetic material [11].Among others, cationic bolaamphiphiles are attractive targets forthe development of novel agents for nucleic acid delivery. They are aclass of surfactants constituted by two functional hydrophilic head-groups linked by a hydrophobic moiety. Dequalinium, an approvedantimicrobial, is a symmetric bolaform surfactant with two identical cationic headgroups. This molecule can complex plasmid DNA,protecting it from DNAse attack [12,13] and transporting it into cel-lular mitochondria in vitro [14], which share some of the propertiesof bacterial cell walls.In this work, we present a cationic bolaamphiphilic molecule,the symmetric 12-12_-(dodecane-1,12-diyl)-bis(9-amino-5,6,7,8-tetrahydroacridinium) chloride or [12-bis-THA]Cl2(Scheme 1),which has demonstrated the potential of delivering TFDs inpathogenic bacteria in animal models to block essential genes [15].The mechanism of transfection by [12-bis-THA]Cl2is being inves-tigated but it is assumed that binding is driven by electrostaticinteractions between the delocalized charge of the quaternaryammonium and the negatively charged components of the bacterialcell wall. In a similar manner, the interaction between the cationicbolaamphiphile and the negatively charged phosphate backbone ofthe oligonucleotide may lead to strong binding and condensationof the TFD, to render it resistant to nuclease digestion. Here we ana-lyze the physical-chemical properties of an aqueous suspension of[12-bisTHA]Cl2and its complex with a TFD to better understand itsproperties and inform strategies for its successful formulation.

MATERIALS & METHODS

[12-bis-THA]I2was synthesized by Shanghai Chempartner Co.,Ltd. [12-bis-THA]Cl2was obtained by anion exchange of [12-bis-THA]I2(see details in Supporting information).The TFD was manufactured and purified by HPLC at AxoLabs(Kulmbach, Germany). It consists of 77 base pairs deriving fromthe two following sequences: 5'CTT GGT TTT TCC AAG TAA TACGAC AAA ACT AGT TAA ATT TCA TTG AAG GAA TAA AAA TAT AATTAT AGA ATT GAT TA 3'; 5'TAA TCA ATT CTA TAA TTA TAT TTTTAT TCC TTC AAT GAA ATT TAA CTA GTT TTG TCG TAT TAC TTGGAA AAA CCA AG 3'. These oligonucleotides were suspended inwater at concentrations of 5×10^{-7}mol/L, mixed in equal volumesand annealed by heating to 95∘C for 2 min and then allowed tocool to room temperature. They were then ligated with T4 DNAligase overnight at 16∘C to form a monomeric circle, containingthe binding site for the transcription factor, and purified by ethanolprecipitation. Unligated products were removed by an ExonucleaseI digestion step following which the TFD was reprecipitated and itsconcentration adjusted to 1 mg/mL.Ultrapure water was obtained by means of a Millipore Elix®3water purification system.

[12-bis-THA]Cl2solutions were obtained by adding the powderin water and stirring by means of a vortex mixer (dilute solutions) orsonicating in an ultrasound bath (concentrated solutions). [12-bis-THA]Cl2/TFD nanoplexes were prepared by mixing the two aqueoussolutions in the appropriate proportions.

Inductively-coupled plasma atomic emission spectroscopy (ICP-AES) analysis of the residual iodine content in [12-bis-THA]Cl2 was carried out using a Varian 720-ES spectrophotometer (Melbourne,Australia). Elemental analysis was performed using a CHN-S Flash E1112 Thermo Finnigan elemental analyzer. Thermogravimetric Analysis (TGA) was performed on a TA Instruments SDT Q600 (New Castle, DE, USA). Between 4–7 mg of[12-bis-THA]Cl2 dry powder was weighed in an aluminum pan andheated up to 500∘C at 10∘C/min.Surface tension measurements were carried out on a TeclisInstruments (formerly I.T. Concept, Longessaigne, France) dynamictensiometer using the pendant drop method. All measurementswere carried out at 25∘C.Steady-state fluorescence of [12-bis-THA]Cl2solutions was measured on a LS50B spectrofluorometer (PerkinElmer, Italy). Thespectra were recorded in the corrected mode, between 300 and500 nm, with an excitation wavelength of 244 nm and 2.5 nm slits.For each sample, 20 acquisitions were collected at 25∘C and aver-aged.Light Scattering (LS) experiments were performed on aBrookhaven BI9000-AT digital autocorrelator, equipped with agreen laser (lambda = 532 nm; Torus, mpc3000, LaserQuantum, UK). Thescattered intensity was collected at 90∘, using a pinhole aperture of 200 _m. The samples were placed in glass tubes, which wereimmersed in a thermostatic cell filled with decahydronaphtaleneto match the glass refractive index. The scattering intensity ofpure toluene was used as a standard. In the Rayleigh–Gans–Debyeregime [16], the intensity Isof the light scattered by a monodis-perse colloidal solution is a function of the number concentration of particles, c, and the scattering vector→q. The modulus q is equalto:

$$q = \frac{4\pi n_s}{\lambda} sin\frac{\theta}{2}$$

with λ being the wavelength of the incident light I0, ns the refractive index of the medium, and Θ the collection angle of the scattered light. As such, Is can be written:

$$I_s\left(\vec{q}, c\right) = I_0 \frac{f\left(\theta\right)}{R_0^2} cV_s \Delta\rho^2 V_P^2 P\left(\vec{q}\right) S\left(\vec{q}, c\right)$$

where f(Θ) is a geometrical factor, R0 the sample-to-detector distance, Vs and VP the solvent and particle volumes respectively, and_r the "contrast" or difference of refractive indexes between particles and suspension medium. P (→q) and S (→q, c) are, respectively, the form factor and the structure factor (the latter is equal to 1 in very dilute solutions). In a Dynamic Light Scattering (DLS) experiment, we exploit the time correlation function of the random fluctuations in scattered intensity due to Brownian motions in solution[17]. By fitting the autocorrelation function with the appropriate algorithms, one can derive the diffusion coefficient of the particle sand, by assuming a spherical shape, their average hydrodynamic diameter (DH) through the Stokes–Einstein law:

$$D_0 = \frac{k_B T}{3\pi\eta D_H}$$

where kB is Boltzmann's constant, T the absolute temperature, and   the medium's viscosity. Depending on the samples, the fitting of the autocorrelation function of the scattered intensity at 90∘wasperformed either with the cumulant method [17] or by inverting the autocorrelation function with the CONTIN algorithm [18].

ζ-potentials were obtained from electrophoretic mobility measurements, performed on a Brookhaven Zeta PALS (Phase AnalysisLight Scattering) instrument, equipped with a laser operating at 659 nm. The scattered intensity was collected at 15∘to determine the electrophoretic mobility; the   -potentials were then calculatedthrough the Helmholtz–Smoluchowski equation. Cryogenic transmission

electron microscopy (cryo-TEM) exper-iments were carried out at the Institut de Biologie Paris-Seine,Université Pierre et Marie Curie, Paris, France. The specimens wererapidly frozen by plunge-freezing in liquid ethane, cooled by liquidnitrogen (LEICA EM CPC, Wien, Austria). The cryofixed specimenswere mounted into a Gatan cryoholder (Gatan inc., Warrendale,PA) for direct observation at −180◦C in a JEOL 2100HC cryo-TEMoperating at 200 kV with a LaB6 filament. Images were recordedin zero-loss mode with a Gif Tridiem energy-filtered-CCD camera,equipped with a 2k × 2k pixel-sized chip (Gatan Inc., Warrendale,PA). Acquisition was accomplished with the Digital Micrograph software (version 1.83.842, Gatan inc., Warrendale, PA).Circular Dichroism (CD) measurements were performed usinga Jasco J-715 spectropolarimeter. The solutions were contained inquartz cells with optical path lengths of 1 cm or 1 mm dependingon the sample. CD spectra were recorded at room temperature inthe 200–400 nm range.UV–vis spectra were acquired at 25◦C on a Varian Cary 100Bio spectrophotometer, equipped with a Varian Cary temperature controller.

RESULT & DISCUSSION

The compound [12-bis-THA]2+is a bolaform cationic surfactantof molecular formula C38H52N4. The iodide salt is soluble in DMSOand methanol, and only sparingly in water. When DMSO solutionsare dispersed in water by vigorous stirring, a suspension of crystal-lites is formed, which precipitates within some hours. On the otherhand, the chloride salt (Scheme 1) shows higher water solubility,which in principle implies a higher bioactivity. For these reasons,the subject of this investigation was the chloride salt.At 25◦C [12-bis-THA]Cl2dissolves in water up to 1 mg/mL(1.6 × 10−3mol/L). Such difference in solubility with respect tothe iodide salt is expected: indeed, ions with similar polarizabil-ities (e.g. two "soft" ions such as [12-bis-THA]2+and I−) tend toform stronger ion pairs and are therefore less soluble salts. Exam-ples of different phase behaviors arising from a simple counterionexchange are very common in colloid science: for instance, aqueoussolutions of hexadecyltrimethylammonium bromide (CTAB) andchloride (CTAC) are characterized by the presence of large elon-gated micelles in the former case and globular micelles in the latter [19], because of the higher association of the counterion with thecationic assemblies.Below the solubility limit, the system appeared slightlyopalescent as prepared, indicating the presence of self-assemblyaggregates of some kind (large enough to scatter visible light).At higher concentrations, a saturated solution was obtained. Thebinary systems containing 80 wt%, 50 wt%, 30 wt%, and 20 wt%water showed no evidence of formation of liquid-crystallinephases.

Aqueous solution behavior of [12-bis-THA]Cl2

Bolaform surfactants are known to self-assemble in solution,leading to a wide range of aggregate morphologies dependingon their chemical structure: tubules, fibers, ribbons, micellesand vesicles are among the common nano- and micromet-ric supramolecular structures reported for bolaamphiphiles [20].Dynamic Light Scattering (DLS) was used here to infer thehydrodynamic size of [12-bis-THA]Cl2assemblies. For relativelyconcentrated dispersions (1.0 × 10−3mol/L), the DLS analysisshown in Fig. 1 yielded Z-average hydrodynamic sizes of120 ± 20 nm, with elevated polydispersity (∼30%).For samples more dilute than 4.5 × 10−4mol/L, the scatteringintensity was only slightly higher than that of pure water. This islikely due to a combination of low concentration and low contrastbetween the surfactant assemblies and water (see Eq. (2)). There-fore, the size and polydispersity of [12-bis-THA]Cl2aggregates indilute solutions cannot be determined by means of DLS. Similarly,the very low scattering invalidated any attempts to reliably mea-sure the _-potential

of these colloidal objects. Even synchrotronsmall-angle X-ray scattering (SAXS) experiments on aqueous solu-tions of [12-bis-THA]Cl2at maximum solubility were unsuccessful,yielding spectra that were very similar to those of pure water.For these reasons we turned to cryogenic transmission electronmicroscopy (cryo-TEM) in order to determine the type of aggre-gates formed by [12-bis-THA]Cl2. Imaging of a freshly preparedsolution (1.8×10^{-4}mol/L, Fig. 2) showed that this surfactant formsa mixture of elongated colloidal structures and (most probablymonolayer) vesicular assemblies termed bolasomes. The formerappear to be short needle-like objects [21], while the latter haveirregular shapes and diameters around or below 100 nm, and theyappear sometimes aggregated in groups of 2–3 vesicles. Despitethe difference in [12-bis-THA]Cl2concentration, this compares wellwith the DLS analysis. Similarly, in a work involving aqueous solu-tions of Dequalinium, a cationic bolaamphiphile with a very similarchemical structure to [12-bis-THA]Cl2, the authors observed bolasomes with sizes of 300 nm (determined by negative-staining TEM)[12].A precise knowledge of the threshold concentration for aggre-gation is of primary importance to formulate amphiphilic drugs inwater or physiologically relevant media; therefore, we focused ourefforts in finding the onset concentration for the formation of [12-bis-THA]Cl2aggregates. Previous literature on symmetric bolaformsurfactants demonstrates that very similar thermodynamic princi-ples of self-assembly apply as for classic surfactants [22]. However,the driving force for aggregation is lower than for the correspond-ing single-headed amphiphiles, due to the presence of the secondpolar headgroup, and this leads to generally higher critical aggre-gate concentrations (CAC). We attempted to determine a CAC for[12-bis-THA]Cl2in water by tensiometry with the pendant dropmethod. The surface tension vs. time curves showed a monotoni-cally decreasing trend without reaching the expected equilibriumplateau (see Fig. S2). One tentative explanation might be found bylooking more closely at the structure of the bolaamphiphile: thepositive charge of the headgroups is mostly localized on the centralamino-pyridinium ring, while the benzene and cycloalkyl groupsare less polar. In addition, the alkyl chains connecting the two head-groups should kink to accommodate both polar ends into the watersubphase. Likely, the adsorption at the interface in the energeticallyfavored conformation is a slow process, if compared to traditionalsingle-chained surfactants. While a clear drop in surface tensionis observed, its equilibrium value could not be appreciated in ourexperimental time frame (up to several hours).As mentioned, the very low scattered intensity even in relativelyconcentrated samples prevents a reliable determination of the CACwith static light scattering. Also, the traditional method involvingpyrene as a fluorescent probe for hydrophobic environment [23]could not be used, due to mutual interference between pyrene and[12-bis-THA]Cl2fluorescence.Indeed, [12-bis-THA]Cl2shows absorption and fluorescencebehavior due to its quinolinium subunits [24]. The absorption andfluorescence of aqueous solutions of [12-bis-THA]Cl2at differentconcentrations, ranging from 8.0×10^{-6}mol/L to 1.6×10^{-3}mol/L,were measured and analyzed (Figs. S3–S4). The fluorescence spec-tra were characterized by a relatively broad band centered at373 nm, the intensity of which linearly increased with growing surfactant concentration until 4.0×10^{-5}mol/L and reached itsmaximum at 8.0×10^{-5}mol/L (Fig. 3). This behavior is consistentwith the increase in concentration of isolated fluorophores (surfac-tant monomers). For higher concentrations, the linearity was lostand the intensity began decreasing. This intensity decrease couldbe an indication of the self-assembly of [12-bis-THA]Cl2, where theclose proximity between fluorophores in the ground state results ina significant fluorescence self-quenching. A data analysis through aclassic Stern–Volmer approach [25] could provide important infor-mation about this concentration-dependent behavior. However, inour experimental conditions, the absorption properties of [12-bis-THA]Cl2gave also rise to a strong primary inner filter effect, whichcontributed to the concentration-dependent decrease of fluores-cence intensity. The contributions from the two phenomena aredifficult to separate, making the CAC determination through theStern–Volmer approach very complicated [25].An analysis of previous literature shows

that in some instancesit is not possible to determine a critical micellar concentration(cmc) or a CAC for bolaamphiphilic molecules in solution [26,27].In the case of Dequalinium, isothermic titration calorimetry, laserlight scattering and Monte Carlo simulations cast doubts on theexistence of a clear-cut monomer-to-aggregate transition [28]. Bycomparing our experimental results with the existing literature,we could not identify any clear-cut aggregation threshold, whichcan be outside the concentration range accessible with our exper-imental techniques. Alternatively, the surfactant may aggregate inoligomers undetectable with DLS: in this case, aggregation wouldnot proceed through pseudophase separation, but would rather bea stepwise phenomenon.The slow equilibration processes of [12-bis-THA]2+at theair/water interface prompted us to monitor a possible timeevolution of the size of the self-assemblies in solution. LightScattering (LS) measurements were performed on solutions atdifferent concentrations (9.0 × 10−4, 4.5 × 10−4, 3.0 × 10−4, and1.5 × 10−4mol/L) and temperatures (4◦C, 25◦C and 37◦C). LSexperiments were performed right after the preparation of thesamples (t = 0) and 48 h later (t = 48 h); the results are presentedin Fig. 4.By comparing the I/I0values at t = 0 and t = 48 h for the threebatches of samples, it can be noticed that the solutions stored at 4◦Cretained the scattering intensity observed immediately after prepa-ration (even 7 days later, data not shown). The solutions storedat higher temperatures (25◦C and 37◦C) underwent a dramaticreduction of intensity over time. After ruling out the presence of aprecipitate, the analysis of the autocorrelation functions revealed a decrease of the average size. For example, the mean hydrodynamicdiameter of the aggregates in a 9.0 × 10−4mol/L solution stored at25◦C was reduced by 50% over 24 h, and a new population of scat-tering objects appeared after 5 days with a diameter below 10 nm(Figs. S5–S6). On the other hand, the sample stored at 4◦C retainedthe same average size. We verified the irreversibility of this phe-nomenon by cooling to 4◦C the samples that had been stored at25◦C and 37◦C, and by stirring with a vortex mixer: in neither casedid the systems revert to their initial state.Since we could rule out precipitation, chemical reactions andmolecular cleavage by means of UV–vis and1H NMR analyses(data not shown), the phenomenon originating the loss of scat-tered intensity and the reduction of the apparent hydrodynamicdiameters calculated via the Stokes–Einstein equation must bea morphological transformation of the colloidal assemblies. Theintensity of light scattering scales with the squares volume of thescattering objects (Eq. (2)), so that large colloidal aggregates con-tribute a correspondingly higher scattering intensity. Also, we recallthat cryo-TEM imaging (Fig. 2) evidenced the co-existence of elon-gated objects, probably fibers, with bolasomes. The hydrodynamicsize of a rod-like structure can be calculated according to Eq. (4):

$$R_H = \frac{L}{2s - 0.19 - \frac{8.24}{s} + \frac{12}{s^2}}$$

where L = length of the rod and s = ln (L/r), with r = radius of the rod [29]. Assuming for example a rigid rod of about 100 nm length (suchas those observed in Fig. 2) and 1 nm radius (compatible with [12-bis-THA]Cl2's molecular size), we obtain a hydrodynamic diameterDH≈ 26 nm. If the bolasomes were transient aggregates formedby the bolaamphiphile upon dissolution, converting entirely intofibers over time, this would account for the lower scattering inten-sity and smaller calculated size. Such transformation is clearly ofspontaneous nature, as it proved to be irreversible: this evidencestrongly hints at the fact that the final morphology represents thestate of thermodynamic equilibrium of the system. Turning to geometrical considerations, the type and shape ofself-assembly aggregates in solution can be predicted by the pack-ing parameter of the amphiphile: P = V lc/A0, where V is the volumeof the hydrophobic chain, lcits length, and A0the polar head-group area [30]. In particular, vesicles will form for ½ < P < 1, whileP ≈ 1 leads to infinite flat bilayers. A symmetrical bilayer does nothave a spontaneous curvature, therefore the existence of vesiclesis

thermodynamically unfavorable for a binary system [31]. Nev-ertheless, exceptions to this rule exist. Let us consider a classicexample among single-headed surfactants: NaAOT is characterizedby $P \approx 1$ and it forms lamellar and bicontinuous (locally flat) cubicphases in water. However it also forms stable unilamellar vesi-cles at very low surfactant concentration (CVC = 7.8×10^{-3}mol/L) [32], where curved vesicles are thermodynamically more stablethan isolated bilayer stacks. In this case, the curvature is allowedthanks to the bilayer asymmetry achieved by packing a lower num-ber of molecules in the inner leaflet of the vesicle [31]. On theother hand, the symmetrical shape of the [12-bis-THA]Cl2molecule would suggest elongated structures such as fibers or ribbons ratherthan spherical vesicles. The vesicular aggregates that form withan energy input, such as vortexing or sonicating to facilitate dis-solution, are therefore short-lived and the bolasomes observedwith cryo-TEM in a freshly prepared solution of [12-bis-THA]Cl2are irreversibly destroyed over time to form fibers (Scheme 2). Inthe abovementioned case of Dequalinium, where bolasomes wereobserved, no temporal evolution of these structures was reportedby the authors.The [12-bis-THA]Cl2bolasomes are nevertheless metastable at4◦C, when thermal fluctuations are probably not enough to disruptany intermolecular forces, maybe comprising weak _- _ interac-tions between the aromatic rings, which contribute to the bolasomeassemblies. Therefore, by playing with storage temperature, we areable to control the morphology of [12-bis-THA]Cl2aggregates.

Interaction of [12-bis-THA]Cl2with DNA

DNAAs mentioned, [12-bis-THA]Cl2is mixed with an oligonucleotidetranscription factor decoy to form a novel type of antimicrobial;therefore, the interaction between the bolaamphiphile and DNAwas studied using a model TFD constituted by a double-strandedoligonucleotide, composed of 77 base pairs and especially rich inthymine and adenine.Aqueous solutions of this TFD and [12-bis-THA]Cl2were mixedin proportions leading to a positive-to-negative charge ratioZ+/−= 11 (with the bolaamphiphile at 1.8×10^{-4}mol/L). The disper-sion became opalescent, and LS experiments showed a scatteredintensity about 20 times higher than for the neat bolaamphiphile.DLS analysis confirmed the presence of aggregates in suspension,with hydrodynamic sizes of 150 ± 20 nm and about 20% polydisper-sity. A cryo-TEM image of these aggregates is shown in Fig. S7. The _-potentials were in the order of 30 ± 2 mV, in agreement with theexcess of positively charged species. These experimental findingssuggest complexation of the TFD by [12-bis-THA]Cl2, as expectedby charge compensation considerations and by the vast body ofliterature about the formation of nanoplexes, i.e. amphiphile-DNAcomplexes, when DNA is added to a cationic surfactant solution.By maintaining an excess of positive charge, the TFD complexationwould be complete, and the interaction of the nanoplex with thebacterial membranes, rich in negatively charged lipids, would befavored. Importantly, the same type of aggregates were obtained regard-less of the "age" of the initial [12-bis-THA]Cl2solution, as shown byDLS analysis in Fig. S8. This proves that the properties of the [12-bis-THA]Cl2/TFD complex are independent on the morphology of theinitial bolaamphiphile assemblies. From an applicative standpoint,this peculiarity could represent a great advantage in the designof the final formulations to be used as antibacterial agents, eventhough other variables, such as time and temperature stability ofthe complex, necessitate further evaluation.When DNA forms complexes upon interaction with cationic sur-factants, it assumes a condensed state and loses its chiral secondarystructure. In this case, the interaction between nucleic acids andthe cationic surfactant can be evaluated using CD spectroscopy,that exploits the differential absorption of left- and right-circularlypolarized electromagnetic radiation by a sample [33]. The TFD,dissolved in ultrapure water at the concentration of 90 _g/mL (cor-responding to 1.9×10^{-6}mol/L), was titrated with the aqueousbolaamphiphile solution up to and above the isoelectric point. The[12-bis-

THA]Cl2concentration in solution varied between 0 and1.7 $\times$ 10−4mol/L, corresponding to a Z+/−variation from 0 to 1.8.These values are far from the Z+/−= 11 used in the earlier tests, sincewe are now looking at the transformations occurring near the iso-electric point. Fig. 5a reports five representative spectra obtainedin this titration experiment (the entire set of data and the corre-sponding absorption spectra are available in Figs. S9–S10).The initial shape of the CD spectrum is characteristic of DNAin B-conformation: it presents a positive peak at 280 nm, originat-ing from base stacking, and a negative peak at 249 nm, due to thehelicity of the double strand. In particular, the negative band is quiteintense, as typical of chiral DNA strands with high A + T base content[33]. The CD signal of the TFD starts changing upon the very firstaddition of [12-bis-THA]Cl2, as the two oppositely charged species interact in solution. The CD spectrum varies slightly even abovethe isoelectric point (Z+/−= 1.0); at Z+/−= 1.5, the bands of the orig-inal spectrum have completely flattened out, and further additionof surfactant does not lead to any change. This evidence is sum-marized in Fig. 5b, where the trend of the CD is plotted, for thewavelengths 249 nm and 280 nm, as a function of Z+/−. It is quiteevident how the intensity reaches zero just above the isoelectricpoint. The same experimental trend has been evidenced for the con-densation of different types of DNA by traditional [34] and geminicationic surfactants [35,36].By observing the absorption spectra in Fig. S10, one can noticethe condensation of the oligonucleotide into packed complexes asthe concentration of bolaamphiphile increases: the higher baselineis a clear signature of the solution's growing opalescence due to thepresence of colloidal aggregates. This behavior can be representedby the plot of the absorbance at 400 nm as a function of [12-bis-THA]Cl2concentration (Fig. 6).To corroborate these results, we monitored the change in fluo-rescence intensity of [12-bis-THA]Cl2upon titration with the TFD.The results are presented in Fig. 7 as a function of 1/Z+/−. Start-ing from a large excess of positive charge (Z+/−= 17, or 1/Z+/−= 0.05in Fig. 7), we witnessed an almost linear increase of fluorescenceintensity up to a plateau; in correspondence of Z+/−= 1, the fluores-cence was quenched and kept decreasing with further increase ofTFD. These results are in good agreement with those obtained byCD spectroscopy.The electrostatic interaction between the oppositely chargedspecies is the main driving force leading to the 1:1 complexbetween [12-bis-THA]Cl2and DNA, similarly to what found byother authors for classic cationic surfactants [37], gemini sur-factants [35,36], and bolaamphiphiles [38,39]. This can be anadvantage compared to certain cationic species which necessitate higher Z+/−values to saturate the DNA: for example, other authorsreported Z+/−= 4 for poly(ethylenimine) [40], Z+/−= 10 for TTAB andZ+/−= 30 for DTAB [34], which necessitate higher amounts of typi-cally cytotoxic compounds to reach full condensation. To accountfor [12-bis-THA]Cl2's higher binding efficiency, it is well knownthat planar aromatic and heterocyclic compounds, even uncharged,can interact with DNA thanks to different types of interactions: ina groove-bound fashion (a mix of hydrophobic, electrostatic, andhydrogen-bonding interactions), or by intercalation between theDNA base pairs [41]. In our case, [12-bis-THA]Cl2presents two pos-itively charged condensed heterocyclic headgroups, each carryingan amine substituent: it is likely that DNA complexation by thisbolaamphiphile results from several forces at play.When DNA is packed into nanoplexes, it assumes a condensedstate similar to denaturation. However, for the TFD to performits therapeutic action, it needs to be renatured. In other words,the cationic carrier must release its DNA payload at the desireddelivery site and in a biologically active form. Here, we show thatthe TFD/[12-bis-THA]Cl2complex is reversible by breaking it withsodium taurocholate (NaTC), a common detergent used for the sol-ubilization of lipids and previously employed for the dissociationof DNA/cationic lipid complexes [42]. Indeed, due to its negativecharge, NaTC competes with the TFD's polyanionic backbone forinteraction with [12-bis-THA]Cl2.The initial aqueous system contained the complex at Z+/−= 1.5([12-bis-THA]Cl2: 2.5 $\times$ 10−4mol/L), a condition in which thebolaamphiphile is in excess and the oligonucleotide is completelycondensed, as demonstrated earlier in this work. The complexwas titrated with aliquots of

NaTC, and the resulting CD andUV spectra are presented in Supplementary information (Figs.S11–S12). At low NaTC concentration, the CD spectra are flat, mean-ing that the detergent is not enough concentrated to displace theTFD/bolaamphiphile complex. The first spectrum where the CD sig-nal starts showing the features of the oligonucleotide is the one for$[NaTC] = 1.4 \times 10^{-2}$mol/L. From this point on, the intensity of thebands with maxima at 248 nm and 278 nm increases; the maxi-mum amplitude is found at $[NaTC] = 4.2 \times 10^{-2}$mol/L, and furtheradditions of detergent do not produce any variation. These trendsare plotted in Fig. 8b, displaying the CD at 248 and 278 nm. Theresults just described are summarized in Fig. 8a, which comparesthe CD spectra for the following representative samples: free TFD;condensed TFD when $Z+/-= 1.5$; the $Z+/-= 1.5$ complex in the pres-ence of 7.0×10^{-2}mol/L NaTC; free TFD with the same amount ofNaTC (but no bolaamphiphile). This figure clearly evidences howthe TFD/[12-bis-THA]Cl2complex is broken in the presence of tau-rocholate, and the free renatured TFD is completely recovered. These results show that the therapeutically active TFD can bereleased from the complex. Such information is important in viewof the biomedical applications of the TFD/[12-bis-THA]Cl2complex.The release mechanism we described in the presence of a simplemolecule as NaTC should be adapted to the conditions found in thebacterial cytoplasm. For example, the complex could be formulatedin a stimuli-responsive fashion (e.g. to pH, redox potential, etc.) inorder to free the TFD only once it has crossed the bacterial cellwall. Further studies are underway to better define the conditionsof existence of this complex and to design the best formulationthat will meet the requirements for therapeutic action at the sitesof infection in the human body.

CONCLUSIONS

Antibiotic therapy based on transcription factor decoys is anovel and specific approach to fight antimicrobial resistance byblocking essential genes in pathogenic bacteria. One of the mainchallenges associated with their use is the necessity of finding theright carrier to transfect them through the bacterial cell wall and toprotect them from DNA-degrading enzymes, both in the biologicalfluids and in the bacterial cytoplasm. In this work, we have pre-sented a new cationic bolaamphiphilic surfactant, [12-bis-THA]Cl2which has proven to meet these requirements in vivo. We haveinvestigated its behavior in aqueous solution, proving that it formsa mixture of vesicular and elongated nanostructured assembliesupon dissolution; the latter structure is the thermodynamicallystable morphology into which the system converts entirely overtime. Interestingly, storing the samples at 4∘C allowed us to trapthe system in its metastable vesicular form. Next, we have evalu-ated its interaction with a model TFD, confirming the formation ofnanoplexes: the main driving force for DNA condensation appearsto be the electrostatic interaction between the oppositely chargedspecies, the cationic bolaamphiphile and the polyanionic TFD back-bone. The types of aggregates were independent on the morphologyof the initial bolaamphiphile assemblies.Importantly, we have demonstrated that this complex can dis-sociate in the appropriate conditions, for example in the presenceof a competing anion such as taurocholate, to release the TFD in itsbiologically active form. These findings evoke the possibility to for-mulate the nanoplexes as smart, stimuli-responsive nanocarriersallowing the controlled release of the TFD. Further work will focuson the elucidation of additional aspects, like time and temperaturestability of the complex, that still need to be clarified, in view of thedesign of [12-bis-THA]Cl2-based formulations that will accomplishtransport, protection, transfection and release of TFDs to perform asuccessful therapeutic action.

References

[1] L.L. Silver, Challenges of antibacterial discovery, Clin. Microbiol. Rev. 24(2011) 71–109, http://dx.doi.org/10.1128/CMR.00030-10.

[2] D.J. Payne, M.N. Gwynn, D.J. Holmes, D.L. Pompliano, Drugs for bad bugs:confronting the challenges of antibacterial discovery, Nat. Rev. Drug Discov. 6(2007) 29–40, http://dx.doi.org/10.1038/nrd2201.

[3] E. Leung, D.E. Weil, M. Raviglione, H. Nakatani, The WHO policy package tocombat antimicrobial resistance, Bull. World Health Organ. 89 (2011)390–392, http://dx.doi.org/10.2471/BLT.11.088435.

[4] M. Kutateladze, R. Adamia, Bacteriophages as potential new therapeutics toreplace or supplement antibiotics, Trends Biotechnol. 28 (2010) 591–595,http://dx.doi.org/10.1016/j.tibtech.2010.08.001.

[5] A. Extance, Biologics target bad bugs, Nat. Rev. Drug Discov. 9 (2010)177–178, http://dx.doi.org/10.1038/nrd3129.

[6] T.K. Lind, P. Zieli´nska, H.P. Wacklin, Z. Urba´nczyk-Lipkowska, M. Cárdenas,Continuous flow atomic force microscopy imaging reveals fluidity andtime-dependent interactions of antimicrobial dendrimer with model lipidmembranes, ACS Nano 8 (2014) 396–408, http://dx.doi.org/10.1021/nn404530z.

[7] B.L. Geller, Antibacterial antisense, Curr. Opin. Mol. Ther. 7 (2005) 109–113(PMID:15844617).

[8] M.J. Mann, V.J. Dzau, Therapeutic applications of transcription factor decoyoligonucleotides, J. Clin. Invest. 106 (2000) 1071–1075, http://dx.doi.org/10.1172/JCI11459.

[9] M. McArthur, M.J. Bibb, Manipulating and understanding antibioticproduction in Streptomyces coelicolor A3(2) with decoy oligonucleotides,Proc. Natl. Acad. Sci. 105 (2008) 1020–1025, http://dx.doi.org/10.1073/pnas.0710724105.

[10] K. Komagata, K.-I. Suzuki, Lipid and cell-wall analysis in bacterial systematicsMethods in Microbiology, vol. 19, Academic Press Ltd., 1987.

[11] D. Simberg, D. Danino, Y. Talmon, A. Minsky, M.E. Ferrari, C.J. Wheeler, et al.,Phase behavior, DNA ordering, and size instability of cationic lipoplexes:relevance to optimal transfection activity, J. Biol. Chem. 276 (2001)47453–47459, http://dx.doi.org/10.1074/jbc.M105588200.

[12] V. Weissig, J. Lasch, G. Erdos, H.W. Meyer, T.C. Rowe, J. Hughes, DQAsomes: anovel potential drug and gene delivery system made from DequaliniumTM,Pharm. Res. 15 (1998) 334–337, http://dx.doi.org/10.1023/A:1011991307631.

[13] J. Lasch, A. Meye, H. Taubert, R. Koelsch, J. Mansa-ard, V. Weissig, DequaliniumTM vesicles form stable complexes with plasmid DNA which are protectedfrom DNase attack, Biol. Chem. 380 (1999), http://dx.doi.org/10.1515/BC.1999.080.

[14] V. Weissig, C. Lizano, V.P. Torchilin, Selective DNA release fromDQAsome/DNA complexes at mitochondria-like membranes, Drug Deliv. 7(2000) 1–5, http://dx.doi.org/10.1080/107175400266722.

[15] M. McArthur, Transcription factor decoys for the treatment and prevention ofinfections caused by bacteria including clostridium difficile. US Patent App.13/802, 103, 2013.

[16] H.C. van de Hulst, Light Scattering by Small Particles, Dover Publications, NewYork, 1991.

[17] P.N. Pusey, Dynamic light scattering, in: Peter Lindner, Thomas Zemb (Eds.),Neutrons X-Rays Light Scattering Methods Applied Soft Condensed Matter,North Holland, 2002.

[18] S.W. Provencher, A constrained regularization method for inverting datarepresented by linear algebraic or integral equations, Comput. Phys. Commun.27 (1982) 213–227, http://dx.doi.org/10.1016/0010-4655(82) 90173-4.

[19] J. Ulmius, B. Lindman, G. Lindblom, T. Drakenberg, 1H, 13C, 35Cl, and 81BrNMR of aqueous hexadecyltrimethylammonium salt solutions: solubilization,viscoelasticity, and counterion specificity, J. Colloid Interface Sci. 65 (1978)88–97, http://dx.doi.org/10.1016/0021-9797(78) 90261-8.

[20] N. Nuraje, H. Bai, K. Su, Bolaamphiphilic molecules: assembly andapplications, Prog. Polym. Sci. 38 (2013) 302–343, http://dx.doi.org/10.1016/j.progpolymsci.2012.09.003.

[21] L. Ziserman, H.-Y. Lee, S.R. Raghavan, A. Mor, D. Danino, Unraveling themechanism of nanotube formation by chiral self-Assembly of amphiphiles, J.Am. Chem. Soc. 133 (2011) 2511–2517, http://dx.doi.org/10.1021/ja107069f.

[22] R. Nagarajan, Self-assembly of bola amphiphiles, Chem. Eng. Commun. 55(1987) 251–273, http://dx.doi.org/10.1080/00986448708911931.

[23] K. Kalyanasundaram, J.K. Thomas, Environmental effects on vibronic bandintensities in pyrene monomer fluorescence and their application in studiesof micellar systems, J. Am. Chem. Soc. 99 (1977) 2039–2044, http://dx.doi.org/10.1021/ja00449a004.

[24] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third edition, Springer,2006.

[25] A. Memoli, L.G. Palermiti, V. Travagli, F. Alhaique, Effects of surfactants on thespectral behaviour of calcein(II): a method of evaluation, J. Pharm. Biomed.Anal. 19 (1999) 627–632, http://dx.doi.org/10.1016/S0731-7085(98)00229-5.

[26] F. Menger, S. Wrenn, Interfacial and micellar properties of bolaformelectrolytes, J. Phys. Chem. 78 (1974) 1387–1390, http://dx.doi.org/10.1021/j100607a600.

[27] S. Yiv, K.M. Kale, J. Lang, R. Zana, Chemical relaxation and equilibrium studiesof association in aqueous solutions of bolaform detergents. 1.Dodecane-1,12-bis(trimethylammonium bromide), J. Phys. Chem. 80 (1976)2651–2655, http://dx.doi.org/10.1021/j100565a006.

[28] J. Lasch, A. Hildebrand, Isothermic titration calorimetry to study cmcs ofneutral surfactants and of the liposome-forming bolaamphiphile Dequalinium®, J. Liposome Res. 12 (2002) 51–56, http://dx.doi.org/10.1081/LPR-120004776.

[29] W. Van de Sande, A. Persoons, The size and shape of macromolecularstructures: determination of the radius, the length and the persistance lengthof rod-like micelles of dodecyldimethylammonium chloride and bromide, J.Phys. Chem. 89 (1985) 404–406, http://dx.doi.org/10.1021/j100249a007.

[30] J.N. Israelachvili, Intermolecular and Surface Forces, 3rd ed., Elsevier Acad.Press, 2011.

[31] V. Guida, Thermodynamics and kinetics of vesicles formation processes, Adv.Colloid Interface Sci. 161 (2010) 77–88, http://dx.doi.org/10.1016/j.cis.2009.11.004.

[32] R. Saha, P.K. Verma, R.K. Mitra, S.K. Pal, Structural and dynamicalcharacterization of unilamellar AOT vesicles in aqueous solutions and theirefficacy as potential drug delivery vehicle, Colloids Surf. B Biointerfaces 88(2011) 345–353, http://dx.doi.org/10.1016/j.colsurfb.2011.07.012.

[33] J. Kypr, I. Kejnovska, D. Renciuk, M. Vorlickova, Circular dichroism andconformational polymorphism of DNA, Nucleic Acids Res. 37 (2009)1713–1725, http://dx.doi.org/10.1093/nar/gkp026.

[34] V.M. Jadhav, R. Valaske, S. Maiti, Interaction between 14mer DNAoligonucleotide and cationic surfactants of various chain lengths, J. Phys.Chem. B. 112 (2008) 8824–8831, http://dx.doi.org/10.1021/jp8017452.

[35] T. Zhou, G. Xu, M. Ao, Y. Yang, C. Wang, DNA compaction to multi-molecularDNA condensation induced by cationic imidazolium gemini surfactants,Colloids Surf. Physicochem. Eng.

[36] Z. Pietralik, R. Krzyszto´n, W. Kida, W. Andrzejewska, M. Kozak, Structure andconformational dynamics of DMPC/Dicationic surfactant andDMPC/Dicationic Surfactant/DNA systems, Int. J. Mol. Sci. 14 (2013)7642–7659, http://dx.doi.org/10.3390/ijms14047642.

[37] D. Luo, M.W. Saltzman, Synthetic DNA delivery systems, Nat. Biotechnol. 18(2000) 33–37, http://dx.doi.org/10.1038/71889.

[38] B. Sohrabi, V. Khani, A.A. Moosavi-Movahedi, P. Moradi, Investigation ofDNA–cationic bolaform surfactants interaction with different spacer length,Colloids Surf. B Biointerfaces 110 (2013) 29–35, http://dx.doi.org/10.1016/j.colsurfb.2013.04.032.

[39] M. Khan, C.Y. Ang, N. Wiradharma, L.-K. Yong, S. Liu, L. Liu, et al.,Diaminododecane-based cationic bolaamphiphile as a non-viral gene deliverycarrier, Biomaterials 33 (2012) 4673–4680, http://dx.doi.org/10.1016/j.biomaterials.2012.02.067.

[40] K. Kunath, Low-molecular-weight polyethylenimine as a non-viral vector forDNA delivery: comparison of physicochemical properties, transfectionefficiency and in vivo distribution with high-molecular-weightpolyethylenimine, J. Controlled Release 89 (2003) 113–125, http://dx.doi.org/10.1016/S0168-3659(03)00076-2.

[41] E.C. Long, J.K. Barton, On demonstrating DNA intercalation, Acc. Chem. Res. 23(1990) 271–273, http://dx.doi.org/10.1021/ar00177a001.

[42] C. Boulanger, C. Di Giorgio, J. Gaucheron, P. Vierling, Transfection withfluorinated lipoplexes based on new fluorinated cationic lipids and in thepresence of a bile salt surfactant, Bioconjug. Chem. 15 (2004) 901–908, http://dx.doi.org/10.1021/bc049942+.
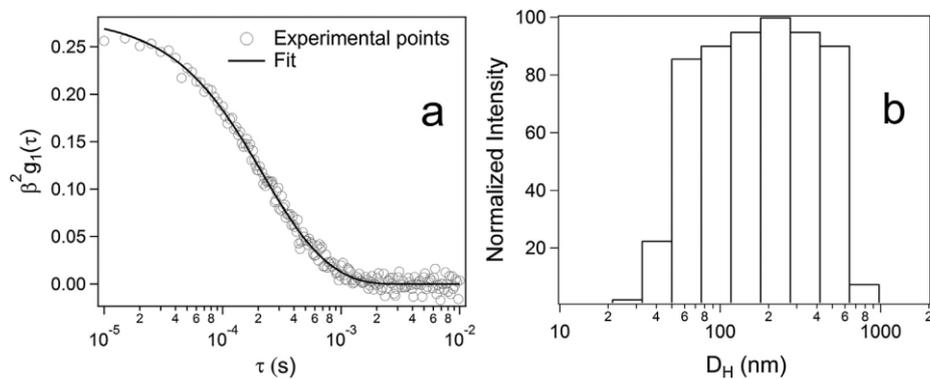
FIGURES

Fig. 1. a) DLS autocorrelation function corresponding to a solution of 1.0×10^{-3} mol/L [12-bis-THA]Cl2, and fit of the experimental data with the cumulant method. b) Intensity-weighed hydrodynamic size distribution obtained by CONTIN analysis of a).
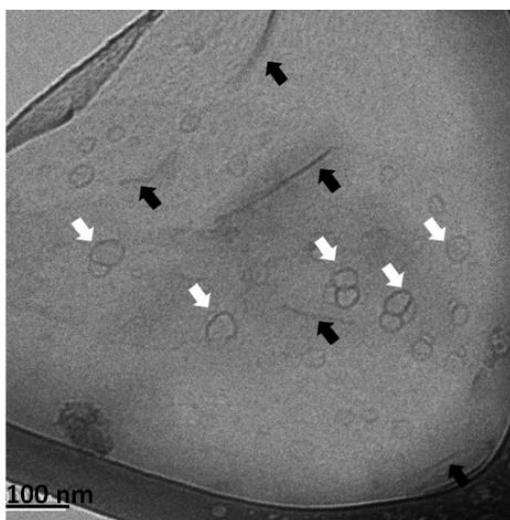
Fig. 2. Cryo-TEM image of an aqueous solution of [12-bis-THA]Cl2, 1.8×10^{-4} mol/L, as prepared. White arrows: vesicular structures; black arrows: needle-like structures.
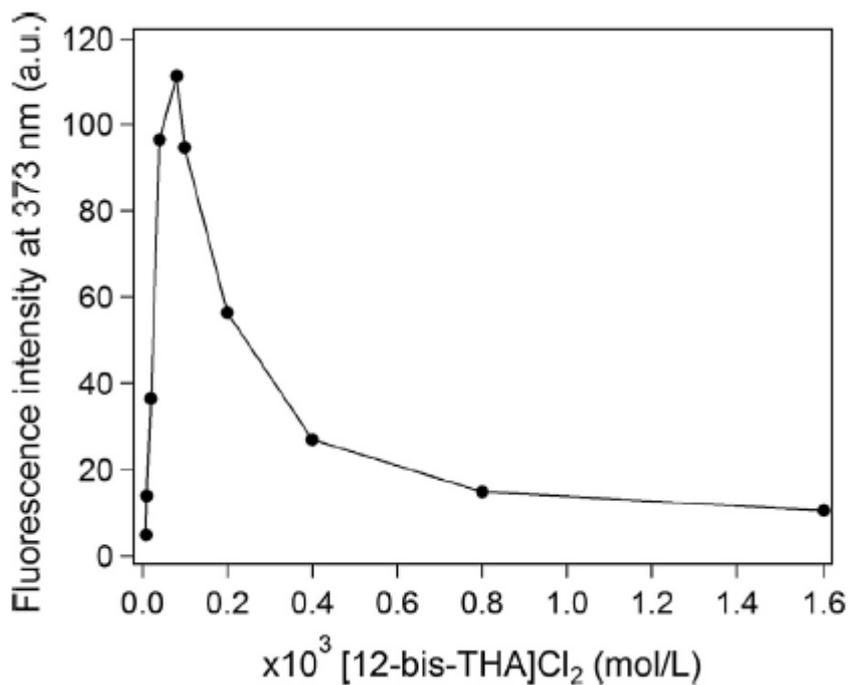
Fig. 3. Steady state fluorescence intensity at the emission maximum (373 nm) as afunction of [12-bis-THA]Cl2concentration in water.
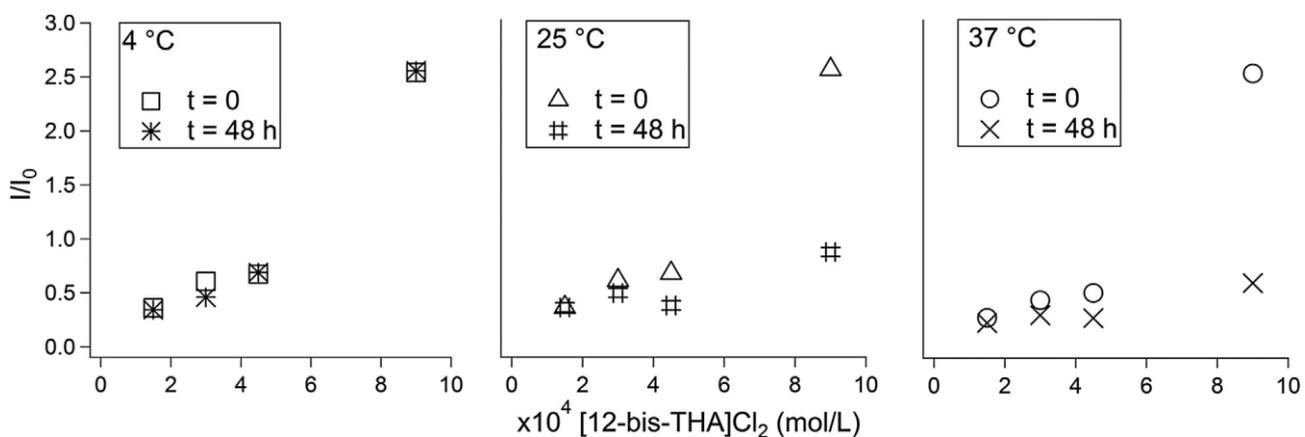
Fig. 4. Normalized scattering intensity (I/I0, where I = sample and I0= toluene) of aqueous solutions of [12-bis-THA]Cl2.
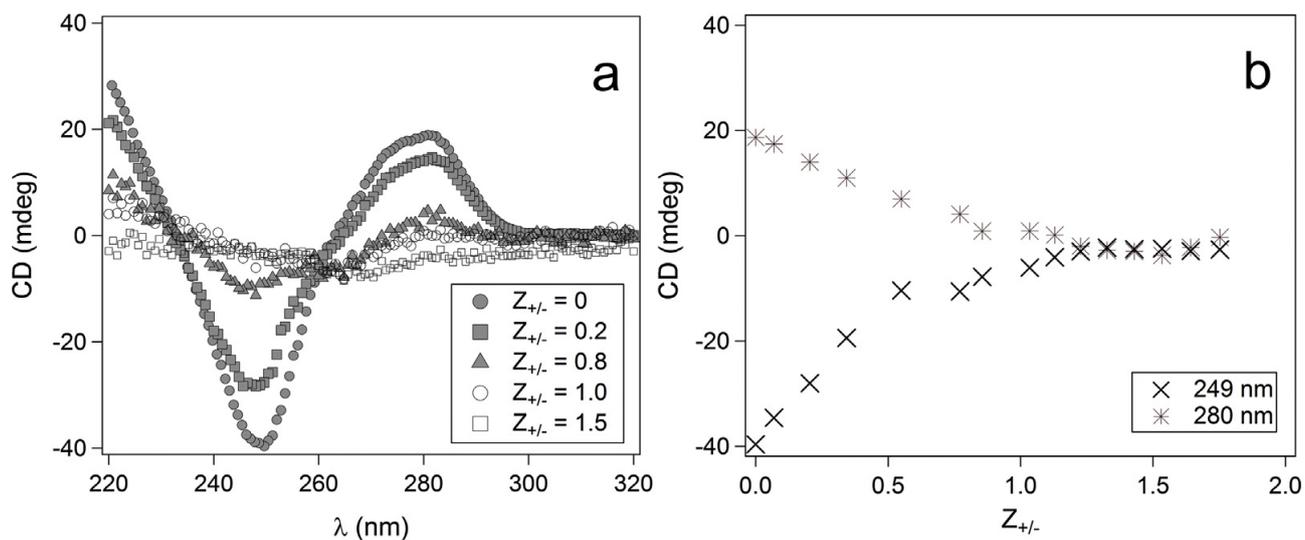
Fig. 5. Titration of a 77-bp TFD (90 _g/mL, or 1.9×10^{-6} mol/L) with [12-bis-THA]Cl2. a) CD spectra for five significative positive-to-negative charge ratios ($Z_{+/-}$). For the sakeof clarity, 1 in every 8 points were traced. b) Plot of the CD values at 249 and 280 nm, from spectra in Fig. S9, as a function of $Z_{+/-}$.
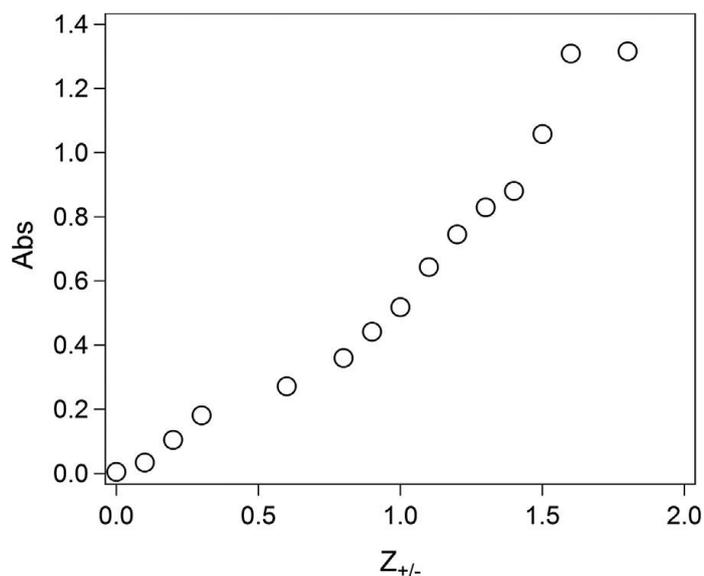
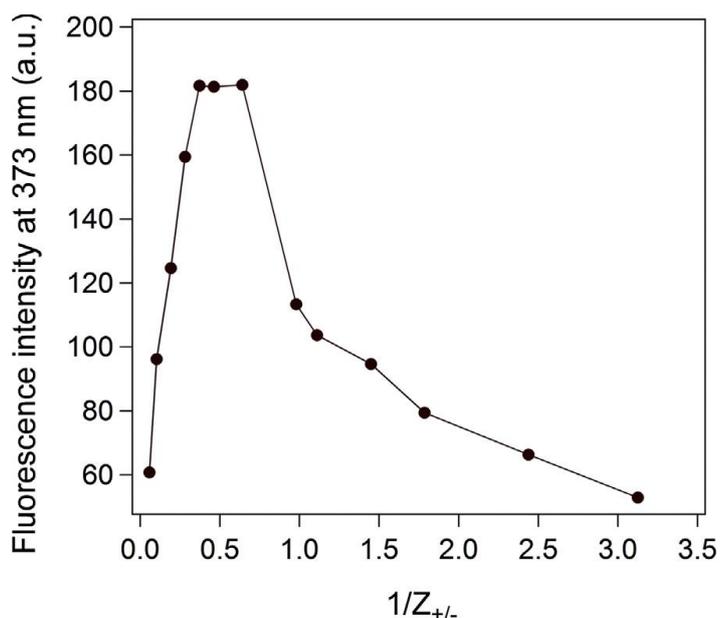Fig. 6. Plot of the absorbance values at 400 nm from the absorption spectra in Fig.S10 as a function of $Z_{+/-}$.

Fig. 7. Plot of the fluorescence intensity at 373 nm as a function of the reverse ofthe positive-to-negative charge ratio, 1/Z+/−. Here 1/Z+/−was used instead of Z+/−tobetter render the trend of the fluorescence intensity over the course of the titration.
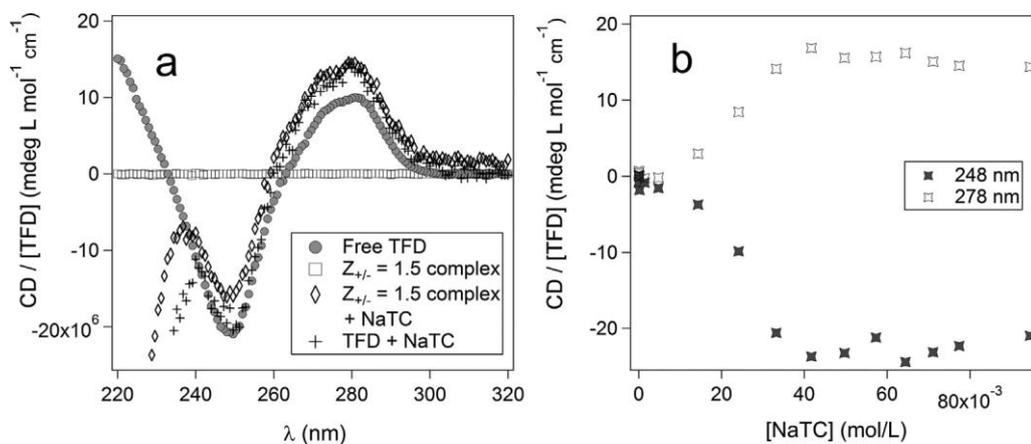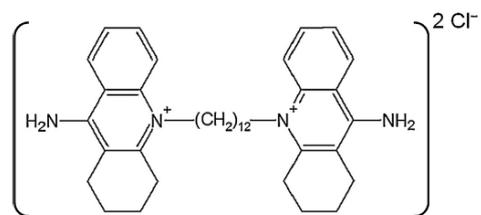

Fig. 8. a) CD spectra showing the disruption of the 1:1.5 complex between the TFD and [12-bis-THA]Cl2using sodium taurocholate (NaTC, 7.1×10^{-2}mol/L). All spectra arenormalized by the concentration of TFD. For the sake of clarity, 1 in every 8 points was traced for each spectrum. b) Plot of the CD values from spectra in Fig. S11 at 248 nmand 278 nm vs. NaTC concentration.

SCHEMES

Scheme 1: Chemical structure of 10,10_-(dodecane-1,12-diyl)-bis(9-amino-5,6,7,8-tetrahydroacridinium) chloride, or [12-bis-THA]Cl2.
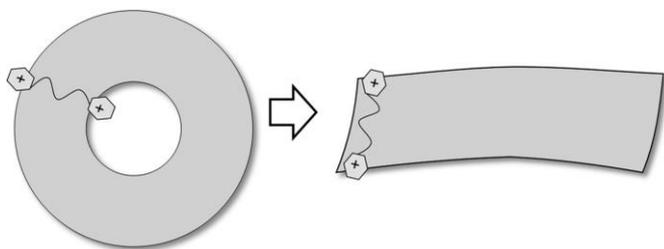
Scheme 2. Representation of the transition from bolasomes to one-dimensional elongated structures (fibers).