# Robust polymeric nanoparticles for the delivery of aminoglycoside antibiotics using carboxymethyldextran-*b*-poly(ethyleneglycols) lightly grafted with n-dodecyl groups†

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Aminoglycoside antibiotics are effective in the treatment of infections caused by aerobic Gram negative bacilli, but their widespread use is hampered by serious side effects that may be alleviated through the use of tailored delivery systems. Robust polyion complex (PIC) micelles, incorporating up to 50 weight % drug, were prepared using two aminoglycosides: paromomycin and neomycin, and a dihydrophilic block copolymer consisting of a poly(ethyleneglycol) (PEG) chain linked to a carboxymethyldextran fragment (CMD) lightly grafted with n-dodecyl groups. The micelles were stable under physiological conditions (pH 7.4, 150 mM NaCl), in contrast to micelles formed by the unmodified CMD-PEG and the aminoglycosides or their guanidinylated derivatives. The aminoglycosides were released from the n-dodecyl-CMD-PEG micelles in a pharmacologically active form as indicated by their ability to kill test micro-organisms in culture. This study opens up new opportunities in the biomedical applications of PIC micelles with inherently enhanced stability.



## 1. Introduction

The intelligent self-assembly of functional polymers is used extensively in the fabrication of soft materials and devices with applications in medicine, pharmaceutics, cosmetics, biosensing, and food technology.[1–3] Much thought needs to be given to the design of the macromolecules themselves, so that, in a given environment, they associate in a predictable and repetitive manner. Although recent advances in polymer synthesis, in particular the advent of controlled free radical polymerization, allow one to synthesize at will complex macromolecular architectures, simple diblock copolymers still remain the key building blocks of soft nanoparticles.[4–7] Dihydrophilic copolymers which consist of two suitable water-soluble blocks readily self-assemble in water in response to external stimuli, such as a change of temperature, pH, salinity or a light impulse. If the copolymer has a neutral hydrophilic block and an ionic block, micellization can also be triggered by interaction with an oppositely charged compound. The resulting nanoparticles, known as polyion complex (PIC) micelles, have a core-shell morphology: the core entraps charged guests neutralized by the oppositely charged blocks, while the shell is formed by the neutral blocks which confer colloidal stability to the nanoparticles *via* steric interactions.[8–10] PIC micelles are effective delivery vehicles for charged hydrophilic compounds, such as drugs, proteins, plasmid DNA, and siRNA.[5,11,12] They present a number of advantages for biomedical applications, including ease of fabrication, excellent colloidal stability in aqueous media, high drug loading capacity, small size, and narrow size distribution. They are thermodynamically stable and resist disintegration upon dilution as long as their concentration exceeds their critical association concentration (CAC), which usually is very low. PIC micelles often have a poly(ethyleneglycol) (PEG) corona, which prolongs their circulation time in blood allowing them to accumulate passively in tissues of leaky vasculature, such as tumors and inflamed tissues.[13,14]

We reported recently the use of the dihydrophilic anionic copolymer carboxymethyldextran-*b*-PEG (CMD-PEG) (Fig. 1) as host in the formation of PIC micelles with a number of cationic drugs.[15,16] CMD-PEG copolymers are non-toxic and yield PIC micelles of small size, high drug loading capacity and are stable upon freeze drying and in presence of serum proteins.[16] These attractive features prompted us to evaluate CMD-PEG copolymers as delivery agents for aminoglycosides, a group of structurally diverse polyamines frequently used in the treatment of serious infections caused by aerobic Gram negative bacilli, such as pneumonia, urinary tract infections and peritonitis.[17,18] The antibacterial activity of aminoglycosides results from their interaction with prokaryotic ribosomal RNA (rRNA).[19] Unfortunately, aminoglycosides therapy is associated with a host of side-effects due to drug accumulation in healthy non-target tissues resulting in nephrotoxicity and ototoxicity. Moreover, aminoglycosides are mostly administered parenterally or locally, since their poor absorption in the gastro-intestinal tract as a consequence of their highly polar cationic nature prevents their oral administration.[20,21]

In view of the clinical importance of aminoglycosides, much effort has been directed towards their encapsulation into vectors to modify their biodistribution, limit their toxicity, and increase their oral bioavailability. Drug delivery systems tested include liposomes,[22–24] polymeric nanoparticles,[25] solid lipid nanoparticles[26] and polyelectrolyte multilayers.[27] Although liposome-encapsulated aminoglycosides showed enhanced activity against

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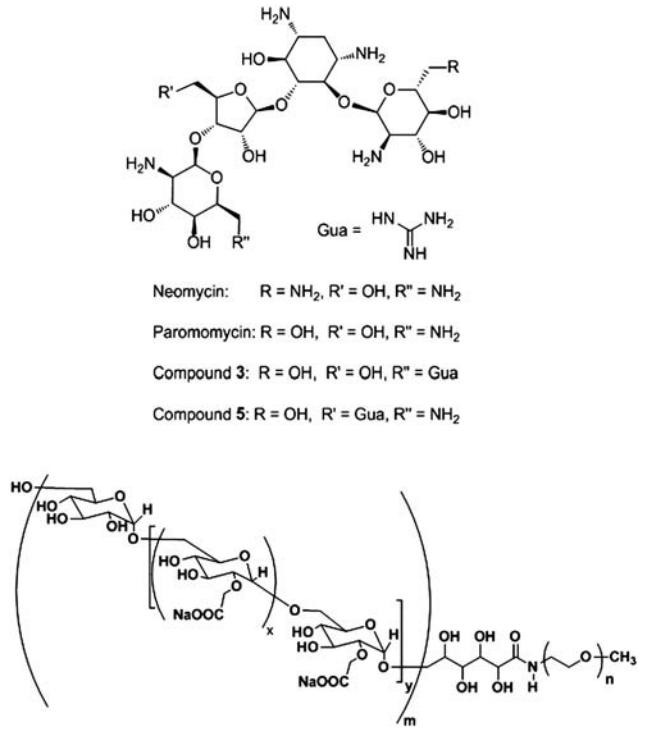



**Fig. 1** Chemical structures of neomycin, paromomycin, guanidinylated paromomycins (top) and the CMD-PEG block copolymer (bottom).

resistant strains of *Pseudomonas aeruginosa* due to enhanced entry into the bacterial cell, their use suffers from several drawbacks, in particular their limited stability in the presence of blood lipoproteins, low encapsulation efficiency, osmotic fragility and poor storage stability.[28,29] Poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles were used for the encapsulation of gentamicin, but their drug loading efficiency was very low (∼ 1 wt%).[25] Gentamicin microspheres, although effective in reducing splenic infections in mice, triggered pulmonary embolism due to particles aggregation.[30]

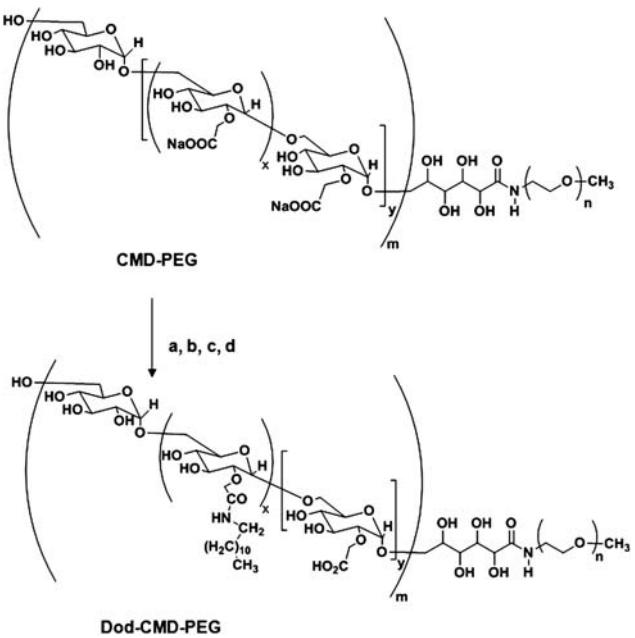
The objectives of this study were to assemble and characterize PIC micelles of CMD-PEG and two aminoglycoside antibiotics: neomycin and paromomycin (Fig. 1), which are examples of 4,5-disubstituted 2-deoxystreptamine aminoglycosides. Their structures differ only by the substituent at the 6′ position, which is a hydroxyl group in paromomycin and an amino group in neomycin. Neomycin and paromomycin are positively charged at pH 7.4, making them suitable for PIC micelles formation with polyanions, such as CMD-PEG. Evidence from isothermal titration calorimetry (ITC), $^1$H NMR spectroscopy, and dynamic light scattering indicated that PIC micelles of CMD-PEG and aminoglycosides form in water or buffers of low salinity. However, to our disappointment, CMD-PEG/aminoglycoside PIC micelles exhibited poor stability against increased ionic strength, severely hampering their *in vivo* applications. Of course, all PIC micelles dissociate above a critical ionic strength, since both the chemical potential of the counterions and the coulombic forces between the ionic block and the charged guest are directly influenced by the external ionic strength. This ionic strength dependent association/dissociation of PIC micelle is useful in some cases. For instance, it has been applied as an on-off control of enzymatic activity.[31]

In our system, however, the critical ionic strength was too low for eventual medical applications and we were brought to modify the design of the aminoglycoside PIC micelles in order to enhance their resistance against increased salinity. We present here the implementation of two strategies towards this goal. One approach was to enhance the ionic interactions between the aminoglycosides and the CMD-PEG carboxylates by introducing a basic functional group in selected positions in the antibiotic structure. The guanidine (Gua) moiety (p$K_a$ of guanidinium = 12.5) was selected in order to increase the basicity of the aminoglycoside.[32] The second strategy was to strengthen the core of the micelles *via* non-covalent interactions between hydrophobic groups linked to the charged block of the CMD-PEG. The formation and stability of the two types of PIC micelles were assessed by $^1$H NMR spectroscopy, dynamic light scattering, and turbidimetry. The kinetics of drug release from the PIC micelles were determined by the dialysis bag method. *In vitro* experiments confirmed that the drugs retained their ability to kill test micro-organisms upon entrapment in the core of PIC micelles. PIC micelles stabilization through hydrophobic modification of CMD-PEG could be applied to other PIC micelles-forming ionic copolymers. This is expected to widen the biomedical utility of PIC micelles since many of their drug-loaded formulations are unstable under physiological conditions.[33,34]

## 2. Results and discussion

### Synthesis and chemical characterization of the copolymers and the guanidinylated aminoglycosides

As previously reported, the dihydrophilic copolymer CMD-PEG was synthesized by end-coupling of ω-dextran-lactone with an




**Scheme 1** Synthesis of Dod-CMD-PEG. Reagents and conditions: (a) EEDQ, 1 : 1 v/v ethanol/water, pH 4.0, 30 min, (b) n-dodecylamine, pH 9.0, 60 min, (c) soxhlet extraction with hexane and (d) cation exchange resin, water.

excess of α-amino-ω-methoxy-PEG, followed by carboxymethylation of the dextran block and extensive purification to remove unreacted α-amino-ω-methoxy-PEG.[35] This polymer was used as such for the preparation of PIC micelles or converted to Dod-CMD-PEG by reaction with n-dodecylamine in aqueous ethanol in the presence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) acting as coupling agent (Scheme 1).[36] The successful outcome of the synthesis was ascertained from the FTIR spectrum of the Dod-CMD-PEG copolymers, in particular the appearance of bands at 1546 $cm^{-1}$ and 1644 $cm^{-1}$, attributed to the amide II and amide I vibration modes (Fig. 2A). The $^1H$ NMR spectrum of the modified copolymers in DMSO-$d_6$ presented signals at 1.18 and 0.78 ppm due, respectively, to the methylene and methyl protons of the n-dodecyl groups, in addition to signals characteristic of the CMD block (δ 3.61–3.78, 4.21–4.32, 4.68 and 4.94–5.01 ppm) and of the PEG block (δ 3.51 ppm) (Fig. 2B). The n-dodecyl grafting density was calculated from the $^1H$ NMR spectra of the copolymers. The chemical characteristics of the copolymers are listed in Table 1. This chemical modification effectively converted CMD-PEG from a double hydrophilic ionic copolymer to an amphiphilic polyelectrolyte. Thus, while CMD-PEG (0.2 g/L) is soluble in water with no sign of aggregation within a wide pH range (pH > 2.0) the modified polymers Dod-CMD-PEG self–assemble in acidic aqueous solution (pH 3.0) into nanoparticles ($R_H \sim 100$ nm). The association of Dod-CMD-PEG in the absence of aminoglycosides can be tuned by the solution pH and salinity, as described in a forthcoming publication.

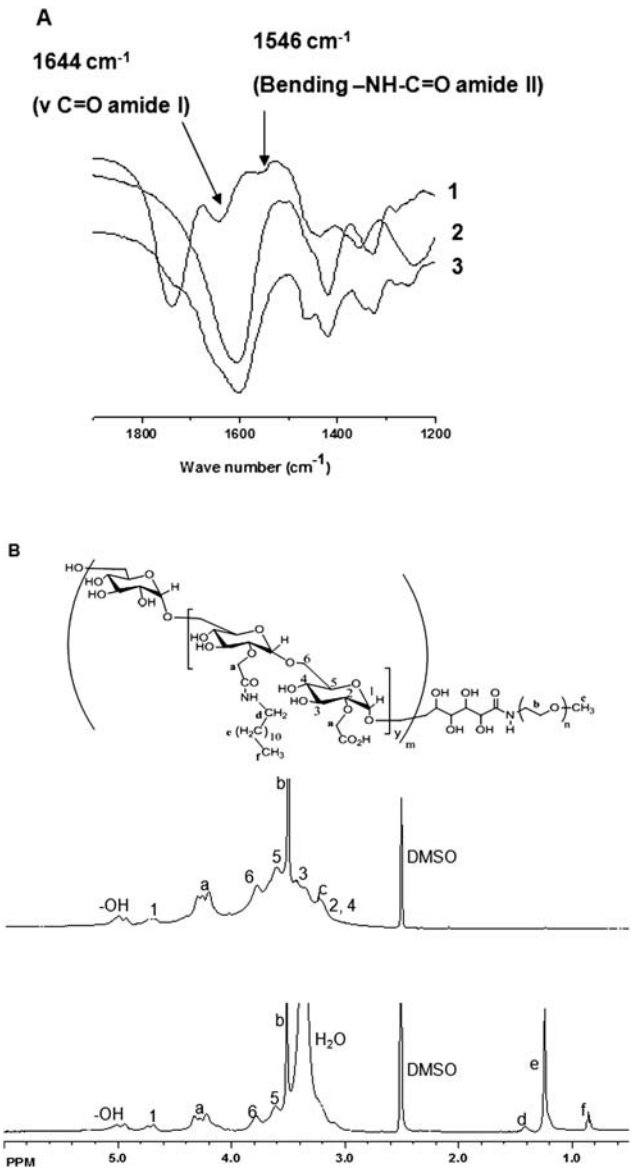



**Fig. 2** A: FTIR spectra of CMD-PEG sodium salt (1), $Dod_{38}$-CMD-PEG free acid (2), and $Dod_{38}$-CMD-PEG sodium salt (3) (powder sample) in the region of 1200–1900 $cm^{-1}$. B: $^1H$ NMR spectra of CMD-PEG (top spectrum) and $Dod_{38}$-CMD-PEG (bottom spectrum) (DMSO-$d_6$, room temperature).

**Table 1** Molecular characteristics of the copolymers prepared

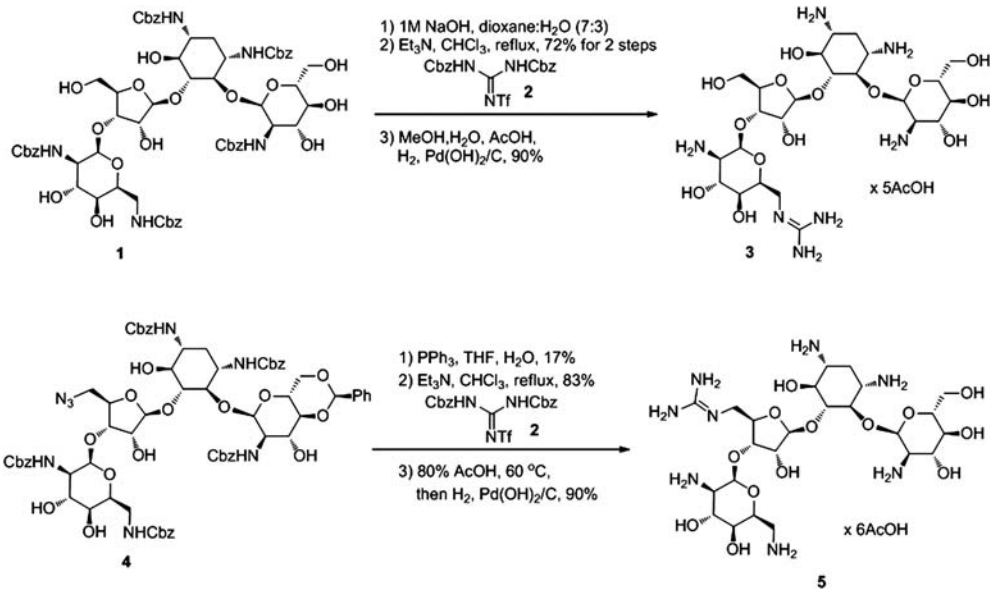
| Polymer | $M_w$[a] (g $mol^{-1}$) | $M_n$[a] (g $mol^{-1}$) | x[b] | y[c] | m[d] | n[e] | % dodecyl[f] |
|---|---|---|---|---|---|---|---|
| CMD-PEG | 14 800 | 10 800 | 0 | 34 | 40 | 140 | — |
| $Dod_{18}$-CMD-PEG | 16 100 | 12 100 | 7 | 27 | 40 | 140 | 18 |
| $Dod_{38}$-CMD-PEG | 17 600 | 13 600 | 15 | 19 | 40 | 140 | 38 |

[a] From GPC measurements in aqueous $NaNO_3$ (0.2 M)/$NaH_2PO_4$ (0.01 M)/$NaN_3$ (0.8 mM); pH 7.02. [b] Number of glucopyranose units bearing an n-dodecyl chain. [c] Number of unmodified carboxymethylated glucopyranose units. [d] Average number of glucopyranosyl repeat units of the CMD chain. [e] Number of $–CH_2$-$CH_2$-O- repeat units of PEG. [f] Mole percent of n-dodecyl-modified glucopyranose units ($^1H$ NMR measurements in DMSO-$d_6$).

Paromomycin possesses five primary amine groups that can be converted into a guanidinyl function in order to increase the basicity of the antibiotic and consequently enhance the strength of its electrostatic interaction with CMD-PEG.[32] We prepared 6‴-G-Par, and 5″-G-Par. The minimum inhibitory concentrations for 6‴-G-Par and 5″-G-Par were comparable to the parent antibiotic paromomycin (MIC = 2–8 μg/mL in both cases). 6‴-G-Par was obtained by treatment of compound **1** with aqueous NaOH to deprotect selectively the 6‴-NH-Cbz group (Scheme 2).[37] The resulting free amino group was guanidinylated with reagent **2**, followed by hydrogenolysis of the *N*-Cbz group to afford the desired 6‴-G-Par.[38] In order to obtain 5″-G-Par, the known compound **4** was treated with $PPh_3$ under Staudinger conditions and the resulting amine was guanidinylated with reagent **2** (Scheme 2).[38,39] Benzylidene deprotection with aqueous AcOH followed by *N*-Cbz hydrogenolysis afforded the desired 5″-G-Par.

### Preparation and characterization of PIC micelles formed by the electrostatically-driven assembly of aminoglycosides and their derivatives with CMD-PEG and Dod-CMD-PEG

Microcalorimetric measurements by ITC were conducted first to ascertain that the thermodynamic parameters of the interactions between CMD-PEG and neomycin sulfate or paromomycin sulfate were favorable to the formation of PIC micelles. Aliquots of a concentrated solution of a drug in a phosphate buffer (10 mM, pH 7.0, 50 mM NaCl) at 25 °C were added in a time-controlled manner to a solution of CMD-PEG in the same buffer also maintained at 25 °C. Interaction between the two entities was accompanied by release of heat, which diminished as the




**Scheme 2** Synthesis of 6‴-guanidino-paromomycin (**3**) and 5″-deoxy-5″-guanidino-paromomycin (**5**).



population of free polyelectrolyte in solution decreased as a result of PIC micelle formation. Eventually, the signal leveled off, indicating completion of the electrostatic interactions that led to micellization. Further addition of drug triggered constant heat absorption due to the dilution of the drug in buffer. Enthalpograms recorded upon titration of each drug into a CMD-PEG solution are presented in Fig. 3, in which the top panels present the heat released/absorbed upon injection of the drug. The bottom panels show the integrated heat data that were used to extract the binding parameters after subtraction of the heat of dilution of the drugs in the same buffer. The data are plotted as a function of the molar ratio of amine groups provided by the drugs to carboxylates provided by the copolymer.

The ITC profiles were fit with a model for one set of binding sites. The thermodynamic parameters resulting from the data

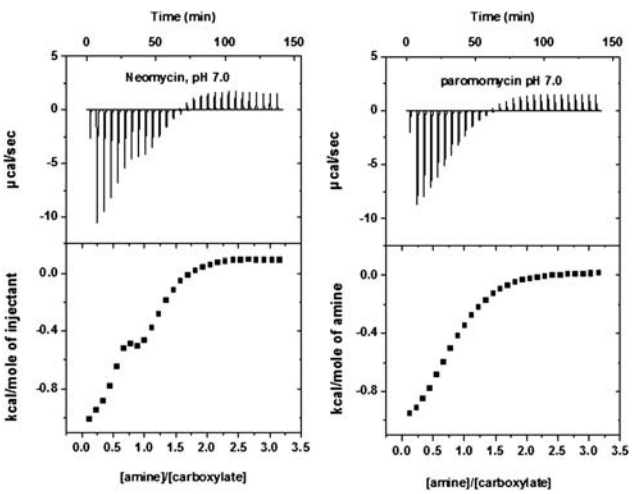



**Fig. 3** Corrected integrated injection heats plotted as a function of the [amine]/[carboxylate] ratio for the titration of either neomycin sulfate or paromomycin sulfate into CMD-PEG copolymer in phosphate buffer (10 mM, pH 7.0) at 25 °C.

fitting are presented in Table 2. The number of binding sites ($N$) was ~ 1, confirming that each CMD-PEG carboxylate group is bound to one amino group of either neomycin or paromomycin. The enthalpy change upon binding of one mol amine was ~ −1.0 kcal in each case, giving a total enthalpy change of ~ −6.0 kcal/mol for neomycin and ~ −5.0 kcal/mol for paromomycin, since neomycin and paromomycin have 6 and 5 amines, respectively. Indeed, the raw ITC data (Fig. 3, top panels) confirm that the binding of neomycin to CMD-PEG is more exothermic than that of paromomycin. This may indicate that the binding of neomycin to CMD-PEG is stronger than that of paromomycin.[40] Stronger neomycin binding is also confirmed by the higher binding free energy ($\Delta G$) of −28.0 kcal/mol for neomycin, compared to −22.7 kcal/mol for paromomycin. In both cases, the binding is mainly entropically driven since the entropic contribution ($T\Delta S$) accounted for ~ 75% of the binding free energy. This observation is in good agreement with a report on the binding of the same drugs to the A site of 16S rRNA for which it was reported that 72% of the driving force for the binding of paromomycin to RNA was derived from entropic contributions.[41] Electrostatic interactions between aminoglycosides and either RNA or CMD-PEG copolymers are associated with counter ions release, which results in entropy gain of the system.[42,43]

The formation of core-shell nanoparticles upon addition of either neomycin sulfate or paromomycin sulfate into

**Table 2** Thermodynamic parameters for the binding of neomycin sulfate and paromomycin sulfate to CMD-PEG (25 °C, 10 mM phosphate buffer, pH 7.0, 50 mM NaCl)

| Parameter | Neomycin sulfate | Paromomycin sulfate |
|---|---|---|
| $N$ | 0.85 ± 0.06 | 0.81 ± 0.01 |
| $K$ (x10$^3$)(M$^{-1}$) | 4.56 ± 2.20 | 3.71 ± 0.41 |
| $\Delta H$ (kcal mol$^{-1}$) | −1.04 ± 0.10 | −1.08 ± 0.03 |
| $T\Delta S$ (kcal mol$^{-1}$) | 3.63 | 3.46 |
| $\Delta G$ (kcal mol$^{-1}$) | −4.67 | −4.54 |

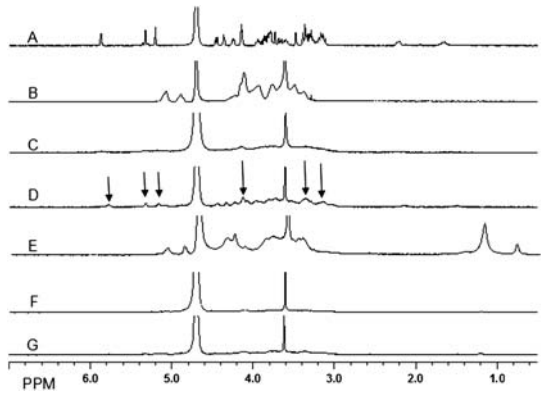



**Fig. 4** $^1$H NMR spectra of neomycin sulfate (A), CMD-PEG (B), neomycin/CMD-PEG micelles (pH 7.4, 0 mM NaCl) (C), neomycin/CMD-PEG micelles (pH 7.4, 150 mM NaCl) (D), $Dod_{38}$-CMD-PEG (E), neomycin/$Dod_{38}$-CMD-PEG micelles (pH 7.4, 0 mM NaCl) (F) and neomycin/$Dod_{38}$-CMD-PEG micelles (pH 7.4, 150 mM NaCl) (G). ($D_2O$: polymer concentration: 2.0 g/L, neomycin concentration: 2.1 g/L and [amine]/[carboxylate] = 2.5).

a CMD-PEG solution was confirmed by $^1$H NMR spectroscopy measurements which take advantage of the fact that signals of protons entrapped within the micellar core are either very broad or do not appear at all, as a consequence of the restricted mobility of such protons in this environment. In contrast, protons of the polymer segments forming the micelle corona maintain their mobility and their signals appear well resolved. The $^1$H NMR spectrum of neomycin sulfate in $D_2O$ (Fig. 4A) presents three singlets at 5.2, 5.34 and 5.87 ppm attributed to the anomeric protons on the three amino sugars. The axial and equatorial methylene protons of the neomycin cyclohexyl ring resonate at 1.66 and 2.2 ppm, respectively. The other neomycin protons resonances appear as a series of signals between 3.16 to 4.45 ppm.[44] The $^1$H NMR spectrum of CMD-PEG (Fig. 4B) in $D_2O$ presents characteristic signals at δ 4.08–4.15, 4.89, and 5.07 ppm, ascribed to protons of the CMD block, and a broad strong singlet centered at δ 3.61 ppm due to the PEG methylene protons (-$CH_2$-$CH_2$-O-).[15] The spectrum of neomycin/CMD-PEG micelles ([amine]/[carboxylate] = 2.5, pH 7.4, [NaCl] = 0 mM) (Fig. 4C) features only a strong signal at δ 3.61 ppm, ascribed to the PEG methylene protons. The signals due to the protons of neomycin and of the CMD segment of the polymer cannot be seen any more, confirming the formation of PIC micelles with a neomycin/CMD core and a PEG corona. Micelles of this composition contain 50 wt% drug, which can be taken as the actual drug loading, since the NMR spectrum for this composition shows no signal due to free drug (Fig. 4C).

Spectra of mixed solutions in the 1.5 ≤ [amine]/[carboxylate] ≤ 2.5 composition range were similar to that presented in Fig. 4C. However, mixed solutions having [amine]/[carboxylate] < 1.5 or > 2.5 showed signals characteristic of free drug and of the CMD block of the copolymer, in addition to the signal at 3.61 ppm due to the PEG protons resonance. This indicates, on the one hand, that micelles do not form until the [amine]/[carboxylate] ratio reaches 1.5 or, if they form, their core is sufficiently loose and hydrated to permit free motion of the drug and CMD protons and, on the other hand, it confirms that maximum drug loading is achieved for a mixture having [amine]/[carboxylate] = 2.5 as seen also by ITC measurements (Fig. 3).

$^1$H NMR spectroscopy was used also to confirm that hydrophobic modification did not affect the ability of the drugs to form core/shell micelles *via* interactions with the carboxylate groups of the Dod-CMD-PEG copolymers. The $^1$H NMR spectra of $Dod_{38}$-CMD-PEG in water in the absence and presence of neomycin ([amine]/[carboxylate] = 2.5) are presented in Fig. 4E and 4F. The spectrum of the mixed solution presents only the singlet at 3.61 ppm associated with the PEG protons. Signals due to the drug protons and to the protons of the $Dod_{38}$-CMD block cannot be detected, as in the case of a mixed CMD-PEG/neomycin solution of identical composition (Fig. 4C).

Dynamic light scattering (DLS) experiments were conducted in order to determine the hydrodynamic size of the PIC micelles formed upon interaction of each drug with the copolymers listed in Table 1, as well as the micelles formed by the guanidinylated aminoglycosides and CMD-PEG. For each drug/copolymer pair, mixed solutions of different [amine]/[carboxylate] ratios were prepared in phosphate buffer (10 mM, pH 7.0) and the hydrodynamic radius ($R_H$) of the resulting micelles was determined (Fig. 5). In all cases, the distributions of nanoparticles were monomodal. Paromomycin/CMD-PEG micelles of [amine]/[carboxylate] = 1.0 had a $R_H$ of ~ 100 nm and a polydispersity index (PDI) ~ 0.3. Increasing the [amine]/[carboxylate] ratio to 1.5 resulted in a drop of $R_H$ to ~ 70 nm and of PDI to 0.2 (Fig. 5A). The $R_H$ of the micelles gradually increased upon further increase of the [amine]/[carboxylate] ratio, probably as a consequence of the incorporation of additional drug in the micellar core. The $R_H$ value levelled off for [amine]/[carboxylate] = 2.5 and remained constant upon further addition of drug, confirming observations by NMR spectroscopy and ITC that the maximum drug loading is achieved at this point. Also, drug/polymer mixtures prepared for [amine]/[carboxylate] < 1.0 did not scatter light indicating the absence of nanoparticles in mixed solutions within this composition range. Similar trends were observed in the DLS analysis of mixed neomycin/CMD-PEG solutions, although neomycin micelles were smaller in size, compared to paromomycin/CMD-PEG micelles (Table 3) presumably as a result of tighter electrostatic interactions in the core of neomycin micelles due to the presence of an additional amino group in neomycin (Fig. 1). This amino group has a p$K_a$ of 9.24, hence it is fully ionized at pH 7.0.[41] ITC experiments

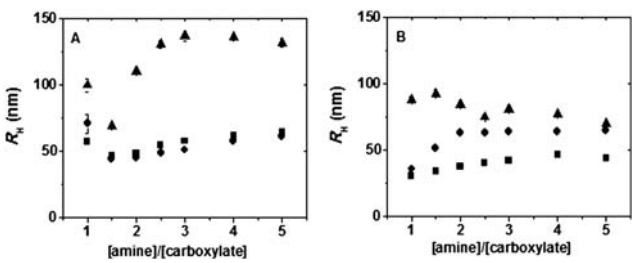



**Fig. 5** Effect of the [amine]/[carboxylate] molar ratio on the hydrodynamic radius of paromomycin sulfate (panel A) and neomycin sulfate (panel B) micelles with: CMD-PEG (▲), $Dod_{18}$-CMD-PEG (●), $Dod_{38}$-CMD-PEG (■); phosphate buffer (10 mM, pH 7.0), polymer concentration = 0.2 g/L.

**Table 3** Characteristics of aminoglycoside/CMD-PEG micelles ([amine]/[carboxylate] = 2.5) in a phosphate buffer (10 mM, pH 7.0), [polymer] = 0.2 g/L

| Micelle | $R_H$[a] | PDI[a] | % Drug[b] |
|---|---|---|---|
| Neomycin/CMD-PEG | 74.9 ± 1.8 | 0.03 ± 0.03 | 50 |
| Paromomycin/CMD-PEG | 130.1 ± 0.5 | 0.08 ± 0.03 | 49.8 |
| Neomycin/$Dod_{18}$-CMD-PEG | 63.3 ± 0.6 | 0.08 ± 0.05 | 50 |
| Paromomycin/$Dod_{18}$-CMD-PEG | 48.5 ± 0.4 | 0.02 ± 0.03 | 49.8 |
| Neomycin/$Dod_{38}$-CMD-PEG | 40.5 ± 0.4 | 0.06 ± 0.03 | 50 |
| Paromomycin/$Dod_{38}$-CMD-PEG | 54.5 ± 1.2 | 0.03 ± 0.02 | 49.8 |
| 6‴-G-Par/CMD-PEG | 110 ± 2.2 | 0.08 ± 0.02 | 50 |
| 5″-G-Par/CMD-PEG | 83.8 ± 2.6 | 0.04 ± 0.05 | 50 |

[a] Mean of six measurements ± S.D. [b] % drug loading = weight of drug/(weight of micelles)×100. $R_H$: Hydrodynamic radius, PDI: Polydispersity index.

also showed stronger interactions between neomycin and CMD-PEG, compared to paromomycin/CMD-PEG.

Solutions of micelles of CMD-PEG and either paromomycin or neomycin in a phosphate buffer (10 mM, pH 7.0, [amine]/[carboxylate] = 2.5, CMD-PEG 0.5 g/L) were diluted serially in the same buffer in order to determine by light scattering the polymer concentration below which micelles do not form, or at least cannot be detected by light scattering. Plots of the changes of the relative scattering intensity, defined as the ratio of the intensity of a solution of given concentration to the scattering intensity of the solution of CMD-PEG = 0.5 g/L, are presented in Fig. 6 together with the hydrodynamic radius of the micelles (Fig. 6 inset). The size of the micelles was not affected by dilution and micelles were detected in solutions of [CMD-PEG] ≥ 0.062 g/L and 0.125 g/L for neomycin and paromomycin, respectively. The minimal CMD-PEG concentrations necessary for micelle formation were determined graphically (see arrows in Fig. 6) as the polymer concentration corresponding to the onset of the increase in scattered light intensity. Both types of micelles exhibit the remarkable resistance against dilution characteristic of PIC micelles.[15,45] This feature is very important for *in vivo* applications where micelles are subjected to drastic dilution upon intravenous injection.

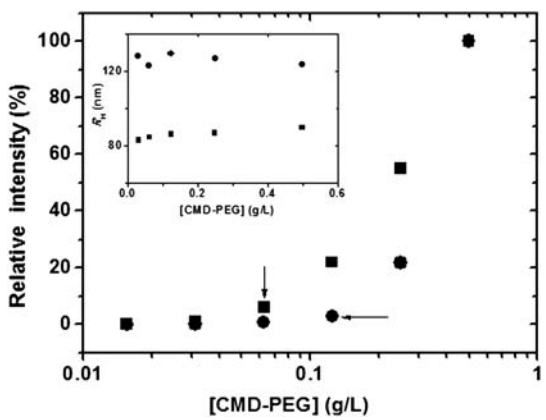



**Fig. 6** Dependence of the relative intensity of scattered light on CMD-PEG concentration for neomycin/CMD-PEG micelles (■) and paromomycin/CMD-PEG micelles (●). Inset: CMD-PEG concentration dependence of the hydrodynamic radius of neomycin/CMD-PEG micelles (■) and paromomycin/CMD-PEG micelles (●). Relative scattering intensity = intensity of scattered light for a given CMD-PEG concentration/intensity of scattered light of a micellar solution of [CMD-PEG] = 0.5 g/L.

We carried out next a light scattering study of micelles formed by hydrophobically modified copolymers and aminoglycosides. In Fig. 5, we present the [amine]/[carboxylate] dependence of the $R_H$ of PIC micelles formed between each aminoglycoside and the hydrophobically modified copolymers, $Dod_{18}$-CMD-PEG and $Dod_{38}$-CMD-PEG at a polymer concentration of 0.2 g/L. For identical [amine]/[carboxylate] ratios paromomycin/Dod-CMD-PEG micelles are significantly smaller than micelles formed by CMD-PEG, but no significant difference in size was detected between paromomycin micelles formed with $Dod_{18}$-CMD-PEG and $Dod_{38}$-CMD-PEG copolymers. Neomycin micelle formation followed similar trends, except that neomycin/$Dod_{38}$-CMD-PEG micelles were significantly smaller than neomycin/$Dod_{18}$-CMD-PEG (Table 3). The core of aminoglycosides/Dod-CMD-PEG micelles may be less hydrated than the core of micelles formed by CMD-PEG due to the presence of hydrophobic dodecyl chains which can act as internal non-covalent crosslinking point.[46] Gao *et al.* reported similar trends in a comparative study of lysozyme/poly(1-tetradecene-alt-maleic acid) and lysozyme/poly-(isobutylene-alt-maleic acid) micelles. The smaller size of the former micelles was attributed to hydrophobic interactions between the tetradecyl chains.[47]

Both 6‴-G-Par and 5″-G-Par form PIC micelles with CMD-PEG, as expected since the guanidinyl group was selected on the basis of its stronger basicity, compared to the amine groups. Micelles formed in mixed solutions of CMD-PEG and both 6‴-G-Par and 5″-G-Par for all [amine]/[carboxylate] > 1. They were significantly smaller than the micelles formed by this copolymer and unmodified paromomycin, independently of the [amine]/[carboxylate] ratio (Figure SI1† and Table 3 for data on micelles with [amine]/[carboxylate] = 2.5). Particularly striking is the decrease in size triggered by a guanidinyl substituent when it is linked to the 5″ position (Table 3). It should be recalled here that a primary –OH group in 5″-G-Par was converted into a guanidine group whereas in 6‴-G-Par a primary $–NH_2$ was converted into a guanidine group. This makes 5″-G-Par more basic than 6‴-G-Par allowing stronger electrostatic interactions with CMD-PEG carboxylate groups.

### Effects of electrolyte concentration on the size and integrity of micelles of aminoglycosides and their derivatives with CMD-PEG and Dod-CMD-PEG

Evidence of the remarkably enhanced stability against increased salinity of aminoglycosides/Dod-CMD-PEG micelles, compared to micelles formed by CMD-PEG, was obtained from $^1$H NMR experiments. Micelles of neomycin were prepared in $D_2O$ with either CMD-PEG or $Dod_{38}$-CMD-PEG (polymer concentration: 2.0 g/L, [amine]/[carboxylate] = 2.5) in the absence of salt. The spectra of the micellar solutions were recorded. For both solutions, only the signal at δ 3.61 ppm attributed to the PEG protons was detected (Fig. 4). Then, we performed the same experiments with micellar solutions containing NaCl of physiological concentration (150 mM). The spectra recorded are presented in Fig. 4. The spectrum of the neomycin/$Dod_{38}$-CMD-PEG micellar solution in the presence of 150 mM NaCl (Fig. 4G) was

identical to the spectrum of the micellar solution in $D_2O$ in the absence of salt (Fig. 4F), indicating that the micelles form and persist even in the presence of salt. In contrast, the spectrum of a neomycin/CMD-PEG solution (150 mM NaCl) presents signals characteristic of neomycin, indicated by an arrow in Fig. 4D as well as small signals due to protons of the CMD block. This observation implies that neomycin and CMD fragments exist as soluble species rather than entrapped within the micellar core, and, consequently that the formation of micelles in this case was severely impeded by the presence of salt.

We determined also by light scattering experiments the effect of added salt, from 0 to 200 mM, on the intensity of the light scattered by micellar solutions and on the size of micelles (Fig. 7). The intensity of the light scattered by the micellar solutions (200 mM NaCl) was $\sim$ 40% and $\sim$ 80%, respectively, for neomycin/$Dod_{18}$-CMD-PEG and neomycin/$Dod_{38}$-CMD-PEG micelles (Fig. 7A). Neomycin/$Dod_{18}$-CMD-PEG and neomycin/$Dod_{38}$-CMD-PEG micelles maintained their initial size at [NaCl] = 200 mM (Fig. 7B). Also, neomycin/$Dod_{38}$-CMD-PEG micelles prepared under physiological conditions ([NaCl] = 150 mM and pH 7.4) showed no signs of disintegration after a three-months storage at room temperature.

Hydrophobic modification of CMD-PEG had a less pronounced effect on paromomycin micelles stability against increase in salinity. Yet, micelles of paromomycin/$Dod_{38}$-CMD-PEG maintained their initial size and their solution kept $\sim$ 80% of their initial scattered light intensity up to [NaCl] = 100 mM (Fig. 7C and D). Paromomycin/CMD-PEG micelles did not form at all at this salt concentration. From these results it can be concluded that the level of CMD-PEG hydrophobic modification, as well as the basicity of the aminoglycoside amino groups are major factors determining micelles stability against increase in salinity. The enhanced stability of neomycin/$Dod_{38}$-CMD-PEG against salt-induced disintegration is probably due to the participation of electrostatic and hydrophobic interactions in the formation of tighter micelle cores.

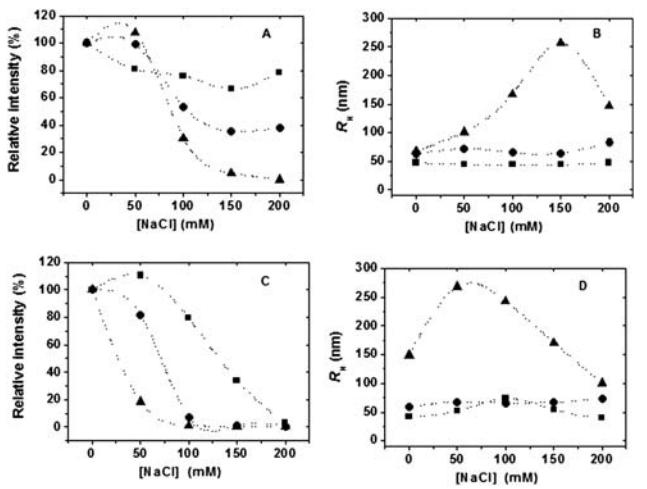



**Fig. 7** Changes in the intensity of scattered light and hydrodynamic radius of neomycin (panels A and B) and paromomycin (panels C and D) micelles as a function of NaCl concentration; Micelles were formed with: $Dod_{38}$-CMD-PEG (■), $Dod_{18}$-CMD-PEG (●), CMD-PEG (▲); phosphate buffer (10 mM, pH 7.0), polymer concentration = 0.5 g/L;[amine]/[carboxylate] = 2.5. Relative scattering intensity = intensity at a given [NaCl]/intensity for [NaCl] = 0.

We assessed also the resistance against salt of micelles formed by the modified drugs, 5″-G-Par and 6‴-G-Par (Figure SI2). Solutions of 6‴-G-Par/CMD-PEG retained $\sim$ 40% of their initial scattering intensity at 50 mM NaCl, compared to $\sim$ 20% for Par/CMD-PEG micelles at the same salt concentration, while 5″-G-Par/CMD-PEG solutions maintained the same scattered light intensity for [NaCl] $\leq$ 50 mM and $\sim$ 30% of their initial scattered light intensity at [NaCl] = 100 mM (Figure SI2). This enhanced stability of guanidinylated paromomycin micelles might result from stronger interactions between guanidine groups of the drug and carboxylate groups of CMD-PEG. Nonetheless, the light scattering data imply that guanidinylation of one position of the paromomycin nucleus is not effective in stabilizing PIC micelles under the salinity conditions imposed by the physiological milieu.

## Drug release study and antibacterial activity of aminoglycoside/CMD-PEG micelles

The release of neomycin from its PIC micelles with CMD-PEG and $Dod_{38}$-CMD-PEG was evaluated by the dialysis bag method (Fig. 8) with micellar solutions in phosphate buffer of various pH values and different salt concentrations since both factors can affect drug release rate from PIC micelles.[15,48] Neomycin rapidly diffused out of the dialysis membrane in the absence of polymers and almost complete release was achieved within 4 h (Fig. 8). In contrast, micelle-encapsulated neomycin exhibited a lower rate of release under all conditions studied. For instance, the lowest release rate was detected in phosphate buffer at pH 7.0–7.4 and 0 mM NaCl. Under these conditions, $\sim$ 30% of neomycin was released after 24 h. The release rate was significantly increased by increasing [NaCl] from 0 to 150 mM. Thus, after 24 h, $\sim$ 70% neomycin was released at pH 7.4, [NaCl] = 150 mM. The higher drug release rate in the presence of 150 mM NaCl confirms that the release involves an ion exchange mechanism,[49] as reported previously.[48] No burst drug release occurred even in conditions of high salt concentration, confirming that the drug is located in the micelles core, and despite higher neomycin release rate under physiological conditions (pH 7.4, [NaCl] = 150 mM), the micelles were still able to sustain drug release for more than 24 h (Fig. 8). DLS studies of the solution recovered inside the dialysis bags after a 24-hr dialysis against phosphate buffer (pH 7.0, [NaCl] = 150 mM) confirmed the presence of nanoparticles ($R_H$ $\sim$ 230 nm), with an estimated neomycin content $\sim$ 30% of the initial value. Changes in pH from 7.0 to 7.4 did not affect the release rate, in the absence or presence of NaCl (150 mM). Moreover, hydrophobic modification of the copolymer did not have any marked effect on release rates. For instance, the release rates of neomycin from neomycin/$Dod_{38}$-CMD-PEG and neomycin/CMD-PEG micelles were identical, within experimental uncertainty.

The antibacterial activity of free aminoglycosides and aminoglycosides encapsulated in CMD-PEG micelles was evaluated by exposing the test organism *E. coli* XL-1 blue strain to different drug concentrations in order to determine the lowest drug concentration that prevents detectable bacterial growth (minimal inhibitory concentration, MIC). In addition to neomycin,

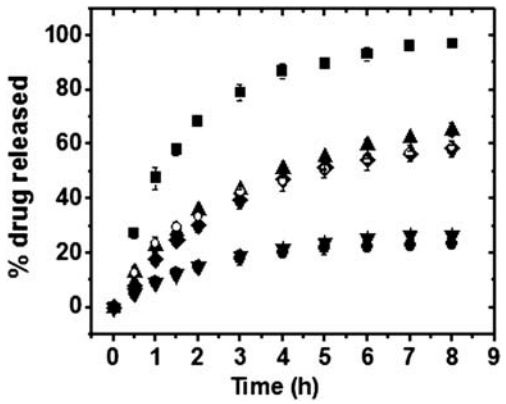



**Fig. 8** Release profiles of neomycin from: a neomycin solution (■); neomycin/CMD-PEG micelles, pH 7.0, [NaCl] = 0 mM (●); neomycin/CMD-PEG micelles, pH 7.4, [NaCl] = 0 mM (▼); neomycin/CMD-PEG micelles, pH 7.0, [NaCl] = 150 mM (◆); neomycin/CMD-PEG micelles, pH 7.4, [NaCl] = 150 mM (▲); neomycin/$Dod_{38}$-CMD-PEG micelles, pH 7.4, [NaCl] = 150 mM (○). ([neomycin] = 2.0 g/L, [amine]/[carboxylate] = 2.5), temperature 37 °C, 10 mM phosphate buffer.

paromomycin and guanidinylated-paromomycin analogues (5″-G-Par and 6‴-G-Par), several other aminoglycosides were tested, including the clinically relevant tobramycin and amikacin. Both 6‴-G-Par and 5″-G-Par showed MIC of 2–8 μg/mL. In no case was the MIC of the drug altered upon encapsulation in CMD-PEG micelles. Thus, whether drugs were free or encapsulated in PIC micelles, MICs were 2–8, 4–8, 2–8 and 2–8 μg/mL for amikacin, neomycin, paromomycin and tobramycin, respectively. Similar results were reported for ciprofloxacin encapsulated in polyethylbutylcyanoacrylate nanoparticles and amphotericin B encapsulated in poly(lactic-*co*-glycolic acid) nanoparticles.[50,51]

## 3. Experimental

### Materials

Neomycin sulfate, paromomycin sulfate and all other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) unless otherwise specified. The block copolymer CMD-PEG (Fig. 1) was synthesized starting with dextran ($M_n$ 6 000 g/mol, Fluka Chemicals) and α-amino-ω-methoxy-poly(ethyleneglycol) ($M_n$ 5 000 g/mol) as described previously.[35] The degree of carboxymethylation of the dextran block, defined as the number of glucopyranose units having carboxymethyl groups per 100 glucopyranose units, was 85%. The average number of glucopyranosyl and of -$CH_2$-$CH_2$-O- repeat units of the CMD and PEG segments, were 40 and 140, respectively. Water was deionized using a Millipore MilliQ system.

### Synthesis of n-dodecyl-carboxymethyldextran-*b*-poly(ethyleneglycol) (Dod-CMD-PEG)

A solution of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (216 mg, 0.87 mmol) in absolute ethanol (120 mL) was added gradually to a solution of CMD-PEG (288 mg, 0.97 mmol carboxylate) in water (120 mL, pH 4.0). The apparent pH of the water/ethanol mixture was adjusted to 4.0 and kept at this value for 30 min. Subsequently, n-dodecylamine (162 mg, 0.87 mmol) was added to the solution. The pH was increased to 9.0 by addition of NaOH (1.0 N). The reaction mixture was stirred for 1.0 h at this pH. Ethanol was removed from the mixture under reduced pressure at 50 °C and the product was recovered by freeze drying. The recovered product was subjected to soxhlet extraction with hexane for 24 h to remove the unreacted dodecylamine and EEDQ. The purified Dod-CMD-PEG was converted to its free acid form by treatment with a cation exchange resin (Amberlite® IR 120). The grafting density (defined as the number of dodecyl chains per 100 glycopyranose units) was determined from the $^1$H NMR spectrum of Dod-CMD-PEG in DMSO-$d_6$ using the ratio of the areas of the signals due to the terminal methyl protons of the dodecyl chain (0.85 ppm) and to dextran anomeric protons (4.66 ppm) (Fig. 2). Two Dod-CMD-PEG samples of grafting densities 18 and 38 were prepared.

### Synthesis of 6‴-guanidino-paromomycin peracetate (6‴-G-Par)

Aqueous NaOH (0.80 g, 20 mmol, in 5 mL $H_2O$) was added to a solution of penta-N-Cbz-paromomycin (compound **1**, 0.50 g, 0.26 mmol) (Scheme 2) in 1,4-dioxane (15 mL).[37,52] The selective deprotection of the 6‴ amine was complete after 16 h, as indicated by TLC on silica plates (mobile phase: $CHCl_3$:AcOEt:MeOH, 20 : 5 : 3) and confirmed by mass spectrometry (MS) analysis (*m*/*z* calcd for $C_{65}H_{77}N_5O_{22}$ [M + H]$^+$: 1280.5, MS found: 1280.6). 1,4-Dioxane was evaporated under reduced pressure. The separated white gum formed was dissolved in MeOH (3 mL). The methanolic solution was transferred in water (50 mL) to obtain a white precipitate that was recovered by filtration and dried by lyophilization. The resulting product was added to a solution of N,N′-diCbz-N″-triflylguanidine (compound **2**, 0.21 g, 0.47 mmol) and $Et_3N$ (0.11 mL, 0.78 mmol) in chloroform (20 mL).[38] The reaction mixture was kept refluxing for 18 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in the minimum amount of $CH_2Cl_2$ and loaded onto a silica gel column eluted with 0 to 5% MeOH in $CH_2Cl_2$ to yield N-Cbz protected guanidinylated paromomycin (0.41 g, 72%). *m*/*z* calcd for $C_{72}H_{84}N_7O_{26}$ [M + H]$^+$: 1462.5, MS found: 1462.7. Water was added to a solution of N-Cbz protected guanidinylated paromomycin (0.41 g, 0.28 mmol) in MeOH (5 mL) until the solution became cloudy. 20% $Pd(OH)_2$/C (80 mg) and few drops of AcOH were added to the cloudy solution and the suspension was stirred under hydrogen atmosphere (hydrogen balloon) until the conversion of the starting material into the product was completed as indicated by MS analysis (6 h). The mixture was filtered through a layer of Celite on cotton, concentrated under vacuum, washed with $CH_2Cl_2$ twice, dissolved in water and lyophilized to afford 6‴-G-Par (240 mg, 90%) as a peracetate salt. *m*/*z* calcd for $C_{24}H_{48}N_7O_{14}$ [M + H]$^+$: 658.3, MS found: 658.4.); $^1$H NMR (400 MHz, $D_2O$), δ (ppm): 5.69 (m, 1H), 5.33 (m, 1H), 5.11 (m, 1H), 4.28 (m, 1H), 414.18 (m, 1H), 4.02 – 3.98 (m, 3H), 3.88 – 3.72 (m, 4H), 3.68 – 3.01 (m, 11H), 2.29 (m, 1H), 1.87 (s, 15H), 1.61 (m, 1H), 1.12 (m, 1H); $^{13}$C NMR (101 MHz, $D_2O$), δ (ppm): 180.6, 156.9, 109.4, 95.6, 95.3, 83.9, 81.1, 77.4, 75.5, 73.3, 73.0, 72.3, 68.8, 68.5, 67.3, 65.9, 62.1, 60.1, 53.5, 50.6, 49.3, 48.4, 46.2, 41.4, 28.1, 22.7.

### Synthesis of 5″-deoxy-5″-guanidino-paromomycin peracetate (5″-G-Par)

Water (0.1 mL) and $PPh_3$ (0.27 g, 1.0 mmol) were added to a solution of (penta-N-Cbz-4′,6′-*O*-benzylidene-5″-deoxy-5″-azido-paromomycin) (compound **4**, 1.2 g, 0.86 mmol) in THF (30 mL) (Scheme 2).[39] The mixture was kept at room temperature for 18 h. The solvent was evaporated under reduced pressure. The residue, dissolved in the minimum amount of $CH_2Cl_2$, was loaded on a silica gel column eluted with 4 to 8% MeOH in $CH_2Cl_2$ yielding the corresponding 5″-amino compound (0.20 g, 17%, *m*/*z* calcd for $C_{70}H_{81}N_6O_{23}$ $[M + H]^+$: 1373.5, MS found: 1373.8. Subsequently, $Et_3N$ (0.041 mL, 0.30 mmol) and reagent **2** (0.80 mg, 0.18 mmol) were added to a solution of the 5″-amino compound (0.20 g, 0.15 mmol) in $CHCl_3$ (20 mL). The reaction mixture was refluxed for 18 h. The residue recovered after evaporation of the solvent under reduced pressure was dissolved in the minimum amount of $CH_2Cl_2$and loaded onto a silica gel column eluted with 2 to 7% MeOH in $CH_2Cl_2$ yielding the desired N-Cbz protected guanidinylated paromomycin (0.20 g, 83%, *m*/*z* calcd for $C_{87}H_{95}N_8O_{27}$ $[M + H]^+$: 1683.6, MS found: 1684.0.) The N-Cbz protected guanidinylated paromomycin (0.20 g, 0.12 mmol) was dissolved in 80% aqueous acetic acid (5 mL) and the solution was heated at 60 °C until total conversion of the starting material into the benzylidene deprotected product (5 h) as ascertained by MS analysis. The solvent was evaporated under reduced pressure. The residue was dissolved in MeOH (3 mL) and $H_2O$ was added dropwise until the solution became cloudy. The resulting mixture was treated with 20% $Pd(OH)_2/C$ (40 mg) and a few drops of acetic acid. The suspension was stirred under hydrogen atmosphere (hydrogen balloon) to achieve total conversion of the starting material into 5″-G-Par (6 h), as indicated by MS analysis (*m*/*z* calcd for $C_{80}H_{91}N_8O_{27}$ $[M + H]^+$: 1595.6, MS found: 1595.9). The mixture was filtered through celite, concentrated under vacuum, washed with $CH_2Cl_2$ twice, dissolved in water and lyophilized to afford 5″-G-Par (105 mg, 87%) as a peracetate salt. *m*/*z* calcd for $C_{24}H_{49}N_8O_{13}$ $[M + H]^+$: 657.3, MS found: 657.4. $^1$H NMR (400 MHz, $D_2O$), δ (ppm): 5.71 (m, 1H), 5.22 (m, 1H), 5.13 (m, 1H), 4.35 (m, 1H), 414.27 (m, 1H), 4.14 (m, 2H), 4.06 (m, 2H), 3.87 – 3.11 (m, 15H), 2.28 (m, 1H), 1.76 (s, 18H), 1.63 (m, 1H), 1.11 (m, 1H); $^{13}$C NMR (101 MHz, $D_2O$), δ (ppm): 180.7, 156.8, 109.8, 94.9, 94.0, 83.8, 78.4, 76.7, 76.2, 73.6, 72.5, 71.8, 69.8, 69.3, 68.7, 67.2, 66.9, 59.8, 53.3, 50.4, 49.2, 48.5, 43.5, 40.0, 28.0, 22.8.

### Preparation of aminoglycoside/copolymer micelles

Stock solutions of CMD-PEG or Dod-CMD-PEG (1.0 g/L) and aminoglycosides (4.96 g/L, 6.94 mmol/L and 5.27 g/L, 5.79 mmol/L for paromomycin sulfate and neomycin sulfate, respectively) were prepared in a phosphate buffer (10 mM, pH 7.0). Specified volumes of the aminoglycoside solution were added dropwise to a magnetically stirred polymer solution over a 10-min period to obtain solutions ranging in [amine]/[carboxylate] ratios from 1.0 to 5.0. The volume of each sample was adjusted to 2.5 mL using the same buffer. Samples had a copolymer concentration of 0.5 g/L, unless otherwise specified. The effect ionic strength on the stability of aminoglycoside/copolymer micelles was studied using solutions (copolymer concentration: 0.5 g/L; [amine]/[carboxylate] = 2.5) in 10 mM phosphate buffer (10 mM, pH 7.0). Aliquots of a NaCl stock solution (2.5 M) in the same buffer were added to the micellar solutions in volumes such that [NaCl] in the sample ranged from 10 to 400 mM. The mixtures were stirred for 5 min and the volume of each sample was adjusted to 2.5 mL with the same buffer. The pH of solutions of [NaCl] = 150 mM was increased to 7.4 by the addition of aqueous NaOH (1.0 N). The samples were stirred overnight prior to analysis.

### Aminoglycoside release studies

A micellar solution (3.0 mL, neomycin: 2.0 g/L, [amine]/[carboxylate] = 2.5) was introduced into a dialysis bag (MWCO = 6.0–8.0 kDa), which was immersed into a phosphate buffer (10 mM, 25 mL) kept at 37 °C and containing either no NaCl (pH 7.0 or 7.4) or 150 mM NaCl (pH 7.0 or 7.4). At predetermined time intervals, 5 mL aliquots were taken from the release medium and replaced by an equal volume of fresh buffer. A control experiment to determine the drug diffusion through the dialysis membrane was carried out starting with a neomycin solution in the absence of the polymer. The neomycin concentration in each aliquot was determined spectrophotochemically after treatment with *o*-phthaldialdehyde.[53] Each sample (1 mL) was treated with a *o*-phthaldialdehyde solution in isopropanol (1 mL, 1 mg/mL), diluted with isopropanol (1.5 mL), and the sample volume was adjusted to 5.0 mL by addition of aqueous borate buffer (50 mM, pH 9.0). The samples were allowed to stand for 1 h at room temperature. Neomycin concentration was determined from the solution absorbance at 335 nm and using a calibration curve. The cumulative percent neomycin released was plotted as a function of the dialysis time.

### Minimal inhibitory concentration (MIC) determination

Free aminoglycosides or aminoglycosides micelles (aminoglycoside concentration: 0.3 g/L, [amine]/[carboxylate] = 2.5) in deionized water were diluted using sterile Luria-Bertani media (LB) in a 96 wells plate to reach drug concentrations of 0.25, 0.50, 0.75, 1.00, 2.00, 4.00, 8.00, 16.0 and 32.0 μg/mL. *E. coli* XL-1 blue strain was grown at 37 °C in 2 mL sterile LB to mid log phase (until absorbance at 595 nm reaches 0.6). The resulting suspension was shaken for homogeneity. Aliquots (1 μL) were placed in the wells containing the micellar solutions. Blank samples were prepared without *E. coli* XL-1 blue strain. After shaking the plate at 37 °C for 5 h, the absorbance at 595 nm of each well was monitored. The lowest concentration at which the absorbance at 595 nm was the same as the blank samples was taken as the MIC. MIC determination was done in triplicates in all cases.

### Measurements

$^1$H NMR spectra were recorded using a Bruker AV-400 MHz spectrometer operating at 400 MHz. Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm). FTIR spectra were recorded on a Perkin Elmer Spectrum One spectrometer with a resolution of 8 $cm^{-1}$. Lyophilization was performed with a Virtis (Gardiner, NY) Sentry Benchtop (3L) freeze-dryer. UV–vis absorption spectra were recorded with an Agilent 8452A photodiode array spectrometer. Dynamic light

scattering experiments (DLS) were performed using a CGS-3 goniometer (ALV GmbH) equipped with an ALV/LSE-5003 multiple-τ digital correlator (ALV GmbH), a He-Ne laser (λ = 632.8 nm), and a C25P circulating water bath (Thermo Haake). A cumulant analysis was applied to obtain the diffusion coefficient (D) of the micelles in solution. The hydrodynamic radius ($R_H$) of the micelles was obtained using the Stokes–Einstein eqn (1),

$$D = \frac{k_B T}{6\pi\eta_s R_H} \quad (1)$$

where $\eta_s$ is the viscosity of the solvent, $k_B$ is the Boltzmann constant, and T is the absolute temperature. The constrained regularized CONTIN method was used to obtain the particle size distribution. The data presented are the mean of six measurements ± S.D. Solutions for analysis were filtered through a 0.45 μm Millex Millipore PVDF membrane prior to measurement. Isothermal titration calorimetry (ITC) measurements were carried out with a Microcal VP-ITC instrument. Solutions of neomycin sulfate, paromomycin sulfate and CMD-PEG were prepared in a phosphate buffer (10 mM, pH 7.0). Prior to measurements all solutions were degassed under vacuum for about 10 min to eliminate any air bubbles. Aliquots of the neomycin sulfate solution (10 μL, 6.0 g/L, 6.6 mM, 39.6 mM amine) or paromomycin sulfate solution (10 μL, 5.65 g/L, 7.92 mM, 39.6 mM amine) were injected from a 300 μL continuously stirred (300-rpm) syringe into a solution of CMD-PEG (1.43 mL, 0.75 g/L, 2.61 mM carboxylate) at 25 °C. Heats of dilution and mixing were determined in control experiments by injecting aliquots (10 μL) of each drug solution into the same buffer (1.43 mL). A total of 28 aliquots were injected into the sample cell in intervals of 300 s. The calorimetric data were analyzed and converted to enthalpy change using Microcal ORIGIN 7.0.

## 4. Conclusion

Electrostatic interactions between two aminoglycosides: neomycin and paromomycin and different CMD-PEG copolymers led to the formation of PIC micelles with a drug/CMD core and a PEG corona. The interactions were entropically driven and stronger in the case of neomycin/CMD-PEG compared to paromomycin/CMD-PEG. Aminoglycosides/CMD-PEG micelles were unstable under physiological conditions (pH 7.4, [NaCl] = 150 mM). Micelles stability was significantly improved by hydrophobic modification of CMD-PEG or guanidinylation of paromomycin. Neomycin/$Dod_{38}$-CMD-PEG micelles resisted salt-induced disintegration for up to 200 mM. Smaller micelle size and better stability against salt were observed for drugs having more cationic groups and polymers having both carboxylate and dodecyl groups. The proposed approaches of micelle stabilization could be applied to other unstable PIC micelles.

## Acknowledgements

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