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Attachment of antibiotics to nanoparticles: preparation, drug-release and antimicrobial activity in vitro

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Summary

Conditions for attachment of ampicillin and gentamicin to nanoparticles were studied. A freeze-dried formulation was also developed. The release rate of ampicillin from nanoparticles was studied both with and without esterases. For polyisobutylcyanoacrylate nanoparticles, the liberation of the drug was enhanced in esterase-rich medium. By contrast, drug release as a consequence of enzymatic degradation of the polymer was not observed with polyisohexylcyanoacrylate nanoparticles. Finally, the antimicrobial activities of both ampicillin and gentamicin remained unaltered upon linkage of these molecules to the nanoparticles.

Introduction

Most intracellular infections are difficult to eradicate because bacteria are protected from antibiotics inside lysosomes (Horwitz, 1982). Infected cells may also constitute a "reservoir" for microorganisms, which are released from time to time causing the recurrence of systemic infections. The need for intracellular chemotherapy has been recognized for many years (Tulkens, 1985).

Resistance of intracellular infections to chemotherapy seems to be related to the low intracellular uptake at commonly used antibiotics or to their reduced activity of the acidic pH of lysosomes. Thus, antibiotics with basic activity — aminoglycosides — lead to lysosomal overloading whereas they display a reduced activity in acidic environment (Tulkens and Trouet, 1978). Conversely, acidic antibiotics — β-lactams — do not diffuse through the lysosomal membrane because of their ionic character at neutral extracellular or cytoplasmic pH (Tulkens, 1977). Finally, certain antibiotics which go into the cell more rapidly and to a larger extent, such as clindamycin (Johnson et al., 1980), are poorly retained in cells (Prokesh and Hand, 1982; Martin et al., 1985); hence activity is not expected to be very long-lasting in this case.

To overcome these difficulties, the development of endocytozable drugs was newly approached by simply associating antibiotics to particulate carriers such as liposomes (Stevenson et al., 1983;

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Nacucchio et al., 1985; Bakker-Woudenberg et al., 1985). Interesting results were obtained in the treatment of listeriosis with ampicillin in mice. The mechanism by which liposomes improve the therapeutic index of ampicillin is presumed to be an increased delivery of the drug to macrophages of the liver (Bakker-Woudenberg et al., 1985). Although these are encouraging data, problems associated with stability and manufacture of liposomes remain unresolved. Therefore, due to the satisfactory stability of nanoparticles both in vitro and in vivo (Couvreur et al., 1979), to the ease with which they are made (Verdun et al., 1986), as well as to their significant capture by Kupffer cells in vivo (Lenaerts et al., 1984), we considered the possibility of developing an alternative endocytozable antibiotic formulation. Thus, the present study describes conditions of attachment of ampicillin to biodegradable polyalkylcyanoacrylate nanoparticles, as well as the release behavior of the drug from these polymeric supports.

In addition, because nanoparticles can modify the distribution profile of drugs (Grislain et al., 1983) and therefore reduce their toxicity (Couvreur et al., 1982), gentamicin was also chosen as a drug model for attachment to the cyanoacrylate carrier.

Materials and Methods

Materials

Isobutylcyanoacrylate monomer was obtained from Ethnor (Paris, France). Isohexylcyanoacrylate monomer was synthetized by Weil Chemische Fabrik (Mannheim, F.R.G.). Various chemicals including dextran-70, D-glucose, theophylline and methanol were purchased from Prolabo (Paris, France). Ampicillin was from Negma (Buc, France) and gentamicin sulfate from Unilabo (Paris, France). The carboxylic ester hydrolase (esterase) was furnished by Boehringer (Meylan, France). Spores of *Bacillus subtilis* (ATCC 6633) were used as inoculum for classical microbiological assay. Assay medium no. 11 was obtained from Difco Laboratory (Detroit, MI 48232, U.S.A.).

Preparation of nanoparticles

Isobutyl or isohexylcyanoacrylate monomer (100 μl) was added under mechanical stirring to 10 ml of an aqueous polymerization medium (dextran 70 1% in 10^{-3} N HCl) containing either ampicillin (conc. 250–4000 μg/ml) or gentamicin (conc. 30 μg/ml). After polymerization of the monomer (2 h for isobutylcyanoacrylate, 6 h for isohexylcyanoacrylate), a milky suspension was obtained, neutralized with 0.1 M NaOH, and brought to isotonicity with glucose.

Ampicillin nanoparticles were then freeze-dried at $-40°C$ using a freeze-dryer (Secfroid, type TS 600, Aclens, Lausanne, Switzerland) for 48 h under vacuum (6×10 mbar). Resuspension of solid nanoparticle formulation was carried out by a single addition of distilled water (10 ml) to each vial.

The size of the nanoparticles was determined before and after freeze-drying by using a laser light scattering method (Nanosizer, Coulter, Margency, France).

Determination of drug content

Measurements of ampicillin loaded to nanoparticles were carried out on the supernatant liquid after ultracentrifugation of the samples at 35,000 rpm for 1 h. Analytical determinations were made using a reverse-phase HPLC method with spectrophotometric determination (224 nm), according to the method of Vree et al. (1978). The apparatus used consisted of an injector (Model, U6K), a solvent delivery system (Model M45) and a variable wavelength detector (model 480LC), all from Waters Associated (Milford, MA, USA). The column 25 cm $\times$ 4.6 mm C18 (Whatman, Clifton, U.S.A.) was protected by a guard column packed with silicium 60 μm (Whatman, Clifton, U.S.A.). The mobile phase consisted of methanol (15%) in phosphate buffer (pH = 4.6). Theophylline was used as an internal standard.

The amount of gentamicin bound to nanoparticles was estimated by a microbiological assay (Sabath and Anhalt, 1980). After 10 ml of nanoparticle suspension were ultracentrifuged at 80,000 $\times g$ (1 h), the sediments (bound drug) were separated from the supernatant liquid (containing the free drug) and dissolved in 10 ml 0.1 N NaOH. Samples (nanoparticles, supernatant and sediment solutions) were diluted with phosphate buffer (pH

= 8) before performing microbiological assays. The inoculum used for these assays were spores of *Bacillus subtilis*. The assay plates were prepared by adding 0.2 ml of *B. subtilis* spore suspension to 300 ml of molten assay medium at 48°C, pouring 50 ml of the uniformly seeded agar into plastic petri dishes (120 × 120 mm) and permitting them to harden at room temperature. Small 5 mm holes were punched in these plates and 50 μl samples were then placed in them. The diameter of each zone of inhibition was measured after incubation of the seeded agar at 37°C for 4–5 h. The results were calculated by forming a standard curve on semilog paper relating the concentration of gentamicin in the standards (log curve) to the diameter (in mm) of the zone of inhibition produced. The sensitivity was 0.025 μg/ml of gentamicin and 0.50 μg/ml of ampicillin. For both ampicillin and gentamicin, the level of drug binding was expressed as the percentage of drug associated with the carrier with respect to the initial amount of drug which was previously dissolved in the polymerization medium.

Drug release from nanoparticles

It has been previously shown that the release rate of drugs from nanoparticles correlates with the degradation rate of the polymer (Lenaerts et al., 1984). Moreover, the existence of an enzymatic degradation pathway consisting of ester hydrolysis has been identified (Lenaerts et al., 1984). For these reasons, the release rates of ampicillin from nanoparticles were estimated after incubation in the presence of carboxylic ester hydrolases. Actually, ampicillin adsorbed onto nanoparticles was incubated at 37°C in a phosphate buffer (pH = 7.4) in the presence or in absence of esterases. The concentration of ampicillin in the incubation medium was 200 μg/ml (1000 μg/ml in nanoparticle sample). Esterase concentration was 150 μg/ml. The pH was regularly controlled and eventually readjusted with 0.1 N NaOH. Samples were taken at different time intervals, ultracentrifuged (80,000 × g; 1 h), and the release of ampicillin in the supernatant liquid was estimated according to the HPLC method described above.

Measurement of the antimicrobial activity

Antimicrobial activity of ampicillin-loaded nanoparticles was compared to free ampicillin using *B. subtilis* spores as inoculum (Sabath and Anhalt, 1980). The same experiment was carried out after addition of esterases in order to induce drug release as a consequence of polymer erosion. Unloaded nanoparticles were also tested as a control.

Antimicrobial activity of gentamicin-loaded nanoparticles was determined by the same method.

Results and Discussion

Particle size analysis

The average diameters of unloaded nanoparticles were determined to be 132 nm for polyisobutyl- and 149 nm for polyisohexylcyanoacrylate nanoparticles. The size of these particles was significantly increased when they were loaded with ampicillin (Table 1). In fact, the higher the concentration of ampicillin in the polymerization medium, the greater was the size of the polymeric carrier. Likewise, the size of nanoparticles was increased after loading with gentamicin (132–281 nm).

Antibiotic content in nanoparticles

In the case of use of polyisobutylcyanoacrylate nanoparticles, 75% of the quantity of ampicillin initially dissolved in the polymerization medium (up to 1000 μg/ml) was associated with nanopar-

TABLE 1

Size [a] of polyisobutyl- (PIB) and polyisohexylcyanoacrylate (PIH) nanoparticles after incorporation of ampicillin or gentamicin

	Size (nm)	
	PIB	PIH
Initial amount of ampicillin (μg/ml)		
0	132 ± 10	149 ± 15
250	145 ± 10	–
500	165 ± 9	–
1000	180 ± 9	200 ± 30
2000	200 ± 10	245 ± 30
Initial amount of gentamicin (μg/ml)		
30	281 ± 8	–

[a] Each value represents the mean ± S.E.

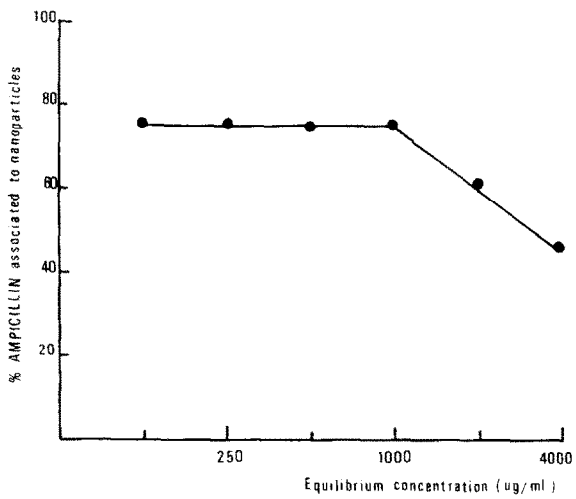

Fig. 1. Drug entrapment into polyisobutylcyanoacrylate nanoparticles (%) as function of the logarithm of the drug concentration in the polymerization medium (pH = 7.4).

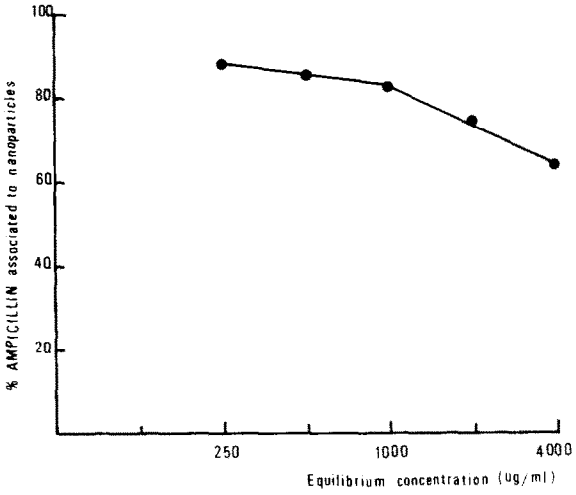

Fig. 2. Drug entrapment into polyisohexylcyanoacrylate nanoparticles (%) as function of the logarithm of the drug concentration in the polymerization medium (pH = 7.4).

ticles (Fig. 1). This percentage was reduced to 60%, when the initial concentration of ampicillin was 2000 μg/ml. For polyisohexylcyanoacrylate nanoparticles, 82% and 75% of the quantity of ampicillin were bound to nanoparticles, for initial concentration in the polymerization medium of, respectively, 1 mg/ml and 2 mg/ml (Fig. 2). The percentage of drug associated to nanoparticles decreased at higher concentration. When expressed in terms of absolute amount of drug linked, the carrier capacity of nanoparticles was found to increase as a function of drug concentration in the polymerization medium. Maximum carrier capacity was 184 μg of ampicillin/mg of polymer for polyisobutylcyanoacrylate nanoparticles and 256 μg of ampicillin/mg of polymer for polyisohexylcyanoacrylate nanoparticles. For a 30 μg/ml concentration of gentamicin in the polymerization medium, 70% (±10%) of the drug was associated to polyisobutylcyanoacrylate nanoparticles.

Freeze-drying of ampicillin-loaded nanoparticles

After the freeze-drying process, ampicillin-loaded nanoparticles were easily resuspended in water. Furthermore, comparative size measurements showed no significant modification of the carrier dimensions (Table 2). Likewise, the level of drug binding to nanoparticles was not altered by the freeze-drying process (Table 2).

Drug release from nanoparticles

A characteristic of cyanoacrylate polymers is that their rate of degradation is dependent on the length of their alkyl chain (Léonard et al., 1966). Indeed, bio-erosion is prolonged with increasing alkyl chain length. Furthermore, it has been previously demonstrated that drug-release could be a consequence of polymer degradation which occurs probably through an enzymatic pathway (Lenaerts et al., 1984). Consequently ampicillin release was tested from polyisobutyl- or polyisohexylcyanoacrylate nanoparticles. Ampicillin release from nanoparticles was weak in an esterase-free medium (Fig. 3). However, no difference was found in the release rate from polyisobutyl- and polyiso-

TABLE 2

Particle size [a] and drug binding [a] before and after freeze-drying of ampicillin [b]-loaded polyisobutyl- (PIB) and polyisohexyl-(PIH) cyanoacrylate nanoparticles

	Size		Drug binding (%)	
	PIB	PIH	PIB	PIH
Before freeze-drying	180 ± 9	205 ± 28	72 ± 4	78 ± 4
After freeze-drying	185 ± 10	217 ± 30	70 ± 5	82 ± 5

[a] Each value represents the mean ± S.E.

[b] Concentration of ampicillin in polymerization medium: 1000 μg/ml.

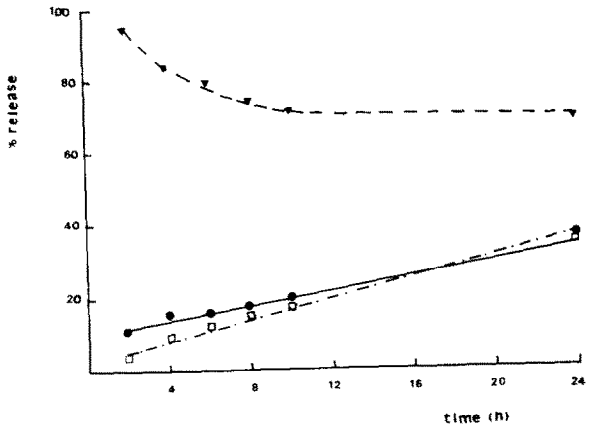

Fig. 3. The release profiles of ampicillin from polyisobutyl- (□) and polyisohexylcyanoacrylate nanoparticles (●) in absence of esterases. Free ampicillin was used as a control of stability (▼).

hexylcyanoacrylate nanoparticles. Furthermore, in both cases, release appeared slightly biphasic with an initial fast release rate, which was probably due to the external adsorption of the ampicillin onto the nanoparticles, after which liberation of ampicillin followed a zero-order kinetic (Fig. 3).

In the presence of esterases (150 μg/ml), release of ampicillin was considerably increased (Fig. 4). For polyisobutylcyanoacrylate nanoparticles 60% of the nanoparticle drug content was released after 10 h incubation. In fact, it is conceivable that ampicillin was released from nanoparticles to a larger extent, since it was found that free ampicillin, incubated as a control under the same conditions, became partly degraded (Fig. 4). Thus, 75% of the amount of free ampicillin remained still intact after 8 h of incubation. By contrast, the presence of esterases did not seem to modify the drug liberation profile from polyisohexylcyanoacrylate nanoparticles. Indeed, 35% of the drug content was released after 24 h, in presence of esterases and 34% without them. This could result from a reduced accessibility of the ester function to the enzymes because of the steric overcrowding due to the longer alkyl chain.

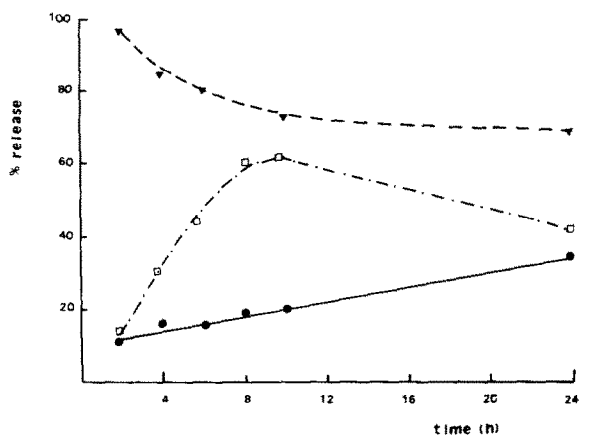

Fig. 4. The release profiles of ampicillin from polyisobutyl- (□) and polyisohexylcyanoacrylate nanoparticles (●) in presence of esterases. Free ampicillin was used as a control of stability (▼).

Antimicrobial activity

Fig. 5 shows the antimicrobial activity of 4 progressive dilutions of ampicillin-loaded nanoparticles, compared to both free ampicillin and ampicillin-loaded nanoparticles which were predegraded by previous addition of esterases. For all tested samples, the zones of inhibition were obviously equivalent. These observations confirmed that antibiotic activity of ampicillin was preserved after linking to nanoparticles.

This suggests that even without esterases, the drug was released from nanoparticles within the

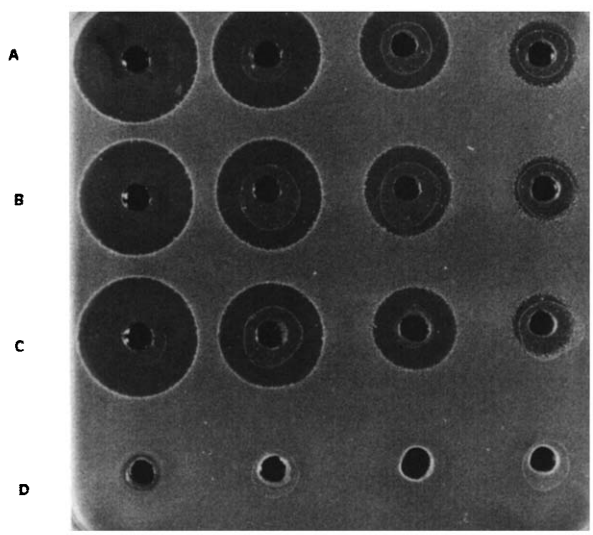

Fig. 5. Antimicrobial activity of ampicillin (A), ampicillin-loaded nanoparticles (B), ampicillin-loaded nanoparticles pretreated with esterases (C) and unloaded nanoparticles (D).

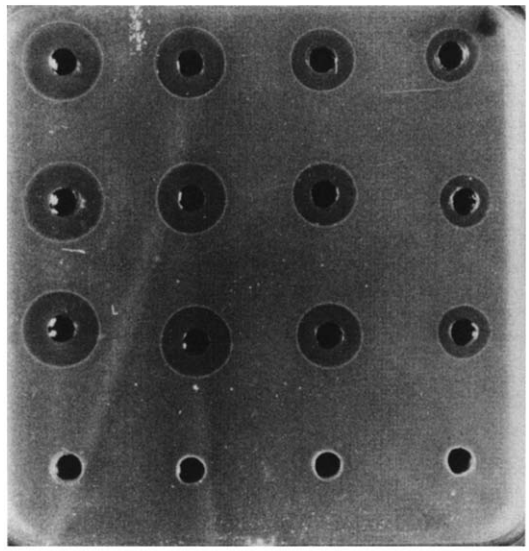

Fig. 6. Antimicrobial activity of gentamicin (A), gentamicin-loaded nanoparticles (B), gentamicin-loaded nanoparticles pretreated with esterases (C) and unloaded nanoparticles (D).

incubation time. By contrast, absence of inhibition was noted with ampicillin-free nanoparticles, except for the nanoparticles with the higher dose, which displayed a slight bacteriotoxicity.

The same observations were made in experiments using gentamicin as a drug model (Fig. 6), leading to similar conclusions as for ampicillin.

Conclusion

The results presented in this work have established experimental conditions for attachment of both ampicillin and gentamicin to polyalkylcyanoacrylate nanoparticles. The carrier capacity of these nanoparticles can achieve up to 256 μg ampicillin/mg polymers. Due to the possible degradation of ampicillin in aqueous acidic polymerization media, a freeze-dried formulation was also developed. It was shown that such a formulation was reproducible with respect to both size and drug content.

Concerning the release of ampicillin from nanoparticles without esterases in the medium of incubation, no difference was noted between polyisobutyl- and polyisohexylcyanoacrylate nanoparticles. This observation does not seem to confirm previous studies made with polyalkylcyanoacrylates. Indeed, drug release from nanoparticles was described to follow polymer degradation (Lenaerts et al., 1984), whereas, on the other hand, polymer degradation rate was found to be dependent upon alkyl chain length (Léonard et al., 1966; Couvreur et al., 1979).

In the presence of esterases, the release of ampicillin was, however, significantly increased for polyisobutylcyanoacrylate nanoparticles. This result confirmed the hypothesis that polyalkylcyanoacrylate nanoparticles are mainly degraded by an enzymatic process leading to more and more hydrophilic compounds (polycyanoacrylic acid) (Lenaerts et al., 1984). Considering that most intracellular infections are localized inside esterase-rich lysosomes, these lysosomes could be an ideal target for antibiotic-loaded polyisobutylcyanoacrylate nanoparticles. Drug release as a consequence of enzymatic degradation of the polymer was, however, not confirmed for polyisohexylcyanoacrylate nanoparticles, for which no difference was noted concerning drug-release with or without esterases. The steric overcrowding of the ester function, due to a longer alkyl chain, is probably the reason for this surprising observation.

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