ELSEVIER

International Journal of Pharmaceutics 105 (1994) 181–186

international journal of pharmaceutics

Note

Gelatin microspheres as a new approach for the controlled delivery of synthetic oligonucleotides and PCR-generated DNA fragments

Rita Cortesi [a], Elisabetta Esposito [a], Enea Menegatti [a], Roberto Gambari [b], Claudio Nastruzzi [a,*]

[a] *Department of Pharmaceutical Sciences,* [b] *Biotechnology Centre, Ferrara University, Via Fossato di Mortara 19, I-44100 Ferrara, Italy*

(Received 5 October 1993; Accepted 14 October 1993)

Abstract

The present paper reports the preparation and characterization of gelatin microspheres containing (a) a 44-mer single-stranded synthetic oligonucleotide, complementary to the HLA-DRA gene (ssDNA-44) and (b) a double-stranded fragment, 144 bp in length, prepared by the polymerase chain reaction (PCR) mimicking a region of the HIV-1 LTR (dsDNA-144). Spherical gelatin microspheres were obtained by a coacervation method, showing a high percentage of encapsulation yields (over 85%). Size distribution analysis of the microspheres produced resulted in an average diameter of 22 μm. In order to analyse the release profiles of both ssDNA-44 and dsDNA-144 from microspheres, in vitro studies were carried out by using a flow-through cell method. The chemical stability of dsDNA-144 to the encapsulation procedure steps was in addition demonstrated by PCR amplification of the DNA eluted from the gelatin microspheres. The reported results indicate that gelatin-based microspheres offer excellent potential as carrier systems for the in vivo administration of both single- and double-stranded DNA molecules.

Key words: Synthetic oligonucleotide; PCR-generated DNA fragment; Gelatin microsphere; Controlled release

Antisense oligonucleotides complementary to viral or eukaryotic RNAs have increased in importance because of their ability to block the viral life cycle as well as to control the expression of target genes (e.g., oncogenes and transfected reporter genes) in different cellular systems (Uhlmann and Peyman, 1990; Neckers et al., 1992; To and Neiman, 1992). Accordingly, these newly discovered repressors of gene expression could be proposed as novel pharmacological agents for the therapy of pathological states associated with an altered expression of gene(s) such as viral infections (including AIDS) as well as neoplastic diseases (Mirabelli et al., 1991).

In addition, other small DNA molecules, such as double-stranded synthetic oligonucleotides or polymerase chain reaction (PCR) generated DNA fragments (approximately comprised in a size range between 30 and 300 base pairs), could be

* Corresponding author.

SSDI 0378-5173(93)E0318-K

efficiently employed as molecular tools to study and modulate gene transcriptional activity. These molecules could indeed exert their biological activity mimicking genomic regulatory regions recognised by transcriptional factors (TFs) interacting in this way with specific TFs and finally causing their displacement from the target DNA sequence (Gambari and Nastruzzi, 1993).

These considerations are particularly important, since interactions between TFs and specific DNA regions located in the promoters of eukaryotic and viral genes represent one of the crucial molecular events regulating the transcriptional activity of genes (Mitchell and Tjian, 1989; Faisst and Meyer, 1992).

It should be pointed out that the use of antisense oligonucleotides and PCR-generated DNA fragments could in principle direct the biological effects on the transcription/translation of a limited number of genes, potentially leading to the selective inhibition of protein synthesis.

In spite of these interesting pharmaco-biological properties, both oligonucleotides and PCR-generated DNA fragments are in general rapidly degraded by cellular and extracellular nucleases. These drawbacks greatly reduce their applicability as therapeutic agents since, in order to exert their pharmaco-biological activities, oligonucleotides should remain stable in the extracellular environment and display appreciable half-lives after in vivo administration.

The task of resolving the stability problems of this class of compounds has been mainly approached through chemical changes of the oligonucleotide molecular structure. This can be achieved either by strategies involving the modification of the phosphate ester backbone or based on the reversion of the phosphate anomeric configuration. To this end, methylphosphonates, phosphorotioates and α-oligodeoxynucleotides have been recently proposed as nuclease-resistant compounds (Crooke, 1991).

Alternatively, another interesting approach to reduce degradation and possible toxicity problems related to oligonucleotide use in vivo is offered by their encapsulation in liposomes. The use of liposomes as carriers for DNA molecules has demonstrated that the instability problems of this class of compounds in biological media can be effectively eliminated (Thierry et al., 1992).

In the search for other administration strategies able to increase both DNA stability and activity towards target cells, in the present paper we propose the alternative use of gelatin microspheres (GMs) as a new delivery system for synthetic oligonucleotides and PCR-generated DNA fragments. The pharmaceutical applicability of microspheres as carrier systems has indeed been demonstrated in a variety of medical applications, including drug delivery and vaccine administration (Langer, 1990). In addition, microspheres have been utilized to transport the entrapped agent to selected sites, improving the therapeutic benefit and both the pharmacokinetic and pharmacodynamic profiles of the encapsulated compounds (Davis et al., 1993).

Concerning the polymeric material that we employed for microsphere production, it should be stressed that the use of gelatin in pharmaceutics is particularly attractive by virtue of its biocompatibility and biodegradability together with the total absence of toxicity or allergic problems possibly associated with the use of synthetic polymers (Nastruzzi et al., 1993a).

With these aims, in this report we describe (a) the preparation and characterization of gelatin microspheres containing small DNA molecules and (b) the in vitro release characteristics of the DNA-carrying gelatin microspheres.

To study the feasibility of the entrapment of small DNA molecules in microspheres, we utilized, as model compounds: (a) a 44-mer single-stranded synthetic oligonucleotide, complementary to the HLA-DRA gene (ssDNA-44); and (b) a double-stranded fragment, 144 bp in length, prepared by the polymerase chain reaction, mimicking a region of the HIV-1 LTR (dsDNA-144).

ssDNA-44 was synthesized on a Pharmacia Gene Assembler Plus DNA synthesizer and purified on a Pharmacia biocompatible HPLC system by standard methods.

dsDNA-144 was prepared by PCR using as template DNA the plasmid pBC12/HIV-1/CAT (Zoumpourlis et al., 1991) which contains the entire LTR region of the HIV-1 genome and two specific PCR primers, whose location and se-

quence are reported in Fig. 1. Each PCR reaction was performed in 50 μl (final volume) containing 50 mM KCl, 10 mM Tris-HCl pH 9, 1 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 ng of template DNA, 150 ng of each primer, 1.65 μM dATP, 1.65 μM dTTP, 1.65 μM dGTP, 1.55 μM dCTP plus 30 μCi of [α-^{32}P]dCTP and 2 U/reaction of *Taq* DNA polymerase (Promega, U.S.A.). After initial denaturation for 5 min at 98°C, 30 cycles of amplification were performed consisting of 60 s denaturation at 94°C, 60 s annealing at 55°C and 60 s extension at 72°C.

The microspheres were produced by the coacervation technique as previously described (Nastruzzi et al., 1993a). Briefly, 7 ml of aqueous gelatin solution (15%, w/v; pre-heated at 80°C), containing the synthetic oligonucleotide (ssDNA-44) or the PCR product (dsDNA-144), together with trace amounts of ^{32}P-labelled material, were added at 80°C to 40 g of an oil phase constituted of isopropyl palmitate. The mixture was mechanically stirred under turbulent flow conditions to form a w/o emulsion, after 5 min, the solution was rapidly cooled at 15°C and then, 30 ml acetone were added in order to dehydrate and flocculate the coacervated droplets. Gelatin microspheres containing DNA molecules were isolated from the suspension by filtration through a sintered glass filter. The removal of residual oil was performed by washing the microspheres with 3 × 80 ml aliquots of acetone.

The morphology of GMs was evaluated by observation on optical and scanning electron microscopy (SEM). Microsphere size and size distribution were determined by analysing SEM photomicrographs showing at least 100 microspheres.

Entrapment yields for both ssDNA-44 and dsDNA-144, were determined by detecting the activity of ^{32}P-labelled DNA molecules in microspheres. The in vitro release kinetics of ssDNA-44 and dsDNA-144 from microspheres were determined by a flow-through cell method (Nastruzzi et al., 1993b), quantitating the released ^{32}P-labelled DNA molecules by β-counting.

Concerning the different experimental parameters employed for microsphere preparation (speed of mixing, aqueous to organic phases ratio and temperature), we examined their effect with respect to the oligonucleotide encapsulation yield and particle size. The most critical factor influencing particle size and size distribution was found to be the stirring speed. By using a stirring speed of 1000 rpm, small microspheres with an average diameter of 22 ± 9 μm in the dehydrated state and narrow size distribution were obtained. In Fig. 2, microphotographs of the gelatin microspheres (panels A, B) and their size distribution analysis (panel C) are respectively reported, relative to microspheres containing ssDNA-44. Electron microscopy shows that aggregation phenomena are almost absent and that no pores are present on the relatively rough surface of the microspheres. Similar results were obtained for microspheres containing dsDNA-144 (data not shown). Taken together, these results indicate that the microspheres produced can be consid-

a. ssDNA-44
44-mer single-stranded synthetic oligonucleotide

OLI-44: CTAATGTGCTTCAGGTATAT
CCCTGTCTAGAAGTCAGATT
GGGG

Complementary to the HLA DRA gene

b. dsDNA-144
144-mer double-stranded PCR generated fragment

primer E: ATTTCATCACATGGCCCGAG
primer F: CAGGATCTGAGGGCTCGCC

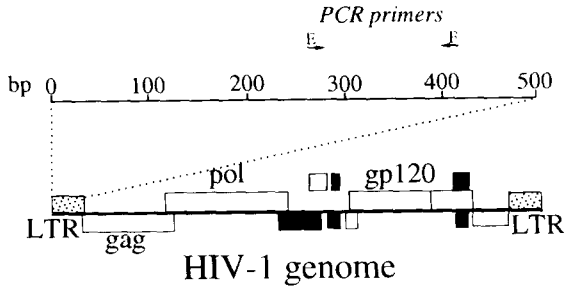

Fig. 1. Synthetic oligonucleotide and PCR-generated fragment employed in the present study. 44-mer single-stranded synthetic oligonucleotide, complementary to the HLA-DRA gene (ssDNA-44) (a) and double-stranded fragment, 144 bp in length, prepared by polymerase chain reaction and mimicking a region of the HIV-1 LTR (dsDNA-144) (b).

ered ideally suitable for encapsulation, release and activity studies of bioactive compounds.

Moreover, the microsphere recovery (the weight percentage of isolated microsphere with respect to the total amount of gelatin used) and encapsulation yields for both single- and double-stranded-oligonucleotides were found to be over 85% under all the experimental conditions utilized for microsphere preparation (see Table 1). This latter point is particularly appealing, since it allows the preparation of microspheres with a high oligonucleotide specific content without loss of material that, especially in the case of expensive compounds (e.g., synthetic oligonucleotides), could profoundly influence the total cost of the microencapsulation procedure

Table 1
Microsphere recovery and encapsulation yield for gelatin microspheres encapsulating single- and double-stranded DNA molecules

	Microsphere recovery (%) [a]	Encapsulation yield (%)
Single-stranded synthetic oligonucleotide ssDNA-44	87.2 ± 2.7	87.79 ± 4
Double-stranded PCR-generated DNA fragment dsDNA-144	93.0 ± 4.5	92.1 ± 8.2

Data represent the average of four independent experiments ± SD.
[a] Weight percentage of isolated microsphere with respect to the total amount of gelatin used.

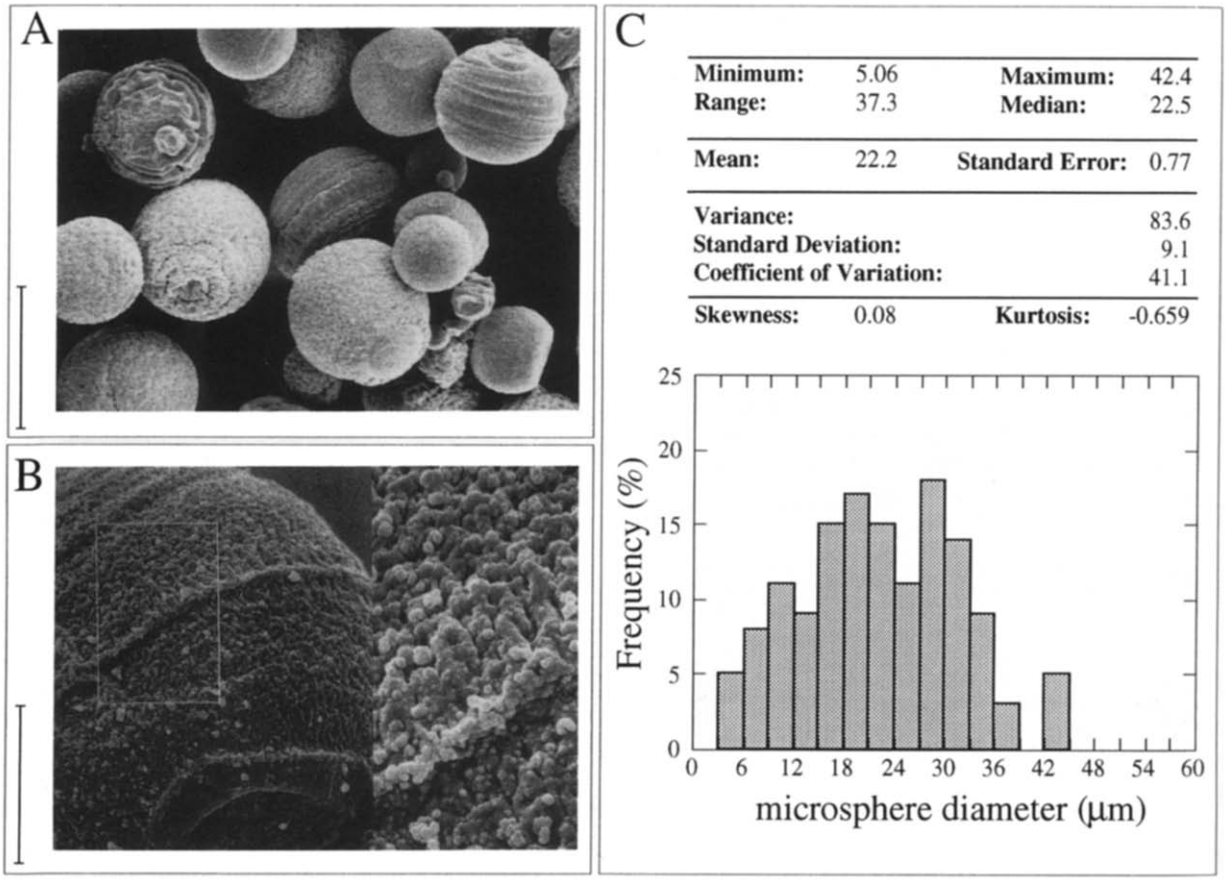

Fig. 2. (A,B) Scanning electron micrographs of gelatin microspheres containing the ssDNA-44 synthetic oligonucleotide. Bars: 25 μm (A) and 10 μm (B), respectively. (C) Size distribution analysis of ssDNA-44 containing microspheres.

Since the therapeutic efficacy of a microencapsulated compound is largely due to its release and local concentration at organ or tissue level, the analysis of both amount and mechanism(s) of drug release, after administration, represents one of the major steps for the development of a microsphere-based delivery system (Magenheim and Benita, 1991). Fig. 3 reports a schematic representation of the experimental system used for the determination of oligonucleotide release kinetics (panel A) together with the complete release profile of the microencapsulated ssDNA-44 and dsDNA-144 molecules. The results presented here demonstrate that the release from microspheres of both ssDNA-44 and dsDNA-144 is generally biphasic, with an initial relatively rapid release phase (a 'burst effect'), followed by a slower release phase.

With the aim of showing that the entire encapsulation procedure does not cause degradation phenomena on the entrapped dsDNA-144, we determined, by PCR technology, after release from microspheres (namely, in the fractions eluted from the release column), whether the full-length double-stranded DNA fragment dsDNA-144 was still present. This was demonstrated by amplification (using primers E and F; see Fig. 1) of the material eluted from the microspheres and analysis of the amplified PCR products by agarose gel electrophoresis (see Fig. 4).

Taken together, these results demonstrate that gelatin microspheres represent an attractive challenge for the in vivo administration of DNA molecules, possibly leading to controlled delivery of both single- and double-stranded oligonucleotides. The complete biocompatibility of the

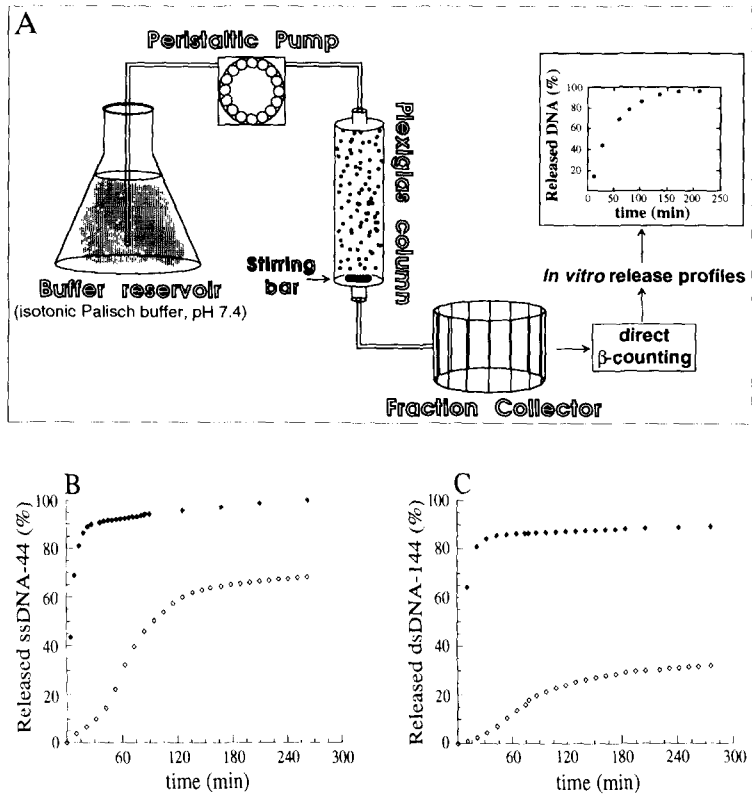

Fig. 3. (A) Schematic representation of the flow-through cell system employed for the determination of the release kinetic profiles. (B,C) Release profiles of ssDNA-44 (B) and dsDNA-144 (C) from microspheres (◇). For comparison, the profiles obtained with the free compounds (♦) are reported.

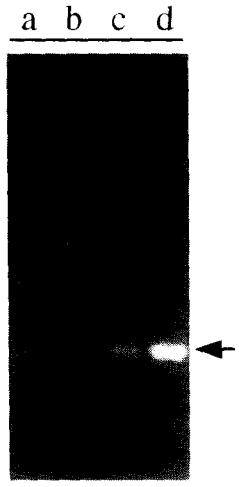

Fig. 4. Polymerase chain reaction (PCR) amplification of the plasmid pBC12/HIV-1/CAT (Zoumpourlis et al., 1991) leading to the production of a fragment mimicking the 281–424 region of the HIV-1 LTR (GenBank entry HIVHXB2CG) (lane a). The 144 bp fragment obtained (dsDNA-144) was utilized for microsphere entrapment. (Lane b) DNA molecular weight marker V; (lanes c,d) PCR amplification of the dsDNA-144 fragments contained in the fractions from the flow-through cell release experiments of free (lane c) or microsphere-encapsulated (lane d) dsDNA-144.

polymer used for the production and the optimal morphological characteristics of the microspheres indicate a promising perspective for their use as drug carriers.

1. Acknowledgements

This work was supported by CNR Target Projects ACRO and Genetic Engineering, by ISS (AIDS Projects 1991–1993) and by Regione Emilia Romagna.

2. References

Crooke, R.M., In vitro toxicology and pharmacokinetics of antisense oligonucleotides. *Anti-Cancer Drug Design*, 6 (1991) 609–646.

Davis, S.S., Illum, L., Moghimi, S.M., Davies, M.C., Porter, C.J.H., Muir, I.S., Brindley, A., Christy, N.M., Norman, M.E., Williams, P. and Dunn, S.E., Microspheres for targeting drugs to specific body sites. *J. Controlled Release*, 24 (1993) 157–163.

Faisst, S. and Meyer, S., Compilation of vertebrate-encoded transcription factors. *Nucleic Acids Res.*, 20 (1992) 3–26.

Gambari, R. and Nastruzzi, C., DNA-binding activity and biological effects of aromatic polyamidines. *J. Biochem. Pharmacol.*, (1993) in press.

Langer, R., New methods of drug delivery. *Science*, 249 (1990) 1527–1533.

Magenheim, B. and Benita, S., Nanoparticle characterization: a comprehensive physicochemical approach. *STP Pharm. Sci.*, 1 (1991) 221–241.

Mirabelli, C.K., Bennet, C.F., Anderson, K. and Crooke, S.T., In vitro and in vivo pharmacologic activities of antisense oligonucleotides. *Anti-Cancer Drug Design*, 6 (1991) 647–661.

Mitchell, P.J. and Tjian, R., Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*, 245 (1989) 371–378.

Nastruzzi, C., Pastesini, C., Cortesi, R., Esposito, E., Gambari, R. and Menegatti, E., Production and in vitro evaluation of gelatin microspheres containing an antitumor tetra-amidine. *J. Microencapsulation*, (1993a) in press.

Nastruzzi, C., Esposito, E., Cortesi, R., Gambari, R. and Menegatti, E., Kinetic of bromocriptine release from microspheres: comparative analysis between different in vitro models. *J. Microencapsulation*, (1993b) in press.

Neckers, L., Whitesell, L., Rosolen, A. and Geselowitz, D.A., Antisense inhibition of oncogene expression. *Crit. Rev. Oncogenesis*, 3 (1992) 175–231.

Thierry, A.R., Rahman, A. and Dritschilo, A., Liposomal delivery as a new approach to transport antisense oligonucleotides. In Erickson, R.P. and Izant, J.G. (Eds), *Gene Regulation: Biology of Antisense RNA and DNA*, Raven Press, New York, 1992, pp. 147–161.

To, R.Y. and Neiman, P.E., The potential for effective antisense inhibition of retroviral replication mediated by retroviral vectors. In Erickson, R.P. and Izant, J.G. (Eds), *Gene Regulation: Biology of Antisense RNA and DNA*, Raven Press, New York, 1992, pp. 261–271.

Uhlmann, E. and Peyman, A., Antisense oligonucleotides: a new therapeutic principle. *Chem. Rev.*, 90 (1990) 543–584.

Zoumpourlis, V., Kerr, D.J. and Spandidos, D.A., Doxorubicin stimulate transcription from the human immunodeficiency virus long terminal repeat sequences. *Cancer Lett.*, 56 (1991) 181–185.