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International Journal of Pharmaceutics 151 (1997) 57–67

international journal of pharmaceutics

# Antisense oligonucleotide delivery to cultured macrophages is improved by incorporation into sustained-release biodegradable polymer microspheres

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Received 22 November 1996; revised 15 January 1997; accepted 21 January 1997

**Abstract**

Macrophages are important reservoirs of infection including the human immunodeficiency virus (HIV) virus and strategies which target therapeutic agents to these cells appear worthwhile. In this study, we have evaluated the use of biodegradable poly(lactide-co-glycolide) (P(LA–GA)) microspheres for the improved delivery of anti-HIV oligonucleotides (ODNs) to macrophage cells in culture. Phosphodiester (PO) or phosphorothioate (PS) sequences, including those antisense to the *tat* gene in HIV, were incorporated into biodegradable P(LA–GA) microspheres (1–2 $\mu$m) using a double-emulsion solvent evaporation procedure. For a given polymer molecular weight and ODN chemistry, entrapment efficiencies and in vitro release rates were dependent on microsphere size. Smaller microspheres (1–2 $\mu$m) released 70% of the entrapped ODN within 4 days compared with 40 days for larger microspheres (10–20 μm), suggesting that this delivery system also offers the potential for sustained release of ODNs. The cellular association of ODNs entrapped within small microspheres was improved 10-fold in murine macrophages compared with free ODNs. Uptake was enhanced when macrophages were activated with interferon-$\gamma$ (INF-$\gamma$) and lipopolysaccharide (LPS) treatment but decreased significantly in the presence of metabolic and phagocytosis inhibitors. Fluorescence microscopy studies with macrophages showed that a more diffuse subcellular distribution of ODNs was observed when delivered as a microsphere formulation compared with free ODNs, which exhibited the characteristic punctate periplasmic distribution. These results indicate that polymer microspheres represent an attractive strategy for the improved cellular delivery of ODNs. © 1997 Elsevier Science B.V.

*Keywords:* Cell uptake; Microspheres; Nucleic acid delivery; Oligodeoxynucleotides; Poly(lactide-co-glycolide) copolymer

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*PII* S0378-5173(97)04887-4

## 1. Introduction

The use of antisense, aptamers and ribozyme oligonucleotides (ODNs) as potential therapeutic agents in anti-HIV (human immunodeficiency virus) therapy is currently being investigated in preclinical and clinical trials studies (Akhtar and Rossi, 1996; Narayanan and Akhtar, 1996; Akhtar and Agrawal, 1997). However, the in vivo therapeutic applications of ODN-based therapies are hindered by their poor biological stability (Wickstrom, 1986; Akhtar et al., 1991), short half-life in the circulation (Agrawal et al., 1992) and limited cellular targeting (Akhtar and Juliano, 1992). Therefore, the successful development of nucleic acid-based therapeutics will require the design of delivery strategies that can improve the cellular targeting and uptake of these agents to diseased cells (Akhtar, 1995).

Since macrophages represent an important reservoir for HIV infection (Roy and Wainberg, 1988; Meltzer et al., 1990), we have been interested in developing macrophage targeted delivery strategies for anti-HIV ODNs. For example, mannose-conjugated ODNs, prepared by an automated synthetic procedure, significantly improved the delivery of these molecules via lectin-mediated uptake in cultured murine macrophages (Akhtar et al., 1995). Biodegradable polymer carriers also represent an attractive strategy (Langer, 1990; Pouton and Akhtar, 1996). Biodegradable polylactide (PLA) matrices can both protect the entrapped ODN or ribozyme from nuclease digestion and also impart sustained release properties (Lewis et al., 1995; Hudson et al., 1996b). PLA and P(LA–GA) copolymers offer several advantages: they are biocompatible and biodegradable in vivo and in cultured macrophages (Kimura et al., 1994) and following their use in surgical sutures have been approved for clinical use in sustained release preparations (e.g. Zoladex, Zeneca Pharmaceuticals, UK). Further, they may offer the potential of targeting ODNs to therapeutic gene products with long half-lives. In the present study, we have investigated the potential use of biodegradable P(LA–GA) copolymer microspheres for the improved delivery of ODNs to murine RAW264.7 macrophages in culture.

## 2. Methods

### 2.1. ODN synthesis and labelling

Phosphodiester (PO) and phosphorothioate (PS) ODN sequences (20-mer) complimentary to the *tat* gene in HIV (5′-ACACCCAATTCTGAAAATGG-3′) and a 3′ hairpin-loop containing (self-stabilized) 20-mer PO ODN complementary to exon 2 of the c-*myc* oncogene (3′-CGAAAGCGTACGGGGAGTTG-5′; hexameric hairpin structure underlined) were synthezised on an automated DNA/RNA syntheziser (Model 392, Applied Biosystems (ABI), Warrington, UK) using standard cyanoethyl chemistry (reagents from Cruachem, Glasgow, UK). In some sequences, fluorescein was added at the 5′-end of the ODN as the terminal coupling step in the automated synthesis cycle by using the FAM-amidite reagent according to the manufacturer's protocol (Applied Biosystems, Warrington, UK). The deprotected ODNs were purified using a NAP 10 column (Pharmacia Biotech) containing Sephadex G25. The ODN was eluted in sterile water, dried under vacuum (DNA Speed Vac, Savant, UK) and then stored at −20°C until use. The concentration of ODN was assessed by optical density measurements at 260 nm (Sambrook et al., 1989). The 5′-end $^{32}$P-radiolabelled ODNs were prepared using bacteriophage T4 polynucleotide kinase as described previously (Coulson et al., 1996).

### 2.2. Microsphere preparation

The required concentration of an aqueous ODN solution (100 $\mu$l) containing 0.4% w/v polyvinylalcohol (PVA), was added to 5 ml of dichloromethane containing 500 mg of P(LA–GA) 50:50 copolymer (molecular mass = 3000 Da; Alpha Chemicals, UK)) and the primary emulsion prepared by vortexing for 5 min. This was then added to 80 ml of aqueous external phase (saline 0.9% w/v, methylcellulose 0.05% w/v and PVA 4% w/v) at 4°C and stirred at 1000 rpm at room temperature using Heidolph stirrers (Lab-Plant, Huddersfield, UK) for 3 h to allow for complete solvent evaporation. The resulting spheres were centrifuged at 4000 rpm for 10 min using a Starst-

edt LCI centrifuge, and washed three times with distilled water to remove any emulsifier and non-loaded ODN. The pelleted spheres were washed and freeze-dried overnight. This procedure resulted in microspheres with diameters in the 10–20 $\mu$m size range as determined by scanning electron microscopy (Stereoscan 90 model, Cambridge Instruments, UK) of gold-coated samples (Emscope SC 500 Sputter-Coater) and by laser light-scattering particle sizing of freeze-dried samples (Malvern Instruments, UK). In order to produce smaller microspheres in the 1–2 $\mu$m size range, a different mixing method was employed. In this case, the primary emulsion was mixed using a Silverson Homogeniser STD 2 (Silverson Machines, Chesham, UK) at high speed (16 000 rpm) with a 3/8 inch sonicating micro-probe for 2 min. The double emulsion was mixed for 5 min using a 1 inch tubular sonicating probe (at 16 000 rpm), prior to stirring at 1000 rpm for 3 h on a Heidolph stirrer to facilitate evaporation of the dichloromethane. The spheres were again centrifuged, freeze-dried and stored as above prior to use.

### *2.3. In vitro release of radiolabelled ODNs from P(LA–GA) microspheres*

Approximately 25 mg of microspheres containing the appropriate concentration of radiolabelled ODNs were suspended in glass vials containing 1.5 ml of phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4) as release media. The vials were shaken on a simple to and fro shaker at 400 rpm at 37°C. Release of radiolabelled ODN was monitored at timed intervals daily over the first 7 days then every second or third day for up to 56 days. At each time interval, the release media was removed and centrifuged for 5 min at 13 000 rpm to remove any suspended microspheres. The supernatant was removed for liquid scintillation counting and the sphere pellet was resuspended in 1.5 ml of fresh release media and replaced into the release vial.

### *2.4. Cellular uptake studies in RAW264.7 macrophages*

We used a 20-mer 5′ end $^{32}$P-labelled hairpin ODN for the uptake studies in the RAW 264.7 murine macrophage cell line (ECCAC, Porton Down, UK) due to the enhanced nuclease stability of hairpin (self-stabilized) ODNs in culture media (Khan and Coulson, 1993; Coulson et al., 1996). Cells were plated at $5 \times 10^5$ per well in a 24-well plate, and the free radiolabelled ODN or ODN-loaded microspheres (at equivalent ODN concentrations) was added approximately 12 h later. At fixed intervals, the apical solutions were collected and the cells washed up to five times with 0.5 ml PBS to remove loosely bound radioactivity with each wash being pooled with the apical sample. Cells were then lysed with 1–3% Triton-X100 and cell-associated radioactivity was determined by liquid scintillation counting.

### *2.5. Sub-cellular distribution of free and microsphere-entrapped fluorescently labelled ODNs*

A 5′ fluorescein-labelled PS ODN was entrapped in the polymer spheres at a concentration of 0.2 $\mu$g/mg polymer. Raw 264.7 cells were plated at a concentration of $1.5 \times 10^5$ onto plastic chamber slides, 8 wells per slide (Nunc, Gibco, UK) and incubated overnight. A sphere suspension (300 $\mu$l) of 5 mg polymer/ml, in Dulbecco's modified Eagle's medium (DMEM) serum-free media, was added to wells in duplicate, and the equivalent free fluorescent PS ODN was added to the control wells. The chamber slide was incubated for a further 24 h. The wells were washed five times with PBS to remove the media and excess spheres, and cells were fixed in 1% paraformaldehyde (in PBS) for 1 h at 4°C. The wells were washed with PBS, the slides removed and mounted in PBS/glycerol mixture (1:1) containing 1% diazabicyclo[2.2.2]octane (DABCO) as an antifading agent (Johnson et al., 1982). Cellular fluorescence in the cells was visualized using an inverted fluorescence microscope (Jenamed, UK) fitted with a 510-nm filter. In some cases, the cells were also counter-stained with the nuclear-

staining dye, propidium iodide. For this purpose, cells were fixed in 200 μl acid alcohol (99 parts 70% ethanol and 1 part 1 M HCl) for 2 min, then washed with PBS four times to remove the acid, before addition of 200 μl propidium iodide (100 μg/ml in PBS) and visualization by fluorescence microscopy.

## 3. Results

The aim of this study was to evaluate the use of P(LA–GA) copolymer microspheres for the entrapment, in vitro release and cellular delivery of antisense ODNs to macrophages in culture.

Fig. 1 shows the typical morphology of P(LA–GA) copolymer microspheres prepared by the double-emulsion method. Microspheres, with and without incorporated ODNs, exhibited a reproducible, smooth surface morphology. Two discrete size ranges of P(LA–GA) copolymer microspheres were prepared and the amount of ODN that could be entrapped within these microspheres was dependent on microsphere size (see Table 1). Entrapment efficiencies of around 67% were obtained for 20-mer PO ODN in the 10–20 μm size range of microspheres whereas entrapment efficiencies of only around 30% could be achieved with smaller, 1–2 μm size range microspheres (Table 1). However, relatively large amounts of ODN could be entrapped provided a high initial concentration was used within the primary emulsion (see Section 2). For initial concentrations of between 0.8 and 8 μg ODN per mg polymer, entrapment efficiencies remained high (at around 67% for 10–20 μm spheres), suggesting that the capacity of these microspheres for entrapping ODNs had not been saturated over this concentration range (data not shown). The exact concentrations required for saturation were not pursued further in the present study due to the large quantities of ODNs needed.

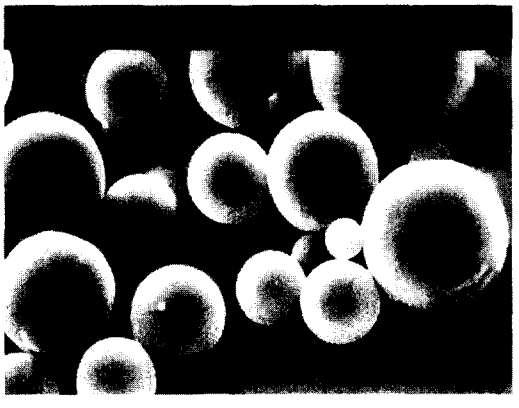

Fig. 1. Typical surface morphology of P(LA–GA) copolymer microspheres as viewed under the scanning electron microscope.

In an attempt to assess the sustained release properties of ODNs from the P(LA–GA) delivery system, in vitro release studies in PBS were performed. The entrapped ODN was released in a sustained manner from both small (1–2 μm) and large (10–20 μm) microspheres in vitro (Table 1). Smaller microspheres yielded relatively rapid release of entrapped ODN compared with the larger spheres. The time taken for 70% of ODN to be released ($t_{70\%}$) was approximately 10-fold slower for the larger particles ( $\approx 40$ days) compared with small spheres ( $\approx 4$ days). Since particle size appears to be an important determinant of ODN release rate, these data suggest that by careful selection of microsphere size and ODN loading, release rates may be tailored for a given therapeutic application using these microsphere delivery systems.

We next investigated if small P(LA–GA) microspheres (1–2 μm) could improve the delivery of ODNs to phagocytic cells in culture as these particles provided the more rapid (1 week) ODN release profile. Fig. 2A shows that uptake of a self-stabilized PO ODN entrapped within small microspheres (1–2 μm) was significantly improved in RAW 264.7 macrophages when compared with the free ODN over a 24-h time period. Less than 1% of this ODN was associated with macrophages after 24 h, whereas incorporation within P(LA–GA) microspheres resulted in an approximately 10-fold improvement in cellular association (Fig. 2A). The polymer microspheres, at the respective doses used in these studies, did not impart any intrinsic toxicity to cultured macrophages as assessed by cell counts, trypan blue exclusion or by MTT assays (data not shown).

Table 1
Release characteristics of oligonucleotide-loaded P(LA–GA) microspheres

| Polymer | Molecular mass (Da) | Microsphere size range ($\mu$m) | Oligonucleotide type and target | Entrapment efficiency (%)[a] | Release time (days)[b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | 30% | 50% | 70% |
| P(LA–GA) | 3000 | 1–2 | 20-mer Phosphodiester vs. *tat* gene | 26.9 | <1 | 2 | 4 |
| P(LA–GA) | 3000 | 10–20 | 20-mer Phosphodiester vs. *tat* gene | 66.8 | 30 | 35 | ≥40 |

[a] Entrapment efficiency is the amount of oligonucleotide incorporated into the polymer microspheres as a fraction of the total amount of oligonucleotide added during preparation and is expressed as a percentage.
[b] Release time in days is shown for when 30, 50 and 70% of the total dose is released from microspheres in vitro in PBS release media.

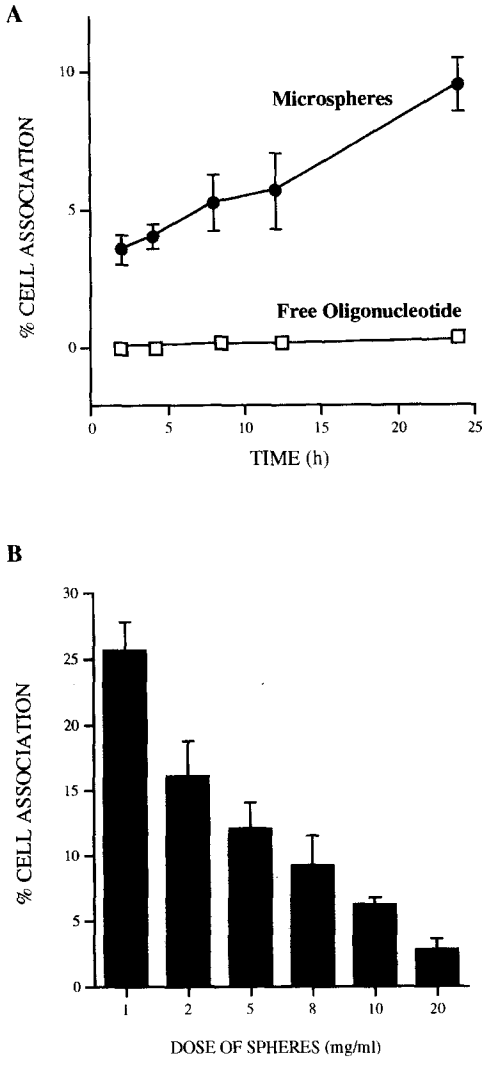


Fig. 2. Cellular uptake of $^{32}$P-labelled ODN-loaded microspheres by cultured murine RAW 276.4 macrophages. (A) Comparison of cellular association of free and 1–2 $\mu$m P(LA–GA) microsphere-loaded hairpin-stabilized PO ODN. A volume of 0.5 ml of a 5 mg polymer/ml solution in serum-free media was incubated with $5 \times 10^5$ cells per well at 37°C. The free ODN was stabilized with a 3′ hairpin loop and added in equal concentration to the entrapped ODN (approximately 4 $\mu$g/ml of ODN was used in each case). (B) The effect of the polymer dose on the % cell association of 1–2 $\mu$m P(LA–GA) microsphere-entrapped $^{32}$P-labelled hairpin-stabilized PO ODN in RAW 264.7 macrophages plated at $5 \times 10^5$ cells per well. Association was determined in serum-containing media at 37°C after 24 h ($n = 4 \pm$ S.D.).

For a given cell density, uptake of particles into macrophages was also dose dependent (Fig. 2B). Using $5 \times 10^5$ macrophages per 24 wells, the most avid uptake was observed with a microsphere dose of 1 mg/ml with a gradual reduction in uptake as microsphere dose was increased further (see Fig. 2B). The relationship was not linear and suggests that microsphere association or uptake by macrophages may be a saturable process. Under the optimal conditions of this experiment, up to 250 $\mu$g of the polymer spheres were associated with $5 \times 10^5$ cells. Hence, with an entrapment efficiency of 30% for these small spheres, approximately 75 $\mu$g of ODN per $5 \times 10^5$ cells were available for release over a period of about a week. This value may be further improved by optimizing the ODN per polymer ratio or by the enhanced phagocytic activity of activated macrophage cells (see below).

Uptake of particles in murine macrophages was temperature dependent (Fig. 3A) and could be reduced significantly in the presence of metabolic inhibitors (10 mM sodium azide and 50 mM 2-deoxyglucose) and by colcemid, a known inhibitor of phagocytosis (Fig. 3B), thereby suggesting that particles are taken up by macrophages via an endocytic or phagocytic mechanism. This is further supported by data on the uptake of ODNs and microspheres in macrophages that have been activated by INF-$\gamma$ and LPS, a treatment known to enhance phagocytic activity (Auger and Ross, 1992) (see Fig. 4). The uptake of free ODN when co-administered with blank polymer microspheres was monitored in both non-activated and activated macrophages. Simple co-administration of blank polymer microspheres improved the cellular uptake of the free ODN ($\approx 115\%$ of control). However, a more significant improvement in uptake of radiolabelled ODN was observed (150%) when the macrophages were in an activated state, presumably due to enhanced endocytosis/phagocytosis. The activation of macrophages was confirmed by detecting elevated levels of nitric oxide (NO) production following stimulation of the inducible form of NO synthase by INF-$\gamma$ and LPS (data not shown).

For sub-cellular distribution studies, fluorescently labelled PS ODNs were used due to their known cellular uptake properties (Akhtar et al.,

1996; Beck et al., 1996). The entrapment of fluorescently labelled ODNs into polymer microspheres also caused the particles to fluoresce and facilitated their detection. Fig. 5A shows the characteristic punctate distribution of free PS ODNs, indicative of vesicular localization following endocytic entry into these cells and is similar to that typically observed for fluid-phase markers such as fluorescently labelled dextrans (Shoji et al., 1991) and to many other types of ODN chemistries (Beltinger et al., 1995; Iversen et al., 1992; Spiller and Tidd, 1992; for review, see Akhtar and Juliano, 1992). In contrast, microsphere-entrapped ODNs exhibited fluorescence within the cytosol and also the nucleus (Fig. 5B). However, it is not yet clear whether this diffuse fluorescence simply arises as a consequence of the fluorescent microspheres becoming entrapped within larger vesicles (e.g. phagosomes) or whether more of the ODN had been released into the cytosol and, in some instances, the nucleus too. The proposed greater release from phagosomes or phagolysomal compartments may result from the improved stability of ODNs within the microspheres (Lewis et al., 1995) compared with free ODNs which are thought to degrade rapidly in lysosomal enzyme-containing compartments (Hudson et al., 1996a). This issue clearly requires further study.

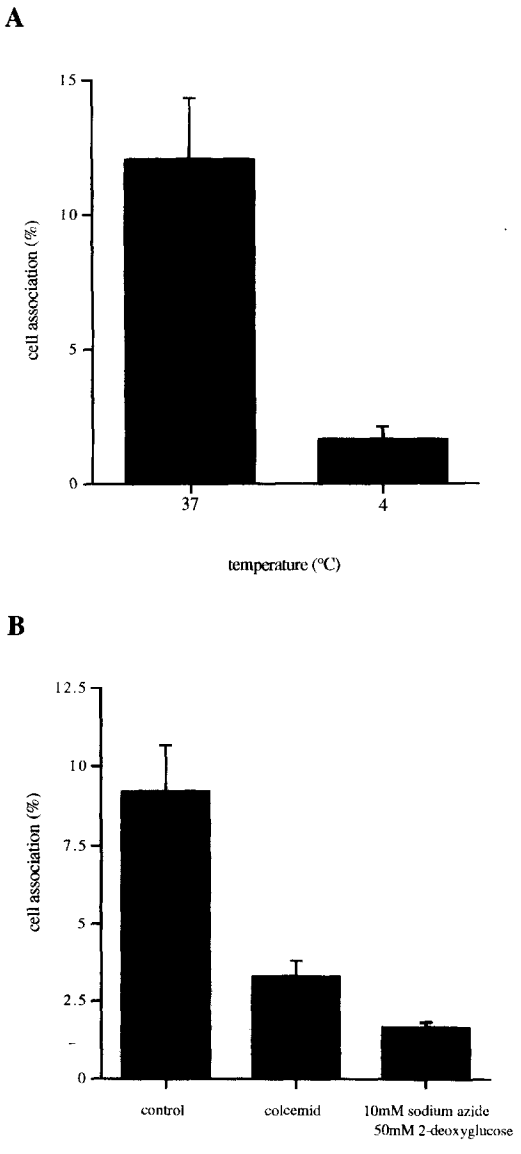


Fig. 3. Temperature and energy-depedendet uptake of $^{32}$P-labelled ODN-loaded microspheres by RAW 264.7 macrophages. (A) The effect of temperature (37 and 4°C) on the association of 1–2 $\mu$m P(LA–GA) microspheres. (B) The effect of metabolic and phagocytosis inhibitors on cell association of 1–2 $\mu$m P(LA–GA) microspheres after 24 h at 37°C. Colcemid at a concentration of 50 $\mu$g/ml or 10 mM sodium azide and 50 mM 2-deoxyglucose were incubated with the $5 \times 10^5$ cells per well for 3 h before addition of $^{32}$P-labelled ODN-loaded microspheres ($n = 4 \pm$ S.D.).

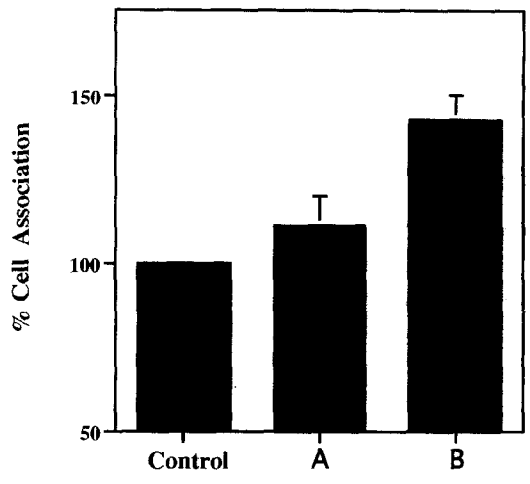


Fig. 4. Uptake of $^{32}$P-radiolabelled ODN and P(LA–GA) microspheres by macrophages stimulated with IFN-$\gamma$ and LPS treatment. The cell association of $^{32}$P-radiolabelled hairpin-stabilized ODN alone (control), expressed as 100%, and in the presence of blank 1–2 $\mu$m P(LA–GA) microspheres (0.5 ml of 5 mg polymer/ml) was monitored after 12 h without (A) and with (B) stimulation of murine macrophages with IFN-$\gamma$ and LPS in serum-free media ($n = 4 \pm$ S.D.).

## 4. Discussion

The macrophage is of particular interest for the delivery of anti-HIV therapeutics as the sequestration of the HIV-1 virus by these cells is thought to be associated with the latency period and the development of acquired immunodeficiency syndrome (AIDS) (Roy and Wainberg, 1988; Meltzer et al., 1990). Macrophages can internalize polymeric microsphere drug carriers by phagocytosis, which is dependent on parameters such as particle size (optimum size of 1–2 $\mu$m), surface charge and hydrophobicity (Yamaguchi and Anderson, 1993; Tabata and Ikada, 1988a,b; Kimura et al., 1994). Indeed, encapsulation of anti-HIV drugs such as the nucleoside analogues, AZT and ddC, in polymeric microspheres consisting of either albumin or polyhexylcyanoacrylate have been shown to reduce HIV infection in macrophages (Bender et al., 1994).

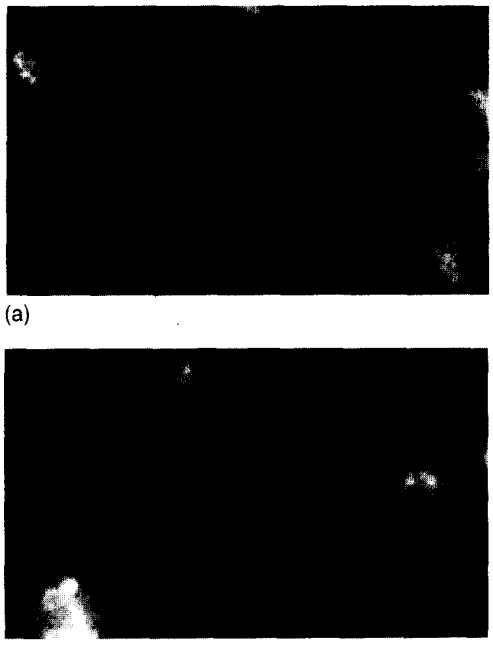


Fig. 5. Sub-cellular distribution of fluorescently labelled ODNs in RAW 264.5 macrophages. (A) Distribution of free fluorescently labelled PS ODN; (B) cellular distribution of fluorescently labelled PS ODN entrapped within 1–2 $\mu$m P(LA–GA) microspheres in cultured RAW 264.7 macrophages. Magnification × 100.

For ODN-based HIV therapy, it has been suggested that after an initial high dose of anti-HIV ODNs, a maintenance dose of only 10% of the loading dose is necessary to maintain antiviral efficacy (Lisiewicz et al., 1993). The potential chronic use of polymer microspheres for delivering a maintenance dose in anti-HIV therapy, therefore, represents an attractive possibility. Furthermore, recent pharmacokinetic and efficacy studies in animals suggest that repeated administration will be necessary for many therapeutic targets in vivo (for review, see Akhtar and Agrawal, 1997). In this study, we have shown that P(LA–GA) microspheres in the 1–2 $\mu$m size range can provide sustained delivery of ODNs from a few days to several weeks and also significantly improve the cellular delivery anti-HIV ODNs to murine macrophages.

The polymer used in the present study, P(LA–GA) 50:50 copolymer (molecular mass = 3000 Da), has been reported to completely degrade within 7 days when internalized by phagocytic macrophages (Tabata and Ikada, 1988a). Increasing the molecular mass of this copolymer to 5000 Da increases the residence time of the particles, and presumably release, to at least 14 days (Kimura et al., 1994), which could be further lengthened by using the more slowly degrading PLA homopolymer (Tabata and Ikada, 1988a). The understanding of such controlling factors as polymer chemistry and molecular weight, microsphere size and ODN loading may allow nucleic acid release profiles to be tailored for a specific therapeutic goal.

A particular advantage offered by biodegradable particulate systems in vivo is the ability to administer them locally to target organs or sites of disease (i.e. site-specific targeting). For example, implantation or parenteral administration of polymeric devices close to a tumour site would facilitate direct and localized delivery of ODNs (Fournier et al., 1991). Alternatively, systemic administration of polymer microspheres via the intravenous route would enable passive targeting to the liver and other reticuloendothelial organs (Illum et al., 1987; Bazile et al., 1992; Davis et al., 1993; Le Ray et al., 1994; Nakada et al., 1996). Furthermore, polymer microspheres of less than

10 μm in size (typically around 1 μm) may traverse the gastro-intestinal tract via the Peyer's patches following oral administration (Eldridge et al., 1990). As some ODN derivatives are thought to be modestly bioavailable after oral administration in animal models (Agrawal et al., 1995), P(LA–GA) microspheres may further enhance the bioavailability of ODNs via this route. The encapsulation of ODNs, or their conjugates, into polymer carriers will in effect 'hide' ODNs from the biological milieu which, in addition to nuclease protection, may also help reduce adverse interactions with non-targeted proteins. For example, the functioning of the dipeptide transporter is non-specifically affected by some lipophilic ODN conjugates (Moore et al., 1997).

The delivery of ODNs using other particulate systems has also been reported recently (Chavany et al., 1992, 1994; Cortesi et al., 1994; Schwab et al., 1994; Godard et al., 1995). The most extensively studied of these are the polycyanoacrylate nanoparticles where the ODNs are adsorbed onto the nanoparticle surface by hydrophobic quartenary ammonium salts and are not entrapped within the matrix core as in our P(LA–GA) microsphere system. These nanoparticles are also reported to provide enhanced delivery of ODNs to cultured cells (Chavany et al., 1994) and to a Ha-ras tumour xenogaft in vivo (Schwab et al., 1994) but the use of some hydrophobic quartenary ammonium salts at the surface of these particles may result in unwanted toxicity (Lherm et al., 1992; Fenandezurrusuno et al., 1995).

In summary, our data suggest that biodegradable polymer devices have a potential role in the delivery of antisense nucleic acids in vitro and in vivo, and require more detailed characterization if they are to be considered for clinical applications of ODN-based therapies.

## Acknowledgements

This work was funded by grants from the MRC AIDS Directed Programme and the Cancer Research Campaign.

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