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Biodegradable polymer matrices for the sustained exogenous delivery of a biologically active c-*myc* hammerhead ribozyme[1]

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Abstract

Ribozymes are potent RNA molecules that can inhibit gene expression in a sequence-specific manner by catalysing the trans-cleavage of target mRNA. The 'hammerhead' motif is the most widely studied ribozyme and is currently being developed as a potential therapeutic agent for cancers and viral diseases. However, the poor biological stability of RNA chemistries typically requires repeated exogenous administration of ribozymes for long-term biological effects. In this study, we have evaluated the potential use of biodegradable poly-(L-lactic acid) (PLA) polymer matrices to improve the biological stability and provide sustained delivery of a biologically active hammerhead ribozyme designed to cleave the mRNA of the c-*myc* proto-oncogene. The in vitro release profile showed that the entrapped hammerhead ribozyme was released biphasically from the polymer films, characterised by an initial rapid burst release, followed by a more sustained release over a period of several weeks. Release was dependent on ribozyme loading but was similar for RNA and DNA oligonucleotides of the same length. The entrapment of a hammerhead ribozyme within solvent cast PLA film matrices improved its biological stability in serum from seconds (for the 'free' ribozyme) to more than 2 weeks when entrapped in PLA matrices. The in vitro catalytic cleavage activity of the polymer-released ribozyme was identical to that of 'free' ribozyme suggesting that the polymer device fabrication procedure did not adversely affect the hybridization and catalytic properties of the hammerhead ribozyme. These data suggest that biodegradable polymer devices appear suitable for the sustained delivery of ribozymes and are worthy of further investigation.

Keywords: Hammerhead ribozyme; Oligonucleotide; Antisense; Stability; Biodegradable polymer; Drug delivery; Release profile; Sustained release; Poly-(L-lactic acid)

1. Introduction

Ribozymes are catalytic RNA molecules which have the ability to cleave target RNA substrates in a sequence specific manner in the absence of proteins or 'traditional' enzymes (for reviews see: Cech and Bass, 1986; Symons, 1994; Akhtar and

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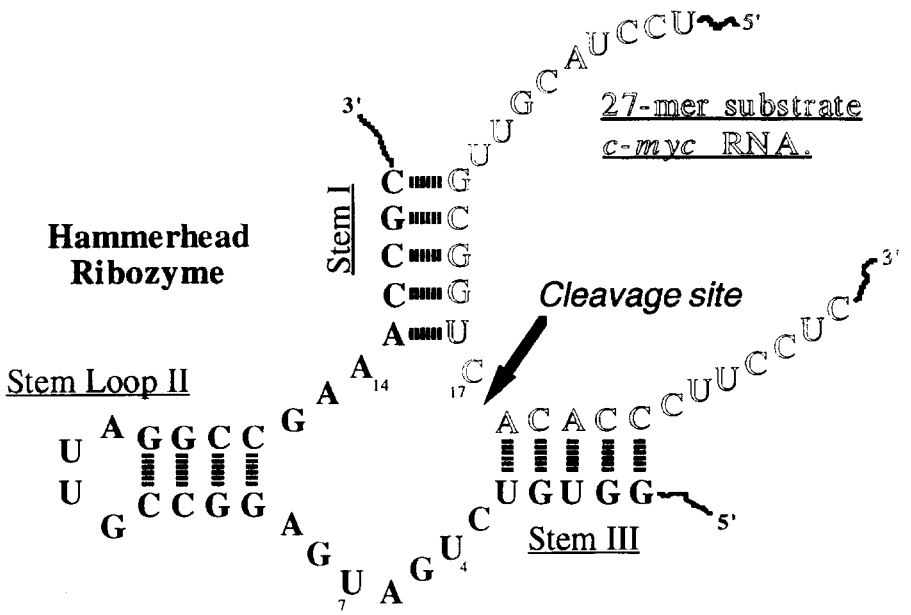

Fig. 1. The sequence of the 32 mer c-*myc* hammerhead ribozyme and its 27 mer substrate, indicating the site of substrate cleavage. The trans-acting form of the hammerhead ribozyme shown is based on the design of Haseloff and Gerlach (1988). This consists of two antisense base pairing stems (I and III) combined with a central catalytic core of non-base pairing conserved nucleotides and an internal loop (Stem II) which is responsible for associating with the divalent metal cation required for catalytic cleavage of the substrate (Tuschl and Eckstein, 1993). Important nucleotides have been numbered according to the standard numbering system agreed for the nucleotides within the catalytic core of the hammerhead ribozyme (Hertel et al., 1992).

Rossi, 1996). A ribozyme can be regarded as essentially an antisense oligonucleotide that contains a catalytic region of conserved nucleotides which, in the presence of divalent cations such as magnesium, can exhibit multiple turnover of substrate mRNA molecules. As such, ribozymes, especially the hammerhead motif, are being actively pursued as novel therapeutic agents for gene inhibition in cancer and viral diseases (Bratty et al., 1993; Akhtar and Rossi, 1996).

The hammerhead ribozyme can potentially be targeted against any substrate mRNA provided it contains a specific trio of nucleotides which allow formation of the correct secondary structure to permit cleavage. Rules are not available for reliably predicting all cleavable sites in a given mRNA although the base triplets; GUC, CUC, GUA and UUC can generally be cleaved providing incompatible secondary structures are avoided (Bratty et al., 1993).

The exogenous delivery of ribozymes would be pharmaceutically desirable in potential therapeutic applications but is limited by the extremely poor nuclease stability of RNA ribozymes in most biological environments. Although chemical modifications to ribozymes can enhance nuclease stability, catalytic activity is often compromised (Buzayan et al., 1990; Ruffner and Uhlenbeck, 1990). Thus, repeated administration is often necessary but is likely to be clinically undesirable in many cases. For these reasons, exogenous delivery systems which can protect ribozymes from ribonuclease digestion and simultaneously provide sustained delivery over extended time periods may be useful for the biopharmaceutical application of RNA ribozymes and other nucleic acids.

In this study, we have evaluated the potential use of biodegradable poly-(L-lactic acid) (PLA) matrices for improving the biological stability and delivery of a 32 mer hammerhead RNA ribozyme targeted against the c-*myc* proto-oncogene mRNA and designed to cleave a GUC base trio located 226 bases 3′ to the AUG initiation codon (Fig. 1). Using the most ribonuclease sensitive (all

RNA) ribozyme, we have shown that ribozyme stability in serum can be improved from seconds to weeks upon entrapment within polymer devices. Furthermore, the entrapped RNA remains biologically active and retains its ability to hybridize and cause catalytic degradation of the c-*myc* RNA substrate. Sustained release over a 4-week period could be obtained in vitro with thin-film matrices of PLA.

2. Materials and methods

2.1. Ribozyme synthesis

An unmodified RNA, 32 mer hammerhead ribozyme 5′ GGU GUC UGA UGA GGC CGU UAG GCC GAA ACC GC 3′ (Fig. 1b) designed against the human c-*myc* oncogene, exon 2 (mol. wt. 10 400), was synthesised for this study. A 27 mer region of the target mRNA substrate molecule 5′ UCC UAC GUU GCG GUC ACA CCC UUC CUC 3′ was also synthesised in order to assess the in vitro catalytic activity of the released ribozyme. These RNA sequences were synthesised on 1.0 μM scale using a Cruachem PS250 synthesiser, 5′-O-(DMTr)-2′-O-(Fpmp) ribonucleoside phosphoramidites and using the recommended synthetic and deprotection protocols (Rao and Macfarlane, 1995). The crude 5′-O-(DMTr)-2′-O-(Fpmp)-protected oligoribonucleotides were released from the solid support by treating with triethylamine in concentrated aqueous ammonia. The ammoniacal solution was evaporated to a pellet under reduced pressure. The partially protected oligoribonucleotides were purified by reverse-phase HPLC and then disolved in 0.5 M sodium acetate buffer (pH 3.25, 500 μl). After 30 h at 30°C, the acidic solution was neutralised with a solution of 3.0 M Tris-base (100 μl) and the fully unblocked RNA sequences were generated by ethanol precipitation without vacuum centrifugation.

2.2. Radiolabelling

The deprotected ribozyme and substrate were separately 5′-end radiolabelled with [^{32}P]-γ-ATP (Amersham, UK) using bacteriophage T4 polynucleotide kinase (Gibco, UK) in 100 mM Tris, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine and 0.2 mM EDTA at 37°C for 30 min as described previously (Akhtar et al., 1991). The radiolabelled RNA were purified by 20% native polyacrylamide gel electrophoresis. The excised bands containing the radiolabelled RNA, as detected by autoradiography, were eluted in 0.1% diethyl pyrocarbonate-treated water (Sambrook et al., 1989), and were concentrated in a Savant Speed Vac. The concentration of the ribozyme and substrate solutions was determined by UV absorption at 260 nm assuming that 1 OD was equivalent to approximately 20 μg RNA (Sambrook et al., 1989). The radiolabelled ribozyme was diluted with concentrated non-labelled ribozyme to achieve the desired concentration to be incorporated into the polymer.

2.3. Preparation of polymer films

Poly-(L-lactic acid) was purchased from Alpha Chemicals, UK (product no. L 214 mol. wt. 690 000). The chemical grade polymer was obtained as almost odourless, almost white granules. The radiolabelled ribozyme was incorporated into a 2% w/w PLA-chloroform solution and mixed well before casting on degreased glass plates as described by Lewis et al. (1995). The chloroform was allowed to evaporate slowly overnight in a drying chamber at room temperature. The films were then dried at 40°C for 48 h. The resulting films were smooth, transparent and flexible, with an approximate thickness of 100 ± 10 μm.

2.4. Release of ribozyme

Pieces of polymer film 5 mm^2 and approximately 2.5 mg were placed in 1.5 ml of either phosphate-buffered saline (PBS), pH 7.4, or citrate phosphate buffer (CPB) at pH 5.5 and were shaken on a mechanical shaker at 37°C. Release of radiolabelled ribozyme from the polymer film was monitored at fixed intervals over a 28-day period. At each timed interval, 1.5 ml release media was removed and placed in 10 ml Optiphase Hi-Safe 3 (Pharmacia, UK) before being

counted for 5 min in a Packard 1900TR scintillation counter. An equivalent volume of fresh media was replaced at each sampling time.

2.5. Stability in serum

In order to investigate the stability of the polymer-entrapped ribozyme, pieces of polymer film (1 cm^2) loaded with ribozyme were placed in foetal calf serum (Gibco, UK) and placed on a mechanical shaker at 37°C. Stability of the ribozyme entrapped in the polymer film was assessed by extracting the remaining ribozyme from the polymer by dissolving the polymer in chloroform and extracting the ribozyme into water treated with 0.1% diethyl pyrocarbonate (Sambrook et al., 1989). The extracted ribozyme and degradation products were analysed by denaturing 20% polyacrylamide gel electrophoresis.

2.6. Biological activity of released ribozyme

Entrapped ribozyme was extracted from the polymer film by dissolving the polymer in chloroform and extracting the ribozyme into water previously treated with 0.1% diethyl pyrocarbonate and drying in a Savant Speed Vac. A 50-μM stock solution of released ribozyme was prepared in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$ which had previously been heated to 90°C. A 2-μM stock solution of the all RNA substrate was prepared in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$ which had previously been heated to 90°C. This yielded a 25:1 ratio of ribozyme to substrate in the final reaction mixture. The cleavage reaction was initiated by the addition of an equal quantity of ribozyme stock solution to the substrate stock solution, both previously incubated at 37°C for 15 min. The reaction was stopped after the required time interval by the addition of an equal volume of 7 M urea and storage at −20°C. Ribozyme, substrate and any cleavage product(s) were separated by 20% polyacrylamide (7 M urea) gel electrophoresis. The intensity of bands on the autoradiographs was estimated by scanning laser densitometry.

3. Results and discussion

The purpose of this study was to evaluate the potential use of biodegradable polymer matrices for the improved delivery of ribozymes. In this regard, we investigated whether we could achieve sustained release of the c-*myc* RNA ribozyme from thin-film PLA matrices, whether the polymer-entrapped ribozyme would remain stable to ribonuclease digestion in serum and whether the polymer-released ribozyme retained its original trans-cleavage activity. In testing these concepts, we utilized an all RNA ribozyme as this was the most nuclease-sensitive chemistry and thus, the most challenging ribozyme from a delivery perspective. Data obtained with this ribozyme would equate to the worst-case scenario and may easily be extrapolated to biologically more stable RNA chemistries, such as 2′-O-alkyl and 2′-amino derivatives (Lamond and Sproat, 1993; Arup et al., 1995).

3.1. In vitro release profiles of hammerhead ribozymes from PLA films

Thin-film matrices of PLA containing ribozyme were prepared as described previously for antisense oligonucleotides (Lewis et al., 1995). In vitro ribozyme release from these matrices was investigated in PBS, so as to reduce the possibility of RNA degradation which could occur in serum-containing biological media (see below). The release profiles over 28 days (Fig. 2) suggested that the entrapped ribozyme was released biphasically from the PLA matrices, characterized by an initial burst effect during the first 48 h followed by a more sustained release. This biphasic release appears to be similar to that obtained with other macromolecules (Heller, 1993), where the burst effect reflects release of the macromolecule from close to the surface whereas the slower phase of release represents efflux of the macromolecule from deeper compartments within the polymer matrix. The amount of ribozyme released from PLA matrices was dependent on polymer loading with higher loadings being associated with greater burst effects and a greater total release of the entrapped ribozyme (Fig. 2). The ribozyme release

profiles at pH 7.4 and 5.5 were similar, suggesting that over this range release is independent of pH (data not shown). These data were in agreement with those obtained with antisense oligonucleotides (Lewis et al., 1995). Indeed, comparison of ribozyme release with a DNA oligonucleotide of the same length (32 mer) at the low loading showed similar release profiles (see Fig. 2) suggesting that RNA and DNA nucleic acids are released at similar rates and probably by a similar mechanism.

3.2. Biological stability of polymer-entrapped hammerhead ribozyme

Free RNA ribozymes degrade instantaneously within serum (data not shown; see also Kariko et al., 1994; Arup et al., 1995; Elkins and Rossi, 1995) and biological instability is likely to be a major barrier in the therapeutic development of even the chemically modified derivatives (Akhtar et al., 1995; Arup et al., 1995). Previous studies in our laboratory have shown that entrapment of unmodified DNA oligonucleotides within PLA matrices significantly improved their biological stability to serum nucleases. To ascertain if a similar protection could be afforded to RNA ribozymes, we performed biological stability tests with an all RNA ribozyme. Fig. 3A shows the stability profile of ribozyme-loaded PLA film matrices exposed to foetal calf serum. In comparison to the free ribozyme which degrades completely within 60 s (data not shown), ribozyme entrapped within PLA matrices was less accessible to ribonucleases present in serum. Some intact ribozyme was still visible after 14 days incubation in foetal calf serum and this represents a significant improvement over the free ribozyme. Although all RNA ribozymes are unlikely to be used clinically because they would still rapidly degrade following release from the polymer device, these data suggest that biodegradable polymer devices would be useful for the improved biological stability of chemically modified ribozymes such as the 2′ O-alkyl modified ribozymes (Arup et al., 1995).

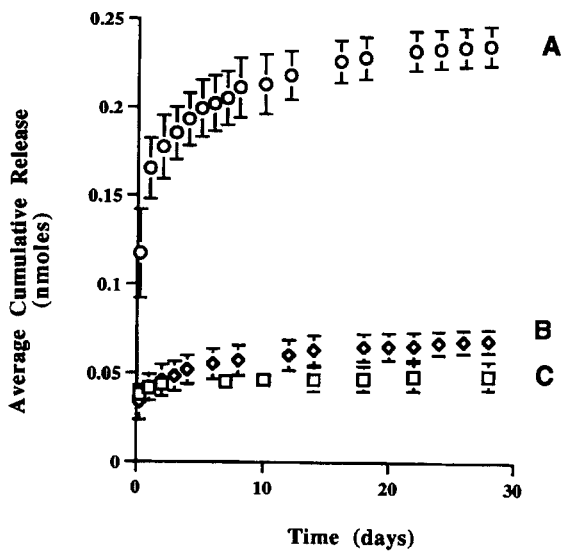

Fig. 2. In vitro release profiles of the 32 mer c-*myc* hammerhead ribozyme from PLA matrices at pH 7.4 and 37°C. Release of ribozyme from matrices containing a loading of (A) 700 ng/mg film and (B) of 250 ng/mg film is compared. Curve (C) shows the release profile of a 32 mer DNA oligonucleotide (250 ng/mg film) for comparison. See Materials and methods for details of release studies. Data points represent average cumulative release, bars show standard deviation, $n = 4$.

3.3. Hybridization and biological activity of ribozyme released from PLA matrices

The successful use of biodegradable polymers as drug delivery systems for ribozymes will also depend on ensuring that device fabrication procedures employed do not adversely modify the biological activity of these macromolecules as may be possible with some solvent casting procedures. To investigate this further for our system, we investigated the capability of the polymer-released ribozyme to hybridize to and biologically cleave its target c-*myc* RNA substrate. The in vitro cleavage activity of the released ribozyme against a 5′ end [^{32}P]-radiolabelled synthetic 27 mer sequence of the target c-*myc* mRNA (for sequence see Fig. 1) is shown in Fig. 3B.

In theory, ribozyme-mediated cleavage of the substrate RNA would proceed via hybridization and the subsequent hydrolysis of the phosphate bond 3′ to the base at C_{17} (see Fig. 1). This would be expected to yield two RNA products; a detectable radiolabelled 15 mer 2′-3′ cyclic phosphodiester product and an unlabelled 5′-hydroxyl 12

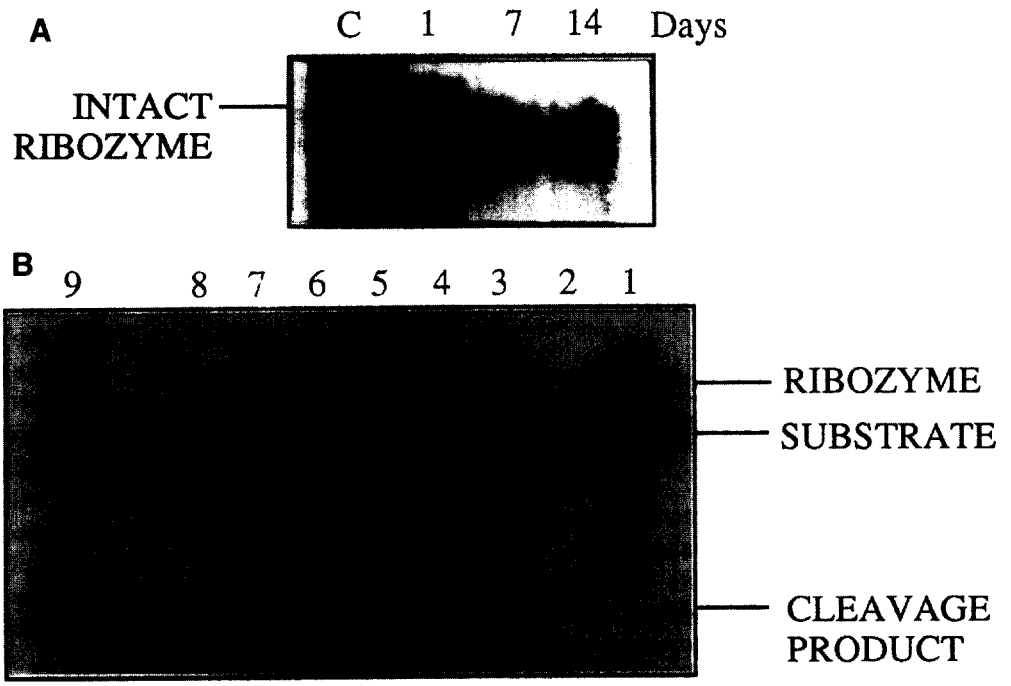

Fig. 3. Stability and biological activity profiles of the PLA polymer-entrapped 32 mer hammerhead ribozyme. (A) The stability of the radiolabelled ribozyme within polymer films was assessed after exposure of matrices to foetal calf serum at 37°C over a period of 14 days. At fixed time intervals, ribozyme was re-extracted from PLA films and analysed by denaturing PAGE (see Materials and methods). Lane C is the control intact ribozyme not exposed to serum, and the other lanes indicate ribozyme exposed to serum for 1, 7 and 14 days. (B) In vitro biological activity of released hammerhead ribozyme from PLA films was examined as described in Materials and methods. Radiolabelled ribozyme and substrate were co-incubated in 25:1 ratio. Cleavage reactions were analysed by PAGE as a function of time. Lanes show increasing incubation time with substrate at 37°C and $[Mg^{2+}]$ of 10 mM. Lane 1 = unreacted ribozyme control; lane 2 = unreacted substrate control; lanes 3–7 show progressive cleavage of the target RNA as a function of time: lane 3 = ribozyme/substrate reaction at time zero; lane 4 = reaction after 2 h; lane 5 = after 4 h; lane 6 = after 8 h; lane 7 = after 18 h. Lane 8 = reaction mixture containing only the substrate after 18 h; no cleavage occurs in the absence of the ribozyme and this also serves as a control to show substrate was not self-cleaving or degrading spontaneously during the reaction, 9 = positive control of cleavage of the same substrate by the same ribozyme taken from a previous reaction run for an extended time period.

mer sequence which would not be detectable by autoradiography. The appearance of the former cleavage product in Fig. 3B suggests that the ribozyme released from the polymer film is still biologically active and capable of cleaving the corresponding 27 mer substrate. There is an increasing concentration of the radiolabelled 15 mer cleavage product visible with increasing reaction time. The catalytic activity of the polymer released ribozyme appears to have been unaffected by the entrapment process as the cleavage ability of the released ribozyme is similar to that of free ribozyme, with 50% of the substrate molecule being cleaved within around 6 h in vitro in both cases. Although the catalytic activity of the ribozyme design used in this study is slightly inferior to that of the most potent ribozymes used by other workers (e.g. Tuschl and Eckstein, 1993), it clearly indicates that the solvent-casting procedure for fabricating PLA matrices did not adversely affect the hybridization and subsequent cleavage activity of the entrapped ribozyme.

In conclusion, these data suggest that biodegradable PLA matrices can improve the biological stability of RNA ribozymes, provide sustained delivery and maintain the cleavage activity of biologically functional RNA sequences. Further studies with chemically stabilized ribozymes

will allow the investigation of these polymer devices in vivo.

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