Poly (DL-lactide-co-glycolide) microparticles as carriers for antimycobacterial drug rifampicin

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Poly (DL-lactide-co-glycolide) polymers were investigated as carriers for the first line antitubercular drug rifampicin. Different formulations of PLG microparticles viz. porous, non porous and hardened exhibited sustained release of rifampicin up to 7 weeks in vitro. However, hardened PLG microparticles exhibited the most sustained release in vivo in different organs up to 6 weeks. In case of free rifampicin, release was detected in vivo only up to 48 hr. In addition, no hepatotoxicity was observed on a biochemical basis (levels of SGPT, ALP and total bilirubin) in comparison to control animals. Taken together, these results suggest that polymer encapsulated antitubercular drug rifampicin may serve as an ideal therapeutic approach for treatment of tuberculous infections.

Mycobacterium tuberculosis, the agent responsible for tuberculosis owes its pathogenicity to its ability to survive and proliferate within macrophages. In recent years, there has been a marked resurgence in this disease due to AIDS pandemic and emergence of multi drug resistant (MDR) strains. The most important factor in the treatment of TB is prolonged chemotherapy, which is often associated with serious unwanted side effects. In the chemotherapy of such intracellular pathogens, it is necessary to achieve relatively high levels of the drug in the blood so that therapeutically effective concentrations of the drug could be achieved in the infected cells or tissues. This presumably leads to adverse and unwanted side effects and thus toxicity over long term repeated administration of the drugs.

Encapsulation of drugs into various carrier systems alleviates many of these obstacles. Poly (lactide-co-glycolide) microparticles constitute a class of biodegradable and biocompatible polymers used to deliver several types of drugs, including antigens, steroids, antibiotics, proteins and peptides. Polymer encapsulated drugs often exhibit reduced toxicity allowing for parenteral administration of much higher doses of the drugs than could be tolerated in the free form. These polymers can be formulated for release up to several weeks or months depending on the type of polymer used and physical and chemical properties of the drug encapsulated. PLG based implantable systems containing entrapped antitubercular drugs such as isoniazid and clofazimine have recently been used for the treatment of mycobacterial infections. Such systems however have inherent disadvantages as they are immobilized at the site of implantation and require surgery for their insertion.

Therefore, the objective of this investigation was to develop a PLG based microparticulate antimycobacterial drug carrier system that is injectable and can continuously deliver therapeutically significant levels of the drugs for prolonged time periods. Rifampicin has been chosen as the drug because it is one of the established first line drugs used for the treatment of tuberculosis.

Materials and Methods

Chemicals and drugs—Poly (DL-lactide-co-glycolide) (50:50) resomer RG 506 (mol wt. 2500) was purchased from Boehringer Ingelheim (Germany). Polyvinyl alcohol, 87-89% hydrolyzed (Avg mol wt. 13-23,000) and rifampicin were obtained from Sigma Chemical Co., USA. Kits for SGPT, ALP and total bilirubin estimation were purchased from Boehringer Mannheim, Germany. All other reagents were obtained from standard companies.

Animals—Mice (Laca strain) of either sex (4 to 5 weeks old) obtained from Central Animal House, PGIMER, Chandigarh were used. The animals were fed a standard pellet diet and water ad libitum.

Preparation of microparticles—Different formulations of PLG microparticles were prepared viz: po-
rous, non porous and hardened. The formulations (poreus, non porous) were named on the basis of their drug release behaviour while, the preparation of hardened microparticles involved the use of greater amounts of the hardening agent so they were called as hardened microparticles.

**Porous PLG microparticles**—Porous PLG microparticles were prepared by double emulsification solvent evaporation procedure as described by Edwards et al. with slight modifications. Briefly, 743.5 mg PLG was dissolved in 10 ml dichloromethane (DCM) and 14.87 mg rifampicin was dissolved in 1 ml phosphate buffered saline (pH 7.2) to prepare an aqueous solution. The drug and polymer ratio was kept at 1:50 (w/w). The drug and polymer solution were emulsified together on ice by probe sonication in dark under slow N₂ pressure for 3-6 cycles of 10-15 sec each on a Sonicator Ultrasonic Processor XL 2020 (Heat Systems Ultrasonic, Farmingdale, NY, USA). The primary emulsion so formed was poured into 50 ml of 1% (w/v) aqueous poly vinyl alcohol (PVA) solution. The microparticles were then continuously stirred overnight to allow hardening of the microparticles and evaporation of dichloromethane. The microparticles were collected by centrifugation, washed several times with PBS, and finally resuspended in PBS.

**Non porous PLG microparticles**—Non porous PLG microparticles were prepared by the single emulsification solvent evaporation procedure as described by Edwards et al. with slight modifications. Briefly, 743.5 mg PLG was dissolved in 10 ml dichloromethane (DCM) and 14.87 mg rifampicin was dissolved in 1 ml PBS. The drug and polymer ratio was kept at 1:50 (w/w). The polymer solution and the aqueous solution were mixed together in a vortex mixer. They were then poured drop wise into 50 ml of 1% (w/v) aqueous PVA solution in dark under slow N₂ pressure while being continuously stirred on a magnetic stirrer. The solution was left for stirring overnight. The microparticles were collected by centrifugation the next day, washed several times with PBS and finally resuspended in PBS.

**Hardened PLG microparticles**—Hardened PLG microparticles were prepared by the double emulsification solvent evaporation procedure as described by Edwards et al. with slight modifications. Briefly, 8.5 mg rifampicin in 1 ml PBS was emulsified on ice into 170 mg PLG in 1 ml DCM by probe sonication in dark under slow N₂ pressure. The drug and polymer ratio was kept at 1:20 (w/w). The primary emulsion so formed was poured into 1 ml of 20% (w/v) aqueous PVA solution. The microparticles were continuously stirred overnight and collected the next day by centrifugation. They were washed several times with PBS and finally suspended in PBS. Empty PLG microparticles for all the formulations were prepared by replacing the drug by normal saline or PBS.

**Determination of drug content of microparticles**—The entrapment of rifampicin in various formulations of the microparticles was determined by lysing them with 5% SDS (w/v) in 0.1 N NaOH. The released rifampicin in the supernatant was evaluated spectrophotometrically as described by Deol and Khuller.

The percentage entrapment was determined by the formula:

\[
\frac{\text{mg/ml of drug recovered in the supernatant}}{\text{mg/ml of drug added initially in microparticles}} \times 100\%
\]

**Size of PLG microparticles**—The size of PLG microparticles was determined on a particle size analyzer, CSI-I, Galai, Israel at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi.

**Drug release studies of PLG microparticles**

*In vitro release studies*—PLG microparticles with entrapped rifampicin were prepared as described before. They were suspended in 1 ml phosphate buffered saline (pH 7.2) and incubated at 37°C. The supernatant obtained after centrifugation of the suspension was collected daily up to 3 days to determine the burst release of the drug and thereafter weekly up to 6-8 weeks. The level of the drug released in the supernatant was estimated by a microbiological assay described by Saito and Tomioka. Briefly, Mueller Hinton (MH) agar plates containing 15 ml of the media were prepared. *Bacillus subtilis* culture (0.5 ml) containing 1.5 x 10⁶ cells/ml was swabbed onto the surface of the MH agar plates using sterile cotton swabs. A filter paper disk (6 mm diam.) was gently placed on the surface of the plates and 5-20 μl of the sample to be assayed was spotted on the disk. After overnight incubation at 37°C, the concentration of rifampicin in the test solution was determined from the diameter of the resulting growth inhibition zone, using standard semilog plots of rifampicin at known concentrations. Rifampicin standards were prepared in PBS and were filter sterilized using 0.22 μ filters before use.

*In vivo release studies*—PLG microparticles containing entrapped rifampicin were prepared as described before. The dose of rifampicin used was
DUTT & KHULLER: PLG BASED MPS AS CARRIERS FOR RIFAMPICIN.

85 mg/kg body wt of mice. Mice were divided into various groups of 5-6 animals each and microparticles were injected subcutaneously. Group 1 was injected free rifampicin, Group 2: porous PLG microparticles, Group 3: hardened PLG microparticles, Group 4: PBS or normal saline, Group 5: blank PLG microparticles.

Mice were sacrificed at various time points and drug concentrations determined in 20% tissue homogenates (i.e., 100 mg tissues homogenized in 0.5 ml PBS) by the microbiological assay as described before. Results were expressed as concentration of rifampicin obtained either in μg/ml of the tissue homogenates or μg/g of the organs at various time points. In free drug group, rifampicin concentrations were determined both in plasma and various tissue homogenates by the microbiological assay as described earlier. Concentrations below 0.5 μg were not detected by the assay and values were expressed <0.5 μg.

Hepatotoxicity studies—Hepatotoxicity studies were carried out by monitoring the plasma levels of serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and total bilirubin in various groups by the standard methods (Boehringer Mannheim kits). Toxicity was monitored in the plasma samples of mice at the 3rd day and then every week post administration of drug containing PLG microparticles.

Results

Encapsulation efficiency of the microparticles—The percentage entrapment of rifampicin in various formulations of PLG microparticles was: 8, 8 and 12-14%, in porous, non porous and hardened PLG microparticles, respectively.

Particle sizing studies—The particle size analysis for the PLG microparticles gave the following results in terms of volume mean diameter (in μm): 40.78, 43.21 and 11.64 for porous, non porous and hardened PLG microparticles, respectively.

Drug release studies in PLG microparticles

In vitro release studies—A sustained release of rifampicin was observed from all the formulations, viz. porous, non porous and hardened PLG microparticles. Peak levels of rifampicin were observed at the 3rd day for both porous and non porous PLG microparticles (Fig. 1 inset) with a sustained release up to 49 days for both (Fig.1). However, hardened PLG microparticles showed peak levels of rifampicin at 12 hr (Fig. 2 inset) followed by a decline in release which however was sustained till 49 days (Fig. 2).

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Fig. 1—Time dependent in vitro release profile of rifampicin from porous and non porous PLG microparticles. All values are mean ± S.D. of 3-4 animals.
In vivo release studies—In vivo release studies of rifampicin from PLG microparticles were carried out by a subcutaneous administration of a single dose of the polymers in mice. Since almost a similar in vitro release profile was observed for both porous and non-porous microparticles; porous PLG microparticles were examined for their in vivo release behaviour. A sustained release of rifampicin was observed in all the organs examined i.e. lungs, liver and spleen up to 7 days. Rifampicin release was even more sustained in spleen up to 27 days post administration of porous PLG microparticles (Table 1). The values were expressed as <0.5 µg/ml of the homogenates as values below this could not be detected by the assay. Concentrations below 0.5 µg/ml of the homogenates would also not be of significance as this would fall below the minimum inhibitory concentration (MIC) of rifampicin which is 0.25 µg/ml.

In case of hardened PLG microparticles, a sustained release of rifampicin was observed in all the organs examined i.e. lungs, liver and spleen up to a period of 42 days after a single dose administration of the microparticles (Table 2).

In free drugs injected subcutaneously in mice at the same dose i.e. 85 mg/kg body wt, peak plasma concentration of 22.5±3.81 µg/ml of rifampicin was ob-

<table>
<thead>
<tr>
<th>Time period (days)</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
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<td>&lt;0.5 (2.5)</td>
<td>&lt;0.5 (2.5)</td>
<td>&lt;0.5 (2.5)</td>
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<tr>
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<td>&gt;0.5 (2.5)</td>
<td>&gt;0.5 (2.5)</td>
</tr>
<tr>
<td>3</td>
<td>&gt;0.5 (2.5)</td>
<td>&gt;0.5 (2.5)</td>
<td>&gt;0.5 (2.5)</td>
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<tr>
<td>7</td>
<td>&gt;0.5 (2.5)</td>
<td>&gt;0.5 (2.5)</td>
<td>&gt;0.5 (2.5)</td>
</tr>
<tr>
<td>21</td>
<td>—</td>
<td>—</td>
<td>&lt;0.5 (2.5)</td>
</tr>
<tr>
<td>27</td>
<td>—</td>
<td>—</td>
<td>&lt;0.5 (2.5)</td>
</tr>
</tbody>
</table>

*Concentration is given as µg/g of the organs in brackets. All values are mean of 3-4 animals.
DUTT & KHULLER: PLG BASED MPS AS CARRIERS FOR RIFAMPICIN.

obtained at 2 hr, which finally reached a minimum of $1.21 \pm 0.38 \mu g/ml$ at 48 hr (Fig. 3). In tissue homogenates examined at 24 hr, the liver contained the maximum amount of the drug i.e. $3.60 \pm 0.4 \mu g/ml$ of the homogenates as compared to the other organs (Fig. 3 inset). At 72 hr, no rifampicin was detected in any of the organs examined.

thus indicating no hepatotoxicity (Table 3).

Discussion

It has been observed that after the injection or ingestion of standard dosage forms, the level of the drug rises in blood, reaches a maximum and then declines after which it has to be readministered to maintain therapeutically effective concentrations in vivo\textsuperscript{18}. In the present study, when rifampicin was administered in a free form, peak levels were observed for only 2-3 hr in plasma, and liver had the maximum concentration of rifampicin at 24 hr. This was expected since the liver being the major metabolic organ and detoxification center for majority of the drugs would naturally concentrate large amounts of the drug as compared to the other organs. However, the most important point was that the entire drug was cleared from plasma and various organs within 48 hr. This is the major disadvantage of the free drugs wherein they have to be administered practically daily to maintain therapeutically significant levels in blood. This leads to the development of non-compliance in patients, a major factor complicating the chemotherapy of tuberculosis. It is believed that the full supervision of the taking of medicament is important to achieve high

<table>
<thead>
<tr>
<th>Time period (days)</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.5 (2.5)*</td>
<td>0.5 (0.5)</td>
<td>&lt;0.5 (&lt;2.5)</td>
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<tr>
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<td>&lt;0.5 (&lt;2.5)</td>
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</table>

*Concentration is given as $\mu g/g$ of the organs in brackets. All values are mean of 3-4 animals.

Fig. 3—Mean concentrations of rifampicin obtained after a subcutaneous dose of 85mg/kg body wt of mice in plasma and at 24 hr (inset) in various organs. All values are mean ±S.D. of 3-4 animals.
Table 3—Levels of alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT) and total bilirubin of mice injected with PLG microparticles. All values are mean ± S.D. of 5-6 animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time period (days)</th>
<th>ALP (IU/litre)</th>
<th>SGPT (IU/litre)</th>
<th>Total bilirubin (mg/100 ml)</th>
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<tr>
<td>Porous microparticles</td>
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<td>32.93±0</td>
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<td>7</td>
<td>62±3.46</td>
<td>35.29±0</td>
<td>0.284±0.01</td>
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<td>14</td>
<td>53.67±0.57</td>
<td>32.05±1.12</td>
<td>0.204±0.01</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>55±0</td>
<td>35.28±1.17</td>
<td>0.205±0.01</td>
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<tr>
<td></td>
<td>27</td>
<td>49±0</td>
<td>32.15±1.79</td>
<td>0.197±0.006</td>
</tr>
<tr>
<td>Hardened microparticles</td>
<td>3</td>
<td>30.83±3.75</td>
<td>44.25±2.67</td>
<td>0.516±0.44</td>
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<tr>
<td></td>
<td>7</td>
<td>40.33±6.3</td>
<td>30.97±4.75</td>
<td>0.224±0.07</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>27±0</td>
<td>36.85±4.75</td>
<td>0.093±0.009</td>
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<tr>
<td></td>
<td>21</td>
<td>53±8.18</td>
<td>33.52±2.02</td>
<td>0.204±0.05</td>
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<td></td>
<td>27</td>
<td>56±5.22</td>
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<td>0.198±0.01</td>
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<td></td>
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<td>62±3.46</td>
<td>33.80±2.61</td>
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<tr>
<td></td>
<td>42</td>
<td>42±8.18</td>
<td>37.93±1.48</td>
<td>0.122±0.01</td>
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<tr>
<td></td>
<td>49</td>
<td>45.6±62.88</td>
<td>36.16±3.76</td>
<td>0.242±0.02</td>
</tr>
<tr>
<td>Normal values</td>
<td></td>
<td>35-49 IU/L</td>
<td>27-38 IU/L</td>
<td>Up to 1mg/100 ml</td>
</tr>
</tbody>
</table>

cure rate(s) in tuberculous chemotherapy\textsuperscript{19} and it has been accorded high priority as directly observed treatment short course (DOTS) by WHO\textsuperscript{20}. But it is difficult and expensive to carry out, particularly so in rural areas. Hence, an effective once weekly or even once monthly regimens that could continuously deliver the drugs would have several operational advantages.

Thus in the present study, 50:50 resomer of PLG has been used as the drug carrier, which is predicted to have a half-life of 1-2 months\textsuperscript{21} appropriate for a one-dose-after-one/two months regimen for TB chemotherapy. A higher dose of rifampicin i.e. 85 mg/kg body wt has been used in this study as compared to the therapeutic dose of 12 mg/kg body wt in humans with the aim of developing a sustained release system that could continuously deliver the drugs over prolonged periods alleviating the requirement for repeated dosages. Also since lower animals such as mice are fast metabolizers of drugs, a higher dose in mice would be equivalent to a therapeutic dose in humans.

Both porous and non porous PLG microparticles exhibited the same percentage entrapment of rifampicin (8%). Also they exhibited a similar particle size of 40.78 and 43.21 $\mu$m, respectively. However, in hardened PLG microparticles, the maximum entrapment of rifampicin (12-14%) was obtained in addition to a smaller particle size (11.64 $\mu$m). The preparation of hardened microparticles involved the use of 20% PVA as an emulsifier. For most of the emulsifiers, the microsphere size decreases with an increase in the emulsifier concentration\textsuperscript{22}. Jeffery et al.\textsuperscript{23} found a reduction in particle size as the concentration of PVA increased in the external phase. This supports our findings as well, in which a lower particle size was obtained for hardened microparticles as compared to the other formulations. This could be due to unstable emulsion droplets at low PVA concentrations resulting in the formation of larger microparticles as compared to those prepared from higher PVA concentration.

The \textit{in vitro} release profile of rifampicin from both porous and non porous PLG microparticles showed a sustained release up to 49 days. Studies with the hydrophilic drug isoniazid indicated porous PLG microparticles to release greater amounts of the drug (isoniazid) both \textit{in vitro} and \textit{in vivo}\textsuperscript{24} (unpublished data). However, in the present study, rifampicin was used for encapsulation into PLG microparticles. Rifampicin is a hydrophobic drug that could probably form strong hydrophobic interactions with the hydrophobic PLG polymer. This could lead to lesser amounts of rifampicin being released from PLG polymer, the release was however sustained till 49 days \textit{in vitro}.

The \textit{in vitro} release profile of rifampicin from hardened PLG microparticles showed a burst release of rifampicin at 12 hr upon incubation of the samples and a sustained release up to 49 days. Microparticles loaded with higher amounts of the drug produce a greater burst release of the drug due to an increase in the number of channels formed in the polymer matrix by the drug\textsuperscript{25}. This could be the reason for a greater burst release of rifampicin obtained at 12 hr in hard-
ened PLG microparticles since the entrapment of rifampicin was more here.

The in vivo release profile of rifampicin from porous PLG microparticles showed a sustained release up to 7 days in all the organs examined i.e. lungs, liver and spleen. The release was even more sustained up to 27 days in the case of spleen has been observed that particles with size range > 10 μm remain at the site of injection forming a depot from where the entrapped contents of the polymer are released slowly. Such depots can show release profiles up to several months till the entire polymer is biodegraded. In the present study also, the formulations examined exhibited a particle size > 10 μm so probably they could effectively function as a depot releasing system that could show a sustained release for prolonged periods.

In hardened PLG microparticles, a sustained rifampicin release was obtained in all the organs up to 42 days. Since these microparticles were prepared using 20% PVA as an emulsifier, this could have resulted in the formation of a thicker and more stable emulsion able to effectively form a depot at the injection site resulting in a prolonged and sustained release of rifampicin.

Recent in vitro and ex-vivo studies using PLG microparticles containing entrapped rifampicin showed release of bioactive rifampicin only up to 7 days. Further, these rifampicin levels were more effective at reducing Mycobacterium tuberculosis growing intracellularly in monocytes than free drugs. However, in the present study, a prolonged and sustained release of rifampicin has been shown from hardened PLG microparticles in vitro and in various target organs up to 49 days. Moreover, the concentrations of rifampicin obtained in the study were higher than the minimum inhibitory concentration (MIC) of the drug. Also the proposed system alleviates some of the disadvantages associated with the use of free drugs such as repeated administration of drugs and increased toxicity.

Hence, use of hardened PLG microparticles with entrapped rifampicin could serve as an ideal therapeutic approach for the treatment of tuberculous infections.

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References


