DETAILS OF THE EXCELLENCE IN RESEARCH WORK FOR THE SUN PHARMA RESEARCH AWARD WITH REFERENCES AND ILLUSTRATIONS

Nanoparticle Shape A New Design Parameter for Splenic Targetting

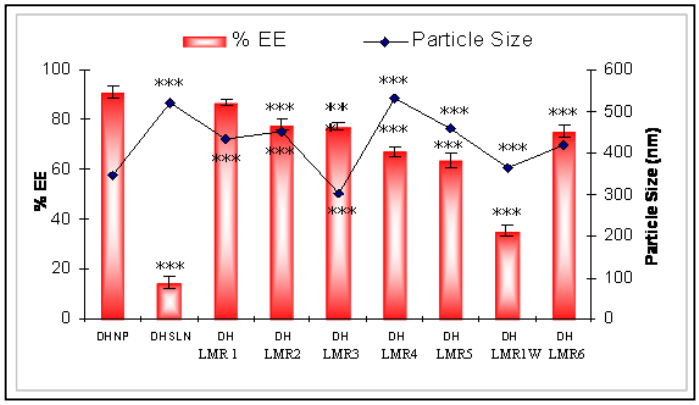
Nanomedicine is a growing field making rapid inroads in therapy and diagnosis. Targeted drug delivery is one major application of nanomedicine. In particular, nanomedicine provides manifold advantages in the therapy of cancer and infectious diseases. This is evident from the formulations which have reached the market, major examples being liposomal Doxorubicin (Lipodox) for various cancers and liposomal Amphotericin B (Ambisome) for leishmaniasis and even mucor mycosis during the current pandemic[1].

Targetting tumours for cancer and macrophages for intracellular infections is well reported. Furthermore, there is abundant literature on targetting different organs like the lungs[2-5], liver[6-10], brain[11-13], colon[14-15] etc. Spleen targetting is a neglected area despite the fact that many infections are spleen resident and are intractable, due to limited drug access to the spleen. Splenotropic drug delivery systems have immense clinical significance for intracellular infections including leishmaniasis, trypanosomiasis, splenic TB, AIDS, malaria, splenic fungal infections[15] and hematological disorders such as hairycell leukemia, idiopathic thrombocytopenic purpura, and autoimmune hemolytic anemia. Number of intractable veterinary infections namely ehrlichiosis, theileriosis and brucellosis are also spleen resident. Splenic drug targetting could provide radical improvement in therapy of such afflictions.

Nanoparticle shape as a design parameter for splenic targetting was a serendipitous finding. The study was initiated with the objective of development of nanoparticles of doxycycline hydrochloride (DH) for targetting the Reticulo-endothelial system (RES) to treat intracellular infections. As RES uptake of hydrophobic nanoparticles (NP) is well reported[17-18] we prepared hydrophobic solid lipid nanoparticles (DH-SLN). Conventional nanoprecipitation, wherein a solution of drug and lipid/polymer is added to an aqueous phase, resulted in low DH entrapment of <15%, attributed to the high aqueous solubility of DH. Modified nanoprecipitation by replacing water with a blend of water and isopropyl alcohol (IPA), enabled only marginal enhancement.

We then conceptualized the innovative Lipomer NP, which was essentially a combination NP of a lipid and a hydrophilic polymer. The objective was to enhance drug entrapment aided by the hydrophilic polymer, while balancing hydrophobicity with the lipid, a vital requirement to ensure targeted RES uptake. Lipomers were prepared with a number of lipids including glyceryl monostearate (GMS), glyceryl distearate, polyglyceryl distearate, glyceryl palmitostearate, hydrogenated vegetable oil and stearic acid. The polymer selected was polymethylvinylether/maleic anhydride) copolymer (Gantrez AN 119), an anionic polymer which could form an ionic complex with cationic doxycycline hydrochloride, to enhance entrapment efficiency[19]. Modified nanoprecipitation enabled Lipomers of average size in the range 391-532 nm, with entrapment efficiency in the range of 35-86%. In the absence of

Gantrez the solid lipid nanoparticles of DH- Glyceryl monostearate (GMS) exhibited poor entrapment of ~15%, while maximum entrapment efficiency was achieved with DH-GMS Lipomer 1 of >80% and was comparable to the polymeric nanoparticles of Gantrez (Fig 1). This confirmed Lipomer as a practical strategy to enhance entrapment efficiency of DH. Further significant enhancement in entrapment efficiency was observed with modified nanoprecipitation (DH-GMS Lipomer 1) compared to conventional nanoprecipitation (DH-GMS Lipomer 1W) as seen in Figure 1.



(mean ± standard error, (n=4), ***p< 0.001 between DH NP and other formulations)

Figure 1: Particle size and Entrapment efficiency% of LMR 1 Glyceryl monostearate; LMR 2 Glyceryl tristearate; LMR 3 Polyglyceryl distearate; LMR 4 Glyceryl Palmitostearate; LMR 5 Hydrogenated vegetable oil; LMR 6 Stearic acid; LMR1W (conventional Nanoprecipitation)

The biodistribution study of Lipomers was carried out in rats by Gamma scintigraphy. DH, DHNP, DH-GMS-SLN and DH Lipomers were radiolabelled using 99^{m}Tc and the process standardized. High RES uptake was evident with all Lipomers as seen from the very high uptake in the liver, spleen and lungs, the major RES organs (Fig 2). High liver uptake as expected was seen with the nanoparticles, except DH-GMS-Lipomer 1, which unexpectedly revealed significantly higher concentration ($p<0.001$) in the spleen[20] This was an unexpected finding, as rapid clearance of intravenously injected nanocarriers by the Kupffer cells of the liver, allows barely 15% of an injected dose to reach the spleen.

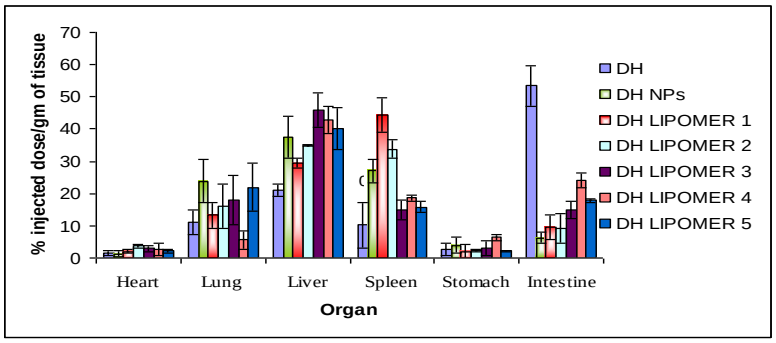


Figure 2: Biodistribution of nanocarriers following intravenous administration in rats

Scintigraphy images are depicted in Figure 3**.** High spleen uptake was seen with DH-GMS-Lipomer 1 (Fig 3D)

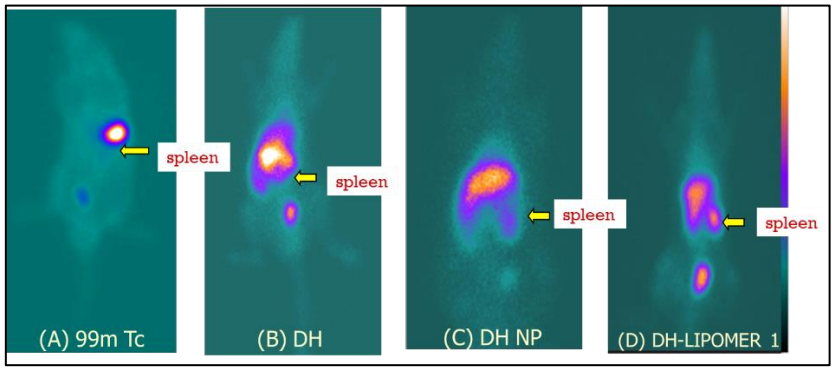


Figure 3: Scintigraphy images A- ^{99m}Tc B - DH C- DH NP D- DH Lipomer 1

Biodistribution was then evaluated by decreasing the GMS concentration (Lipomer 0.5) and increasing the concentration (Lipomer 1.5) in DH-GMS-Lipomer 1. GMS concentration dependent splenic uptake was evident (Figure 3) with Lipomer 1.5 revealing maximum spleen uptake. This strongly suggested GMS as a splenotropic agent. However, Lipomer 1W which had the same composition of Lipomer 1, but was prepared by conventional nanoprecipitation revealed a spleen liver ratio of ~ 0.5 which was comparable with DHNP and not indicative of splenotropy. Furthermore, DH-GMS-SLN where the carrier was only GMS exhibited very low spleen liver ratio (Figure 4), challenging our hypothesis of GMS as a splenotropic agent.

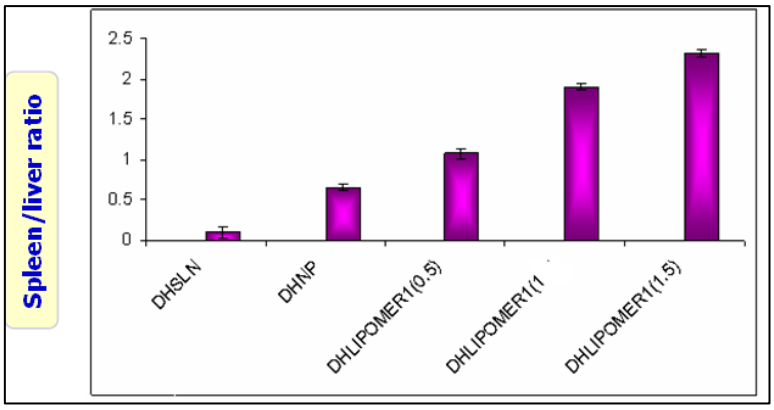


Figure 4: Effect of GMS concentration on Spleen: Liver ratio

The anatomy of the spleen is species dependent. While in humans it is sinusoidal it is non sinusoidal in some animal species. Hence we extended the biodistribution study in three other animal species, mice (non sinusoidal), rabbit(sinusoidal) and dog (sinusoidal) to evaluate biodistribution of Lipomer 1. While in the non sinusoidal mice model spleen liver ratio was <1, in rabbits the ratio was ~3, confirming splenic targetting[21]. The scintigraphy image of rabbit is depicted in Figure 5.

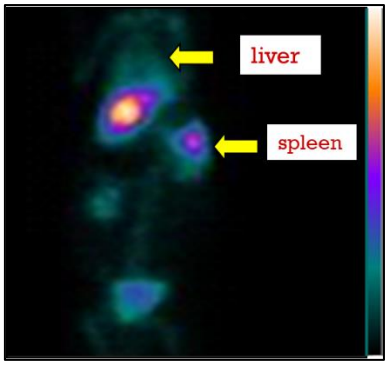


Figure 5: Scintigraphy image of DH-GMS-Lipomer 1 in rabbit showing high spleen targetting

In dogs we evaluated Lipomer 1 and Lipomer 1W which had shown distinct differences in biodistribution in the rat model, with Lipomer 1W revealing low spleen liver ratio. We were amazed when we could actually visualize splenic accumulation with DH-GMS-Lipomer 1 and did not see the same with Lipomer 1W (Figure 6). Further DH-GMS-Lipomer 1 revealed a high spleen liver ratio of >6 confirming very high spleen targetting. On the other hand the ratio was extremely low ~0.5 for DH-GMS-Lipomer 1W clearly indicating high accumulation in the liver with limited concentration in the spleen.

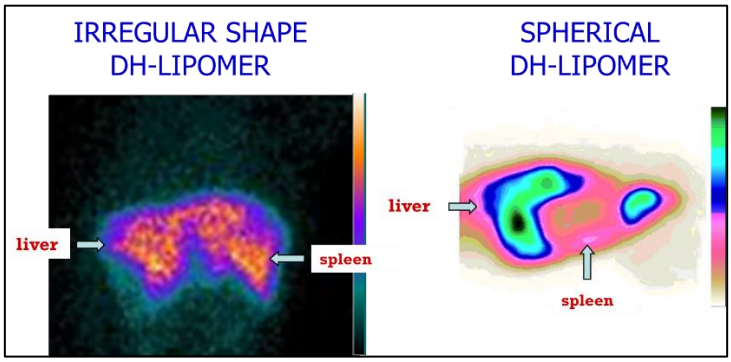


Figure 6: Scintigraphy images of dog

The comparative spleen liver ratio in the different species is shown in Table 1.

Table 1: Spleen :Liver ratio of DH-GMS Lipomer 1 is various animal species

Species	**Spleen/liver ratio**
Mice*	0.91±0.36
Rat	1.95±.98
Rabbit	2.86±1.31
Dog	6.77±2.52
Dog (DH-GMS **Lipomer 1W**)	0.538± 0.46

The TEM images of the Lipomers and SLN are depicted in Figure 7. It is seen from the TEM that SLN, Lipomer 1W and Lipomer 0.5 were near spherical. However, Lipomer 1 was

asymmetric and irregular, while the irregularity was even greater with Lipomer 1.5. This suggested NP shape as a probable explanation for splenotropy.

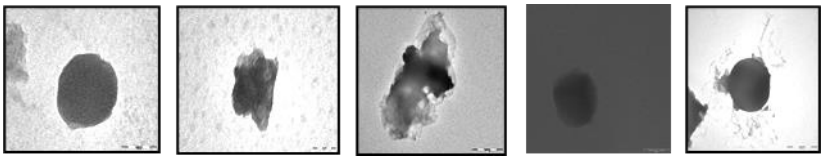


Figure 7: TEM Images of a) Lipomer 0.5 b) Lipomer 1 c) Lipomer 1.5 d) SLN e) Lipomer 1W

A careful analysis of reported data on nanoparticle uptake by RES revealed some reports that demonstrated high phagocytosis of spherical particles and bypass of phagocytosis by irregular particles[22]. High phagocytosis would result in high liver uptake and bypass would mean escaping the liver. Hence the asymmetric/irregular DH-GMS-Lipomer could bypass Kupffer cell uptake, explaining the lower liver uptake.

The rabbit, dog, and rat spleen, like human spleen are anatomically characterized as sinusoidal spleens which have longitudinally arranged endothelial cells and a fenestrated basement membrane. Circulating blood passes through the slits between the endothelial cells and is drained into different parts of the spleen. While micron sized red blood cells routinely pass through the spleen, deformed RBCs are trapped and filtered by the spleen[23]. This proposed that deformity could induce splenic trapping. The enhanced splenic uptake of cholesterol rich liposomes (50 mol% cholesterol; <200 nm) is reported[24] However, the reason for their splenotropic behavior is not discussed in the paper. Cholesterol is known to decrease the fluidity of liposomes and increases their rigidity. Hence, we presumed that rigidity of the cholesterol rich liposomes could have played an important role in their splenic clearance. Further, reports suggest that stealth polymeric nanoparticles >200 nm, which could escape Kupffer cell uptake, accumulated in the spleen[25-27]. However, while spleen accumulation is reported no explanation is provided for the same.

Based on the above, we attribute the splenotropic behavior of irregular GMS LIPOMER to a combination of size (>200nm) and rigidity as it is a solid nanoparticle. Recognition of asymmetric/irregular shaped GMS LIPOMER as a deformity by the splenic vasculature further aided splenotropy. In summary it is now clear that spleen targetting was achieved through a combination of the above three factors. This elucidation provides opportunity for rational development of nanocarriers for spleen targeting

We have also enabled a scientific understanding of role of lipid on the shape of Lipomer, and explained why some Lipomers were spherical while the GMS-Lipomer was asymmetric/irregular Lipomer[28]. Our study therefore provides a strategy to design irregular nanoparticles for spleen targetting.

We are happy to share that the science of asymmetric nanoparticle shape for splenic targetting has been successfully extrapolated for the design of asymmetric SLN of buparvaquone[29], to treat a veterinary intractable spleen resident infection theileriosis, which affects cattle. The technology is licensed and transferred to industry.

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Prof. Padma V. Devarajan

The work on Nanoparticle shape Based Spleen targeting was citeds as cutting edge research in nanotechnology by US magazine ***SCIENTIST***

Project: Designing nanoparticles to treat infectious diseases

User: Padma Devarajan, Professor of Pharmaceutical Sciences and Technology, University of Mumbai, India

Problem: The spleen, a potential target in treating diseases such as splenic tuberculosis, AIDS, and malaria, receives only about 15% of nanoparticles injected into the bloodstream. That's because nanoparticles are rapidly cleared by immune cells within the liver before they reach the spleen. Devarajan wanted to create particles that bypassed the liver.

Solution: Her group stumbled onto a solution in 2008 when they were attaching the antibiotic drug doxycycline to lipid–polymer nanoparticles. By simply increasing the concentration of glycerol monostearate during the process of mixing together the nanoparticles with the drug, they produced two seemingly different types of particles that behaved differently: One lingered in the liver, as expected, but the other bypassed the liver and collected in the spleen. A closer look revealed that the nanoparticles targeting the spleen were irregularly shaped and made with greater concentrations of glycerol monostearate, whereas the ones in the liver were spherical. Data from their work and other labs suggested that the particles' shape determined whether they targeted the spleen (*J Biomed Nanotech* 4:359–66, 2008).

They then tagged the nanoparticles with a radiolabeled dye and observed their biodistribution in vivo. They found high splenic uptake in rats, rabbits, and especially dogs, whose spleen-to-liver uptake ratio was nearly 6, compared to 0.5 for the original preparation (*J Pharm Sci* Jan 20, 2010 Epub ahead of print). "We are currently evaluating methods to standardize the process," Devarajan says.

Considerations: In the past 2 years, scientists have shown that shape plays an important part in nanoparticle design. Devarajan says researchers should try tweaking simple steps in their nanoprecipitation protocols to see whether it helps targeting. Stick with simple, inexpensive methods: "When I am looking at a drug or therapy I should have something that could be scaled up easily; otherwise, it's no use," she notes.

Cooperative Particles

Project: Nanoparticle design for cancer diagnosis and therapy

User: Michael Sailor, Professor of Chemistry and Biochemistry, University of California–San Diego

Problem: Heating a tumor has shown some potential in treating cancer, but as a therapy it is somewhat nonspecific and inefficient. One approach is to use radiowaves, but small tumors do not convert the waves into heat efficiently. "You can get the tumor hot but not hot enough to kill it," Sailor says.

Prof. Padma V. Devarajan