Journal of Stem Cells & Regenerative Medicine
ISSN 0973 - 7154

RESEARCH ARTICLE

JSRM Code: 007020300012

Solid Lipid Nanoparticles Regulate Functional Assortment of Mouse Mesenchymal Stem Cells

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A rapid decline in self-renewability, viability and function, of isolated stem cells are major hurdles in developing cell based therapies. There has been an increasing interest towards identifying a support material for maintaining stem cell features of the isolated cells. Pioneering observations of the present paper, demonstrate functionally diverse potential of Solid Lipid Nanoparticles (SLNs) in deciding the fate & behavior of mouse mesenchymal stem cell. The evidences are provided to show the dual nature of the SLNs for being a scaffold for the stem cell attachment, to retain stemness, and as reagent for inducing stem cell differentiation. Scanning electron microscopic examinations together with expression analysis were used to conform to such observations. Results of the study thus suggest that Solid lipid nanoparticles can be used as a good support material when functionalized to achieve adhesive properties and as a molecular paradigm for studying the adipocytic differentiation. We envisage a new role of SLNs towards regulating stem cell character by orchestrating the structural alignment during preparation of Solid lipid nanoparticles

Keywords: Solid lipid nanoparticles, scaffold, adipogenesis, mesenchymal stem cells.

Introduction:

Nanotechnology brings new possibility to stem cells research and development. Measuring in nanoscale to the design, construction, and utilization of functional structures is the hallmark of the technology [1-4]. Such materials and systems are designed to exhibit novel and significantly improved physical, chemical, and biological properties, phenomena and processes as a result of the limited size of their constituent particles or molecules [5, 6].

Stem cell nanotechnology shows great attracting prospects. In recent years, application of nanotechnology in stem cells has made great advances, and is becoming an emerging interdisciplinary field [7, 8]. It requires intense current knowledge and principles to fabricate novel multifunctional or homogenous nanostructures, their processing, characterization, interface problems, high quality nanomaterials availability and nanomaterials tailoring to suit the requirements in modifying stem cell fates. It is believed that stem cell nanotechnology finds applications in treatment of various degenerative diseases [9-12]. Hence, this necessitates an understanding of basic behavior of stem cells upon its interaction with varied characters of the nanoparticles. In this regard we used the sold lipid nanoparticles and studied their interactive behavior with the stem cells of mesenchymal origin.

Mesenchymal stem cells (MSCs) are a heterogeneous population of plastic-adherent, fibroblast-like cells, which in culture are able to self-renew and differentiate into mesodermal and non mesodermal derived tissues [12-17].

Advancements in understanding tissue specific differentiation of MSCs in conjunction with global genomic and proteomic profiling of MSCs have not only provided insights into their biology but also made MSCs based clinical trials a reality for treating various debilitating diseases and genetic disorders [18-20]. The emerging evidences that MSCs are immunologically well tolerated make them even more attractive candidate for regenerative medicine [21-23].

In the present paper, we, custom prepared the solid lipid nanoparticles to study cellular characteristics of mesenchymal stem cell line ($C_3H10T½$). Observations in the present study, for the first time, lend support to the notion that fate determination of mesenchymal stem cells is a function of SLNs structural assortment.

Materials and method:

CHEMICALS: DMEM (4.5gm glucose per liter, 3.7 gm sodium bicarbonate, sodium pyruvate with L-glutamine.(1X), Trypsin-EDTA solution, fetal bovine serum (FBS), Penicillin streptomycin (penstrap), isopropyl alcohol, TAE buffer, Trizol reagent, agarose were purchased from Hi-Media, India. PCR kit was purchased from invitrogen, India. The plasticware for the cell culture work viz. Falcon tubes (15 ml and 50 ml),

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Received 27 Jan; accepted 16 May; published online 30Oct 2011

Beakers(100ml and 500 ml), T-25and T-75 flasks, Six-well plates, Syringe filters, Syringes (0.5ml), PCR tubes were purchased from Tarsons Ltd., India. The Cell line $C_3H10T½$ was provided by NCCS Pune, India. All the other chemicals purchased from local suppliers were used in the present study.

Preparation & Characterization of lipid nanoparticles: The SLNs in different morphological assortment were prepared in the laboratory as per the method of Kakkar et al [24]**.** Briefly, the solid lipid nanoparticles were prepared by microemulsification method. The lipidic phase (containing lipid-8%) and the aqueous phase (polysorbate 80, soy lecithin and water) were heated ~10 degree above the lipid melting temperature of 70°C. This hot microemulsion was then transferred into cold water to obtain solid lipid nanoparticles. Excess of polysorbate 80 was removed from the particulate dispersion by washing it with distilled water using a dialysis membrane.

The mean diameter of the SLNs was determined using laser diffraction (Mastersizer 2000, Malvern Instruments, UK) and morphology was examined using a Transmission & scanning electron microscope. SLNs emulsion was lyophilized for performing scanning electron microscopy

Transmission Electron Microscopy: A drop of the SLN dispersion was spread on a 500-mesh gold grid coating and the excess droplets were removed with filter paper. After 5 min, a drop of 2% (mass fraction) phosphotungstic acid in ethanol was placed onto the gold grid. The grid was dried at room temperature and then examined under a Transmission Electron Microscopy (Morgagni 268; Phillips, Holland).

Cell culture: The mouse mesenchymal stem cell line ($C_3H10T½$) were maintained in DMEM (with high glucose content), supplemented with 10% FBS (heat inactivated), 100 U penicillin, and 100 µg/ml streptomycin. All the study was performed using third passaged cells. For the experiment purpose the cells were plated at a concentration of $1.5x10^6$ cells per well of the six well plate in the above mentioned growth medium and kept in an incubator maintained at 37 °C in the atmosphere of air and CO_2. All the experiments were performed 24 hrs post establishing the cells in culture and designated this time as 0-day of culture.

Solid Lipid Nanoparticles as Scaffold: Solid lipid nanoparticles (400ng) were added and spread uniformly in two wells of a six well plate. These SLNs were then air dried and UV irradiated overnight to form a thin film. The wells without SLNs served as control. Twenty four hours after formation of the SLN film, equal number of cells (1.5×10^6 cells/well) were plated in a six well plate in the presence of 1.5 ml full growth medium. After 24 hours of incubation, the attachment of the stem cell on SLN film was observed under phase contrast microscope.

Solid Lipid Nanoparticles as adipogenic differentiation medium: Varying amounts of SLNs (0, 1, 4, 8, 16, 20,100 and 200 ng were used to study the effects of SLNs on differentiation of C3H10T1/2 cell line. For this, 24 hrs post establishment the cell (1.5×10^6 cells/well of a six well plate) were treated at the above mentioned concentrations and observed under phase contrast microscope for any morphological characterization.

Oil-Red-O (ORO) staining: To determine the adipogenic potential of SLNs, oil red O staining of intracytoplasmically accumulated lipid was carried out at varying doses of SLNs. Briefly, the cells were fixed for a period of 5 min with 10% formalin followed by washing with 60% isopropanol. The cells were then incubated with Oil-Red-O solution for 10 min at room temperature and then were rinsed with water. Stained cells were visualized microscopically and observations recorded.

RT- PCR Analysis: Total RNA of $C_3H10T½$ cells under different experimental conditions was extracted by Trizol method (invitrogen) according to the manufacturer's instructions. Specific PCR amplification procedures were carried employing forward and reverse primers of Adipsin (forward -5'ATG GTA TGA TGT GCA GAG TGT AG 3'; REVERSE- 5'CAC ACA TCA TGT TAA TGG TGA C 3'), ß-catenin (forward 5'AAG GAA GCT TCC AGA CAT GC 3';Reverse 5'AGC TTG CTC TCT TGA TTG CC 3'), RigS/15 (forward 5'TTC CGC AAG TTC ACC TAC C 3', reverse 5' CGG GCC GGC CAT GCT TTA CG 3') and PPAR gamma (forward-5' ATG GCC ATT GAG TGC CGA GTC TG 3', reverse –5'ACT GGT CCC TCA AGG AGT TTT CGG 3') .The amplified products were analysed on 0.6 % agarose gels.

Results & Discussion:

We prepared the solid lipid nanoparticles with average particle size of approximately 120 nm (10% particles were >188 nm), having a zeta potential -1.45 mV. The scanning electron microscopic (SEM) images revealed their morphological characteristics which were mainly round in shape (Figure.-1a). The shape and surface morphology of these synthetic made solid lipid nanoparticles were studied with TEM (Figure.-1- b & -c).

Figure 1

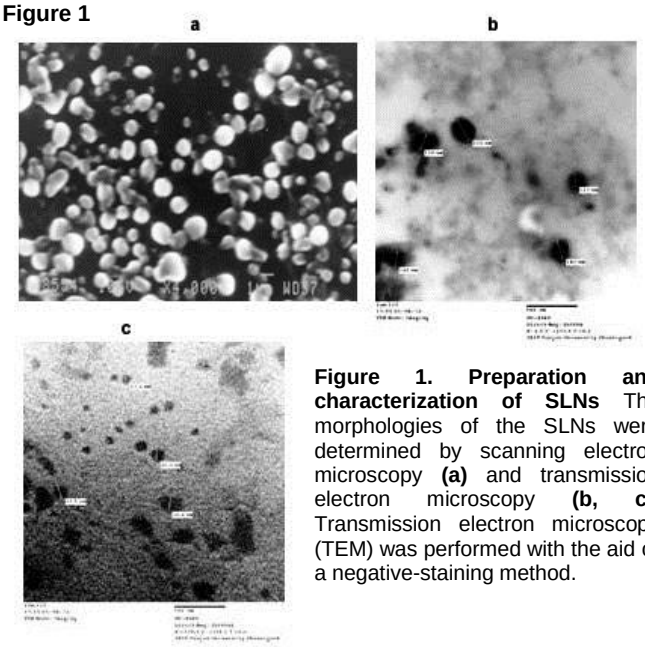

Figure 1. Preparation and characterization of SLNs The morphologies of the SLNs were determined by scanning electron microscopy **(a)** and transmission electron microscopy **(b, c)**. Transmission electron microscopy (TEM) was performed with the aid of a negative-staining method.

An average particle size below 200 nm could be achieved and reproduced. The particles were of spherical nature with smooth surfaces, which could facilitate an even distribution of stem cells on the particles. The negative zeta potential value of SLNs assisted two different arrangements for the SLNs which were found to be dependent upon the concentration of SLN used. This physical assortment of SLNs also significantly altered the behavior of the mesenchymal stem cells in culture.

Concentrations of SLNs ranging from 1-200 ng per ml remained refractory towards any alterations in the morphology of SLNs and these remained rounded as seen in Figure-1a however at higher concentrations (400 ng per ml) these SLNs arranged themselves as sheet like structures on cell culture plastic ware. This sheet like arrangements is shown in photomicrograph (Figure-2 a, & 2b). In spite of the amphipathic nature of the lipids used during SLN preparation, the sheet like arrangement of SLNs in the aqueous milieu remained very sensitive to touch and handling. These sheets of SLN start to break and eventually fall apart with in 36 hrs. Hence, broken sheets with cell attached are shown in these photomicrographs at different magnifications (Figure-2a, 4x & 2b, 10x).

Figure 2

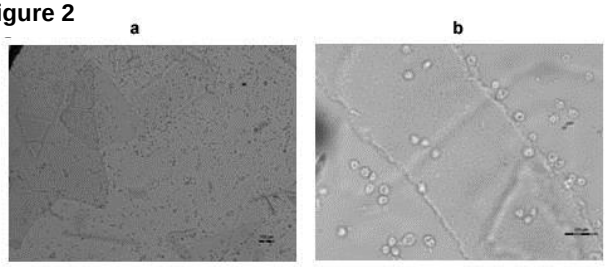

Figure 2. SLNs as sheet: An emulsion of SLNs at a concentration of 400 ng per ml when plated on plastic cell culture plate, with in 24 hrs of plating, emulsion took sheet like appearance which however remained unstable. Cells when plated on this sheet appeared to have a rounded morphology with some attached and some suspended on these sheets [Figure.2 a (4X) and 2 b (10X)]

Parallel to this however, the effect of varied concentration of SLNs in aqueous milieu demonstrated a variable effect on their interaction with the stem cells. As shown in photomicrographs (Figure-3 panels b to f), the concentrations SLNs varying from 1-20 ng adopted a physical arrangements that enabled these particles to behave more like a cell differentiation molecules and triggered the differentiation of the C3H10T½ mesenchymal stem cells to adipogenic lineages as demonstrated by positive staining for the oil O red (Figure. 3b-f) compared to untreated cells (Figure. 3a). Oil O red is an important marker for the adipocytic cells because of its ability to stain the triglyceride component of the cells [25].

Figure 3

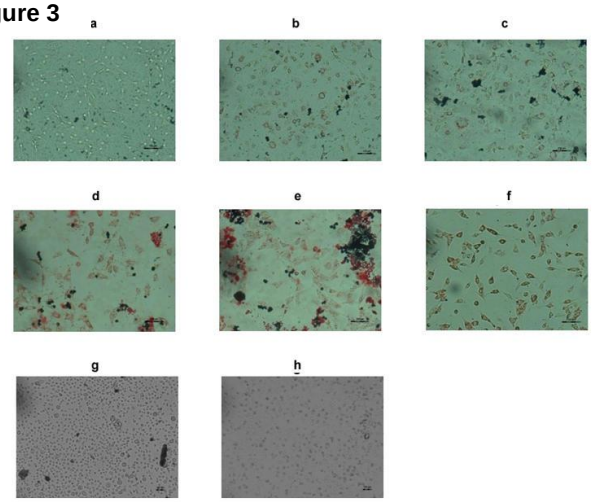

Figure 3. SLNs as differentiation agent Adipocytic differentiation of C3H10T1/2 in the presence or absence of SLNs (day1) stained by oil red O (day2), Photomicrographs of C3H10T1/2 cells either untreated **(a)** or treated with dose **(b)** 1.0 ng, **(c)** 4.0 ng, **(d)** 8.0 ng, **(e)** 16 ng **(f)** 20ng, **(g)** 100 ng, and **(h) 2**00 ng per ml

This potential of the SLNs probably dwells in the nature of the constituent lipids used in their preparation. Lecithin is one of the main constituent components of these nanoparticles which seem to be responsible for the adipogenic differentiation. These observations are corroborated from the studies of Zhang et al [26], that lecithin triggers the adipogenesis and lipogenesis in NIH 3T3 cells and are responsible for triglyceride accumulation. At present it is not known how lecithin could interact with the mesenchymal stem cells and trigger its response. Though, the possibility of the nanoparticle internalizing the stem cells cannot be ruled out. Hence having crossed the cellular bilayer, these particles may bring in response from the constituents lipid of SLNs to favor adipogenic differentiation. SLN internalizing different cell types is already known whereby; unloaded SLNs have been shown to be quickly taken up inside the cells. In one similar study it was demonstrated that SLNs even have the potential to cross the tumor cells, characteristics similar to embryonic stem cells, with in short time span of 2-3 minutes [27-30]. Such observations, therefore, suggest that SLN behavior is a function of the cell type used. The present studies though preliminary, but help to provide an insight into the functional character of the SLNs when used with mesenchymal stem cells.

The concentrations of SLNs beyond 20 ng per ml specifically the amount used in the study i.e. 100 and 200 ng severely affected the cell attachment ability and morphology. At these concentrations SLNs were found to be inhibitory to cell establishment. As shown in Figure-3g (100 ng, SLN conc.) & 3h (200 ng, SLN conc.) the cells attained rounded morphology and were found to be rather floating in suspension. Despite the repeated attempts of washing these floating cells could never got attached to the plate surface. Surprisingly none of these cells (100ng SLN conc.) took up trypan blue suggesting these cells were still live but lost the ability to establish.

Our observations further demonstrated that adipogenic potential of the low concentrations of SLNs towards C3H10T½ is not just a consequence of the constituent lipid accumulation rather a bonafide effect on gene expression. Hence, we sought to determine effects of SLN by using one of the adipogenic differentiation concentration of SLNs (20 ng, same dose as used in Figure- 3f, (oil O red staining) on expression profile of adipsin, a marker gene (31) for adipocytic differentiation. Adipsin, a complement factor D, is expressed at high levels in adipose tissue and is a regulator of lipid accumulation in adipocytes [31-32]. To analyze this we determined expression of adipsin following RT-PCR amplification as shown in Figure-4a. The Lane one (L-1, Figure-4a) did not show adipsin expression in mesenchymal stem cells in the absence of SLNs. Upon treatment (20 ng/ml) with SLN, as shown in lane two ((L-2), Figure-4a), amplification in the adipsin expression could be observed. This expression profile of adipsin goes in parallel with the positive staining for Oil Red O in the SLN treated mesenchymal cells (Figure-3 f), suggesting adipogenic potential of SLNs. Likewise SLN treatment also induced the expression peroxisome proliferator activated receptor (gamma) (Figure4-d lane 2). Activation of PPAR induces adipocyte differentiation and lipid accumulation by adipocytes by modulating numerous genes regulating adipogenesis, lipid uptake and lipid metabolism [33]. At mechanistic level, it has been well documented that wnt/β-catenine pathways play an important role in regulating the process of adipocyte differentiation [35]. To identify whether the SLNs adopt same putative signal transduction of a specific inhibitor of wnt

pathway, i.e. lithium chloride, LiCl. As shown in Figure-4a, lane three (L-3), presence of LiCl produced a significant downregulation of adipsin expression. This observation supported the fact that SLNs also exploit wnt/ β- catenine signaling pathway to bring about adipogenesis. The LiCl is a well established inhibitor of glycogen synthease 3β Kinase (GSK- 3 β) [34-36], that leads to stablilization/accumulation of β-catenine thus enabling β-catenine's forced entry to nucleus to regulate the gene expression [37]. Thus downregulation of β-catenine by SLNs seems a plausible reason to induce adipogenesis. This gets support from fact that control undifferentiated mesenchymal stem cells (lane-1-4b) demonstrated a maximal relative expression of β-catenine which was found to be downregulated in the presence of SLN (L-2, Figure-4b) and started to build up again when treated with LiCl (L-3, Figure-4b). These observations thus point towards an inverse correlation between expressions of β-catenine and adipsin. Such functional character of SLNs is, probably, observed for the first time and their character changes as the concentration of SLNs was increased.

Figure 4

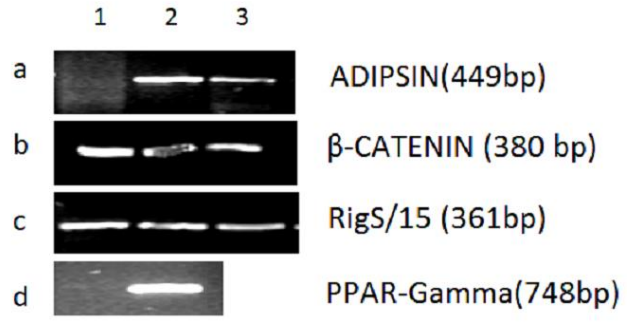

Figure 4. RT-PCR analysis of a) adipsin b) β-catenin c) RigS/15 loading control and d) PPAR–gamma. Lane-1 represents Control (without SLN treatment), lane-2 represents SLNs treated cells, Lane-3: GSK3β inhibitor LiCl, treatment to SLN treated cells.

As shown earlier (Figure-2a & b) st concentration of 400 ng per ml these SLNs formed a rather a thick emulsion which when plated on plastic cell culture plate, acquired a sheet like appearance with in 24 hrs of plating. (Figure.-5b & c). These sheet like structures were very unstable and in spite of repeated trial these could not be kept as single sheet, thus appeared as small broken sheet in culture medium. These sheet like structures, hence, were used to observe the behavior of mesenchymal stem cells. Unlike individual SLN particles, the SLNs in sheet form behaved as support scaffold for the attachment of mesenchymal stem cells (Figure-5b1 & c1). This observed arrangement of SLNs demonstrated a newer character of SLNs, albeit unstable for long cell culture conditions. The studies are thus underway to develop mixed SLNs possessing components of extracellular matrix. Further as shown in this figure (Figure-5 b1 & c1), few of the cells could be seen adhered to these SLN sheets and while few cells remain suspended. All the cell populations, whether attached or suspended, however, remained rounded (Figure-5b1 & c1) and did not retain discernable structural morphology as is seen in cases when these cells are allowed to establish on uncoated cell culture plastic ware, i.e. in a fibroblast -like morphology (Figure-5a1). These observations suggested that SLNs can provide a suitable support that apparently kept the cells in undifferentiated state. This observations of ours was further supported by the fact that the cell attached to SLN sheets also remained refractory to the adipocytic and myocytic cell differentiation cocktail (Figure-5 b-2 & c-2), that otherwise differentiated the cells on plastic ware to respective lineages i.e. adipocytic, positive for oil Red O (Figure-5a-2) and myogenic, elongated morphology (Figure-5a-3). These observations thus suggest a new paradigm for maintaining the stem cell character which is the function of structural assortment of SLNs linked to its concentration in aqueous milieu.

Figure 5

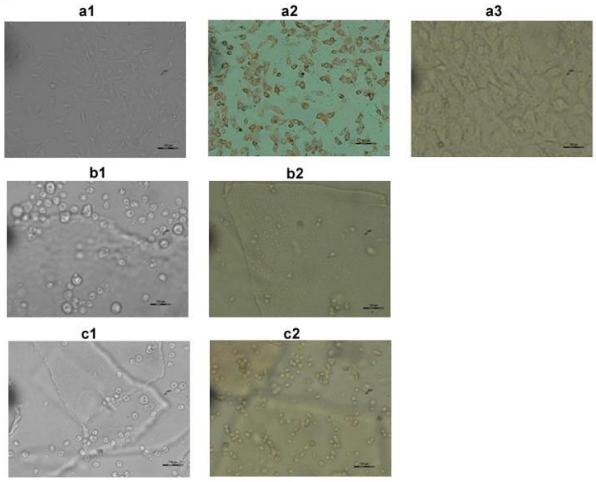

Figure 5. SLNs as Stem Cell Scaffolds: Photomicrographs of C3H10T1/2 cells (a-1-3) plated on six well uncoated plate (control), (b-1-2),(c-1-2) when plated on SLNs coated film in a six well plate and their corresponding picture when treated with adipocytic differentiation dose(MIX) and myogenic differentiation dose (5-azacytidine), respectively. Panel a2 & a3 represents the adipogenic differentiation and myogenic differentiation of cells on uncoated plasticware, respectively. Cells plated on SLNs coated film did not differentiate into either lineage when treated with the differentiation cocktails**.**

Acknowledgments

Authors also acknowledge the help rendered by Miss Nidhi Mahajan. This study was funded by PURSE grant from Department of Science & Technology, New Delhi to Centre for Stem Cell & Tissue Engineering. The facility used at Centre with Potential for Excellence in Biomedical Sciences at Panjab University, Sector-14 Chandigarh is duly acknowledged.

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