BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
48 (2023) 102651

Original Article

nanomedjournal.com

Evaluation of drug carrier hepatotoxicity using primary cell culture models

Güneş Kibar, PhD[a,b,c], Subhadeep Dutta, PhD[d], Kaushal Rege, PhD[d,e,f,*], O. Berk Usta, PhD[a,b,**]

[a]*Center for Engineering in Medicine and Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA*
[b]*Shriners Hospitals for Children, Boston, MA 02114, USA*
[c]*Department of Materials Science and Engineering, Adana Alparslan Turkes Science and Technology University, Adana 01250, Turkey*
[d]*School of Molecular Sciences, Arizona State University, Tempe, AZ 85287, USA*
[e]*Chemical Engineering, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ 85287, USA*
[f]*Biological Design Graduate Program, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ 85287, USA*

Revised 17 November 2022

Abstract

This study aims to establish a primary rat hepatocyte culture model to evaluate dose-dependent hepatotoxic effects of drug carriers (lipopolymer nanoparticles; LPNs) temporal. Primary rat hepatocyte cell cultures were used to determine half-maximal Inhibition Concentrations (IC_{50}) of the drug-carrier library. Drug-carrier library, at concentrations <50 μg/mL, is benign to primary rat hepatocytes as determined using albumin and urea secretions. Albumin, as a hepatic biomarker, exhibited a more sensitive and faster outcome, compared to urea, for the determination of the IC_{50} value of LPNs. Temporal measurements of hepatic biomarkers including urea and albumin, and rigorous physicochemical (hydrodynamic diameter, surface charge, etc.) characterization, should be combined to evaluate the hepatotoxicity of drug carrier libraries in screens.

Keywords: Lipopolymer nanoparticle (LPN); Nanotoxicity; Primary rat hepatocyte; In vitro culture; Hepatotoxicity

Introduction

Nanocarriers have been increasingly studied over the past two decades for facilitating the efficacious delivery of nucleic acids, including plasmid DNA (pDNA)[1], small interfering RNA (siRNA)[2] messenger RNA (mRNA)[3], antisense oligonucleotides[4], and CRISPR/Cas9 constructs[5], in addition to a wide variety of different therapeutic agents[6]. Various nanocarriers including: (i) polymeric carriers (porous nanoparticles, nanogels[7], hydrogels[8], micelles[9]), (ii) carbon-based carriers (nanotubes[10], fullerenes[11]), (iii) inorganic-based carriers (silica-based carriers[12], metal-organic frameworks[13]) and, (iv) lipid-based carriers (liposomes[14], niosomes[15], lipid nanovesicles[16], cubosomes[17], nanogels[7], solid lipid nanoparticles[17], nanostructured lipid carriers[18]), have been generated for the non-viral delivery of nucleic acids, small molecules, and other therapeutic cargo. Lipid-polymer hybrid nanoparticles, including lipopolymer nanoparticles (LPNs), are a novel class of hybrid nanocarriers, and have been drawing increasing attention because of their desirable biological, physicochemical, and/or multifunctional properties[19,20]. Specifically, the conjugation of lipids onto polymer backbones provides stability, imparts the ability to

Acknowledgments and funding support: This research was supported by grants from the National Institutes of Health (NIH 1R21GM136002 and NIH 5R01HL145031), the National Science Foundation (NSF Engineering Research Center, Award # 1941543), and a Massachusetts General Hospital (MGH) Executive Committee on Research (ECOR) Interim Support Fund at Dr. Usta's lab. We also acknowledge the support and use of facilities at the Morphology and Imaging Shared Facility, and Regenerative Medicine Shared Facility provided at the Shriners' Children - Boston. We also acknowledge the Turkish Fulbright Commission for a Fulbright Postdoctoral Scholarship and the Scientific and Technological Research Council of Turkey (Tubitak 2219 Award #1059B191801017) for Dr. Gunes Kibar. We are also grateful to the NIH (Grant R41 TR003247 to Synergyan, LLC and KR) for partially supporting this study at Dr. Rege's lab. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the NSF.

* Correspondence to: K. Rege, Biological Design Graduate Program, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ 85287, USA.
** Correspondence to: O. Berk Usta, Center for Engineering in Medicine and Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA.
E-mail addresses: rege@asu.edu (K. Rege), ousta@mgh.harvard.edu (O.B. Usta).

https://doi.org/10.1016/j.nano.2023.102651
1549-9634/

simultaneously load multiple drugs, and synergistically deliver nucleic acids and small molecule drug. It can also facilitate high efficacy of transgene delivery and expression to cells in vitro and by modulating reticuloendothelial system (RES) uptake *in vivo*[19–21]. Consequently over the past decade, LPNs have emerged as a platform technology for improving the bioavailability of challenging bioactive or therapeutic molecules with fewer side effects[20,21].

Although promising as therapeutic agents, nanocarriers have the potential to trigger cytotoxicity, immunogenic responses, and other adverse effects when administered to patients. This might arise due to either the functional groups they present on their surface, or the possibility of residual organic solvents commonly used in preparation of the formulation[20,22]. Therefore, in vitro *and* in vivo preclinical toxicity assessments of drug carriers are vital for determining the effective dose range and for demonstrating the safe application of nanoscale carriers of therapeutic cargo. Traditionally, animal models are widely used for the evaluation of toxicity of drug carriers in vivo[23,24]. A recent example using a rat model was used to demonstrate high efficacy of novel drug loaded lipopolymers for breast cancer[23]. In another study, nucleic acid-loaded lipopolymers were tested for antitumoral effects on chronic myeloid leukemia in a mouse model to investigate potential use in gene delivery[24]. Although *in vivo* models are important for capturing biodistribution and immunological effects, the cost associated with these studies makes them relatively impractical for evaluating the toxicity of large libraries of drug carriers. In contrast, in vitro cell culture systems that can provide rapid, robust, and reliable evaluation of drug carrier libraries, can be an invaluable part of the drug discovery and delivery pipeline. Many in vivo studies involving systemic (e.g., intravenous) delivery show that nanocarriers often localize to major organs such as the kidney, lung, and liver upon systemic or local delivery. Amongst these, the liver plays a key role in the elimination of xenobiotics from the body and protection against toxicity from exogenous chemicals[25]. The liver is also a major target for injury from potential insults caused by exogenous nanocarriers. Therefore, evaluation of hepatotoxicity becomes a key factor in determining the clinical translation of nanomaterial-based drug carriers.

Despite the critical need to determine the potential hepatotoxicity of nanoparticle drug carriers, only a few, mostly cell-line focused efforts in vitro, have been explored for this purpose. Previous libraries of drug carriers were assessed for cytotoxicity in different hepatic (HepG2[26], SMMC-7721[27], Huh7[28]) and other tissue culture models[29]. However, many of the established hepatic cell lines are based on hepatic cancers and do not reflect the phenotypic and functional characteristics of liver tissue. Therefore, primary hepatocyte culture models have emerged as the benchmark for in vitro testing of hepatoxicity as they can maintain relevant functionality during a 1–3 day period used in hepatotoxicity studies[30,31]. Additionally, they can be utilized for the study of enzyme induction or inhibition and are effective for screening interindividual differences in metabolism[32]. Furthermore, to retain polygonal morphology similar to that seen in vivo, sandwich culture models have been established by placing the hepatocytes between two layers of collagen[33,34]. Recently, Yang et al. found that drugs encapsulated in nanoparticle carriers demonstrated lower toxicities in primary compared to the free, unencapsulated version of the drug[35]. Depletion of macrophages in vivo resulted in a significant increase of nanocarrier-induced hepatotoxicity, which implicated macrophage uptake as one of the determining biological factors for reducing drug carrier hepatotoxicity. Despite these studies, physicochemical and biological factors that contribute to the hepatotoxicity of nanoparticle-based drug carriers are not yet well understood. Therefore, there is an urgent need for establishing physiologically relevant and effective cell culture models for the systematic determination of drug carrier cytotoxicity, particularly for evaluating nanoparticle libraries. Considering that systemic delivery of nanoparticle therapeutic agents leads to their accumulation in the liver[5,36], we reasoned that the development of a primary hepatocyte-based cell culture model for rapid screening of lipopolymer nanoparticles, used as model drug carriers, can lead to establishment of a relevant cell culture model for accelerating the drug carrier discovery pipeline.

We recently developed a new class of aminoglycoside-derived polymers, which were subsequently derivatized with acyl chlorides resulting in the formation of lipopolymer nanoparticle (LPN) libraries[1,37–43]. The parental polymers and derivatized LPNs demonstrated a diverse range of physicochemical properties, including hydrodynamic size, surface charge, and hydrophobicity. Previous studies on the parental and lipopolymer libraries, using in vitro screens or local delivery in vivo, led to the identification of ten candidates that demonstrate promise for the delivery of nucleic acids, small molecules, and/or simultaneous delivery of nucleic acids and small molecule drugs[39,41].

Here, we report an investigation for the determination of drug carrier hepatotoxicity using the parental aminoglycoside-derived polymers and the derivatized lipopolymers as candidate vehicles. Primary rat hepatocyte cultures in 96 well plates were first used to determine the range of hepatotoxicity via imaging. This was followed by detailed studies on IC50 values of each LPN via albumin and urea secretion using 12-well plates. These studies demonstrate that primary cultures are useful for determining the effect of drug carriers on hepatocyte function and are therefore indicators of potential toxicity, which can have a significant role in accelerating the discovery of drug carriers that emerge from discovery pipelines. The use of primary hepatocyte models facilitates a more realistic evaluation of drug carrier hepatotoxicity compared to hepatic cell-line based studies and can be extended as a general model for rapidly determining the hepatotoxicity of a diverse set of drug carriers.

Material and methods

Materials

Paromomycin sulfate, neomycin sulfate, and 3.5 kDa molecular weight cut-off (MWCO) dialysis membranes were purchased from Fisher Scientific. Apramycin sulfate, resorcinol diglycidyl ether (RDE), glycerol diglycidyl ether (GDE), butyryl (C_4), hexanoyl (C_6), octanoyl (C_8), myristoyl (C_{14}) or stearoyl chlorides (C_{18}), triethylamine, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) were purchased from Sigma-

Aldrich. All other chemicals were purchased from Sigma-Aldrich and used without further purification.

Synthesis of aminoglycoside-derived parental polymers

Aminoglycoside-derived polymers were generated following the methods described in our previous studies [39, 46, 48]. The parental polymers, neomycin-RDE (NR), paromomycin-RDE (PR), apramycin-RDE (AR), neomycin-GDE (NG), and paromomycin-GDE (PG) were synthesized by a crosslinking reaction between aminoglycoside monomers and corresponding diglycidyl ethers (see furthered in supplementary information).

Synthesis of aminoglycoside-based lipopolymer nanoparticles (LPNs)

A set of 6 lipid-conjugated nanoparticles was synthesized using the reaction schematics shown in Fig. 1A and the chemical

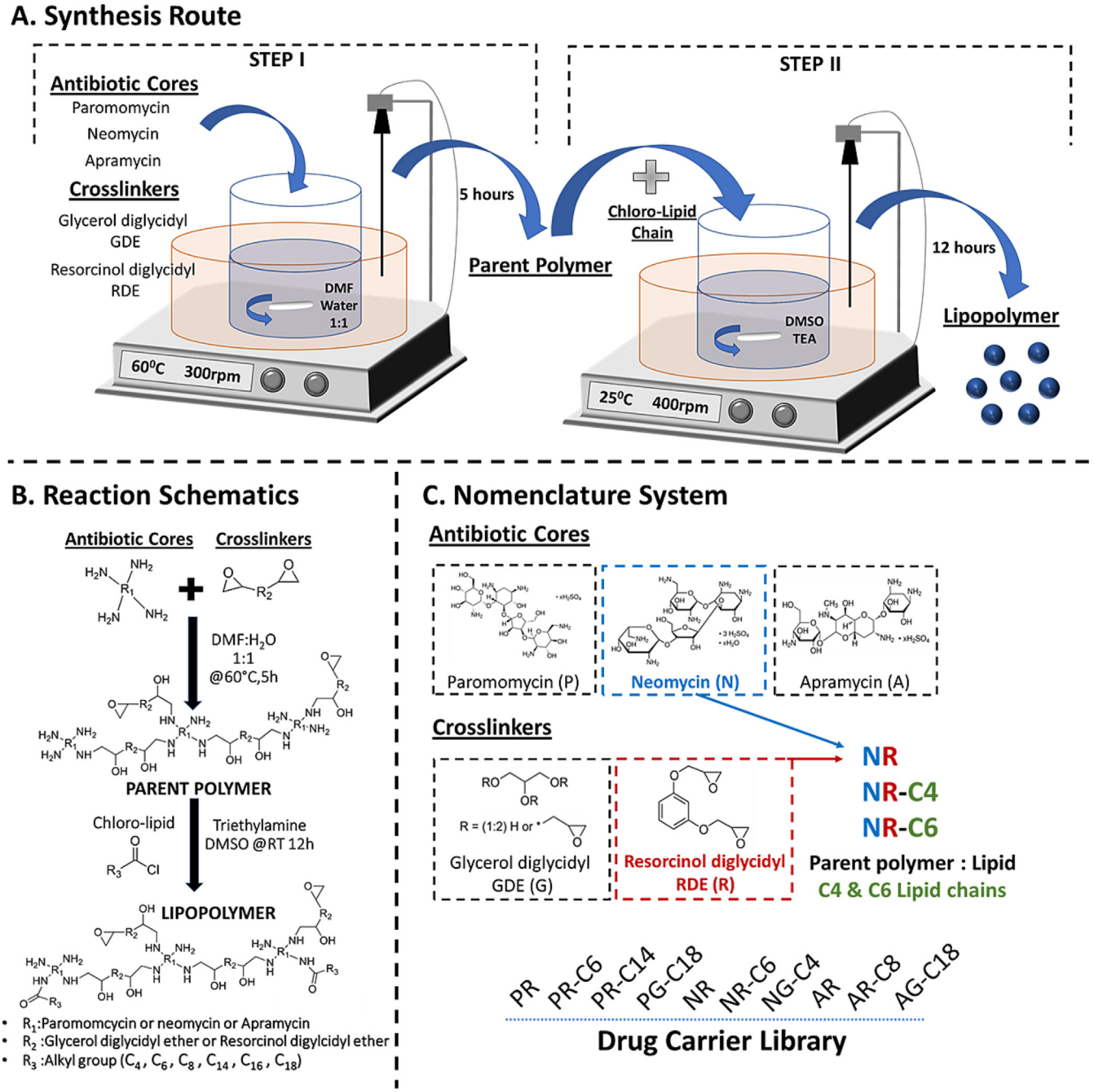

Fig. 1. Aminoglycoside-derived parental polymers and lipopolymer: **A**. Schematic illustrations showing synthesis steps and conditions, **B**. Chemical route of synthesis technique, **C.** Nomenclature system of drug carrier library used for the toxicity tests.

synthesis route in Fig. 1B. Aminoglycoside-derived parental polymers NR, PR, AR, NG, or PG were derivatized with different alkanoyl chlorides of varying chain length of lipids, including butyryl (C_4) chloride, hexanoyl (C_6) chloride, octanoyl (C_8) chloride, myristoyl (C_{14}) chloride or stearoyl (C_{18}) chloride (explained in supplementary information). The standard nomenclature used for all synthesized LPNs is shown in Fig. 1C.

Characterization of drug carrier library

Hydrodynamic diameter and zeta potential measurements of drug carrier nanoparticles

Hydrodynamic diameters and zeta potential values of all LPNs and parental polymers (NR, PR, and AR) in DMEM cell culture buffer (pH 7.5) were determined using a Nano-ZS Zetasizer instrument (Malvern Instruments with SOP values of refractive index 1.59 and adsorption parameter 0.01). Lyophilized polymers (at a concentration of 5 mg/mL) were dissolved in DMEM cell culture media (pH 7.5) with gentle shaking overnight at 4 °C and filtered through a 0.2-micron syringe filter. This filtered dispersion was used to determine the hydrodynamic diameter and zeta potential of the LPNs using dynamic light scattering (DLS).

Transmission electron microscopy (TEM) of drug-carrier nanoparticle

Aqueous dispersions of LPNs (1 mg/mL in Milli-Q water) were placed dropwise onto a TEM grid and, after that, negatively stained using uranyl formate (by incubating for 1 min with a sterile-filtered 0.75 % (w/v) solution prepared in Milli-Q water) and visualized using a Philips CM12-TEM equipped with a Gatan model-791 CCD camera. The diameter analysis of particles was calculated using ImageJ software by counting 40 distinct particles in two independent images.

Determination of drug carrier hydrophobicity

Drug carrier hydrophobicity was determined by calculating the octanol/water partitioning coefficient using previously reported methods[44] with minor modifications described in the supplementary information.

Methods for primary rat hepatocyte culture models

Cell isolation: Primary rat hepatocytes were isolated from 10 to 12 weeks old adult female Lewis rats (Charles River Laboratories, USA) weighing 180–200 g as previously described[45]. The Cell Resource Core (CRC) performed the isolation protocol #2011N000111 approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (MGH) and provided primary hepatocytes with 90–95 % viability (determined by Cellometer K2, Nexcelom, USA).

Cell culture: Freshly isolated primary rat hepatocytes were cultured in 12 and 96 wells with and without a top gel to assess the suitability of these methods for determining drug carrier hepatotoxicity. Cells were cultured in a medium that contains Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, CA, USA) as the base, which is then supplemented with 10 % fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 2 % penicillin-streptomycin, 7.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), and 14 ng/mL glucagon.

The 96-well plate monolayer culture was prepared as follows. A 96-well (96WP) cell-culture-treated plate (Thermo Fisher Scientific, USA) was coated with type I rat tail collagen. Specifically, we used 100 μL dilute rat tail collagen solution (1.25 mg/mL collagen solution diluted 1:24 v/v in 1XPBS) for each well and incubated at 37 °C in 5 % CO_2 for 1 h. Thereafter, freshly isolated primary rat hepatocytes (5×10^5 cells/well in 50 μL culture medium) were seeded in each well in their regular culture medium. The cells were incubated at 37 °C in 5 % CO_2 for 24 h for stabilization. Different concentrations of drug carrier nanoparticles were then introduced to the cells (Fig. S1A, Supplementary Information). This approach was used to assess the preliminary toxic concentration range of the drug carrier library.

The 12-well monolayer (no-top-gel) culture was prepared as follows. The primary rat hepatocytes (6×10^5 cells/well in 500 μL culture medium) were seeded into 12-well tissue culture plates (12WP) in which each well was precoated with 1 mL of a dilute rat tail collagen type I (as described above). They were then incubated at 37 °C in 5 % CO_2 for 24 h for stabilization and confluency. The media was collected, and a fresh medium with varying concentrations of nanoparticles was introduced to cells. The medium was collected every 24 h for further analysis and replaced with fresh media (Fig. 2).

The 12-well sandwich (top-gel) culture builds on the monolayer model described above and was prepared as follows. After the cultures were stabilized for 24 h in monolayers, a top collagen-based hydrogel (collagen gel) was applied in each well to cover the cells[45,46]. This approach enables hepatocytes to polarize and retain hepatocyte-specific functions for longer-term culture. Specifically, a 200 μL top-gel solution (1.25 mg/mL collagen diluted 9:1 v/v in 10× DMEM) was added onto cells and incubated at 37 °C in 5 % CO_2 for 1 h. Thereafter 500 μL culture medium was added to each well and cells were incubated at 37 °C in 5 % CO_2 for 24 h to stabilize the culture. After 24 h, the culture medium was collected, and different concentrations of drug carrier nanoparticles were added to the cells in culture. The medium was collected every 24 h for further analysis and replaced with a fresh medium (Fig. S2A).

Methods for hepatoxicity assessment

Cell viability analysis by live/dead assay

The LIVE/DEAD™ viability/cytotoxicity kit for mammalian cells (Thermo Fisher Scientific, USA) was used to determine the viability of the cultured cells. The cell viability analysis is described in the supplementary information.

Hepatotoxicity analysis via functionality assays

Hepatotoxicity was also assessed by measuring albumin and urea secretion from hepatocytes in culture. The secreted albumin and urea concentrations following nanoparticle treatments were analyzed using samples, collected daily, from three independent primary rat hepatocyte isolation cultures (Fig. 2). The daily collected samples from 12WP cultures were stored at −80 °C until albumin and urea analysis.

Albumin assay

The albumin concentration was measured using an in-house developed enzyme-linked immunosorbent assay (ELISA) method as described earlier[46,47] in the supplementary information.

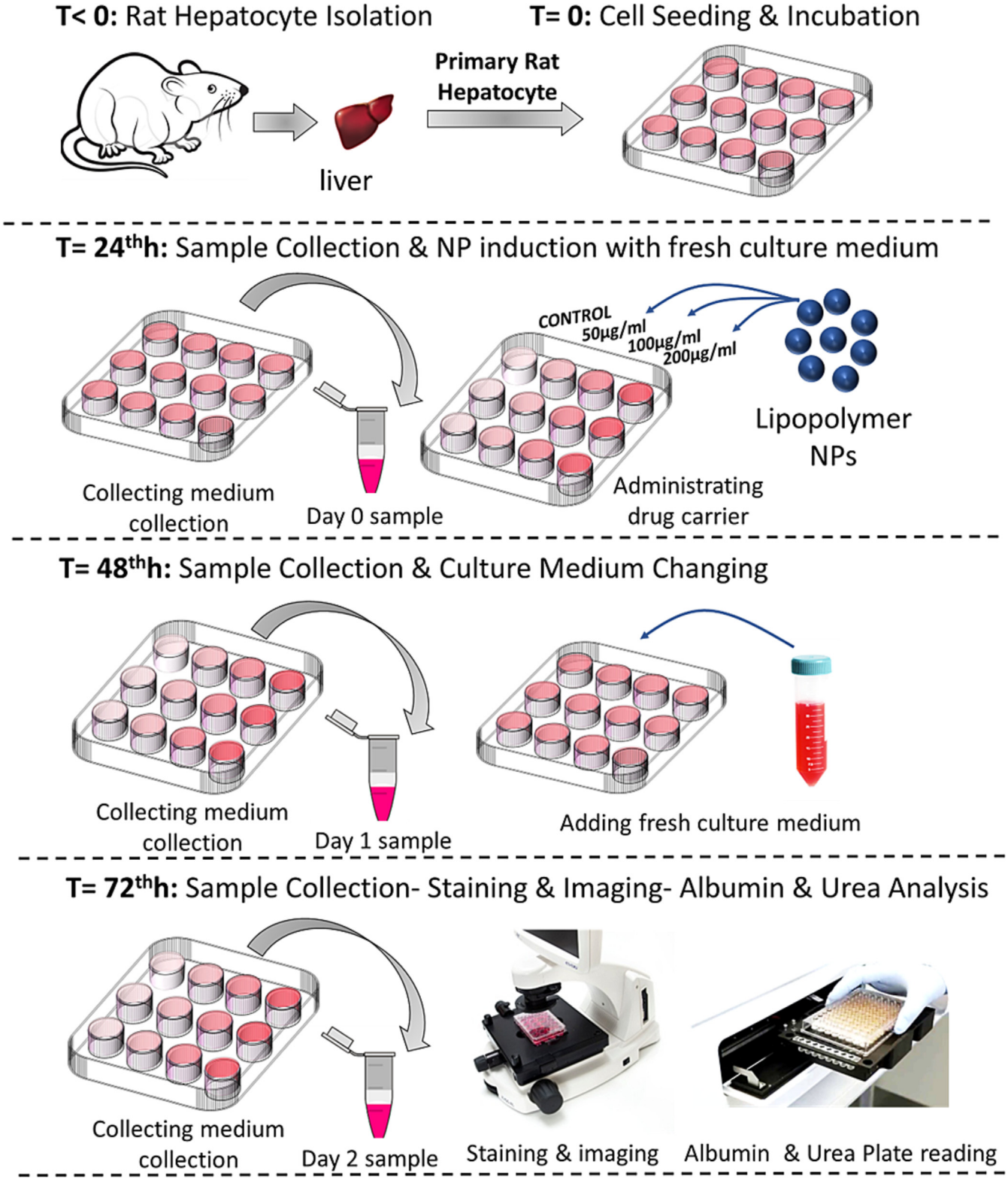

Fig. 2. Schematic of the experimental protocol via no-top-gel 12-well plate (12WP) culture. Each drug carrier nanoparticle was tested using 3 independent primary rat hepatocyte isolations.

Urea assay

The urea concentration was measured using a colorimetric urea detection kit (Stanbio Urea BUN assay kit, TX, USA). Ten microliters of the collected samples and assay standards (0–100 μg/mL urea standards in culture medium) were added into clear bottom 96 well-plate following the manufacturer's instructions. Then, 150 μL of the BUN kit reagent mix was added into each well and incubated at 60 °C for 90 min. After incubation, the plate was kept at room temperature to cool down for 10 min. The absorbance of each well in the plate was measured simultaneously at 520 nm and 650 nm using a Benchmark Plus microplate reader (Biorad Inc., CA, USA). Albumin concentrations in collected samples were calculated according to a constructed standard curve for each plate.

Determination of half-maximal Inhibition Concentration (IC_{50}) of drug carrier nanoparticles

The IC_{50} values of the LNP library were calculated based on albumin and urea concentrations of the control group from 3 independent culture data for each nanoparticle candidate in the library. The calculation method was described in the supplementary information.

Statistical analyses

The results of albumin and urea assays were evaluated for statistically significant differences between the means of the groups. Two-way analysis of variance (ANOVA) with Bonferroni test was performed using OriginPro 2018. ANOVA provides a pairwise comparison of the means[48]. Each candidate in the library was analyzed using 3 wells of cultured cells from 3 independent primary rat hepatocyte isolations (N = 3, n = 3). A threshold for significance, $p < 0.05$ was used for all statistical analyses.

Results and discussion

Generation of aminoglycoside-derived parental polymers and lipopolymer nanoparticles

Aminoglycoside-derived parental polymers, PR, NR, and AR were synthesized by a crosslinking reaction and purified using previous methods described by us[41,44] with an approximate yield ranging from 40 to 50 %. Lipopolymer nanoparticles were then synthesized by reacting primary amines present in parental polymers (PR, NR, AR, PG, or NG) with different lipids (in acid chloride form) with varying chain lengths including C_4, C_6, C_8, C_{14} and C_{18} chlorides (Fig. 1B). Different alkanoyl groups of varying chain lengths were conjugated in order to systematically modulate the self-assembling property and hydrophobicity of the resulting lipopolymers. A total of 6 different LPNs were synthesized and purified with an average yield ranging from 35 to 55 % (calculation of % yields are followed as described in previous literature[41,49]).

Physicochemical characterization of drug carrier nanoparticle library

Parental polymers and lipid-conjugated polymers (lipopolymers) self-assemble to form nanoparticles in aqueous media (DMEM culture media with pH ~7.5). Dynamic light scattering measurements revealed that the parental polymer (NR, AR, and PR) nanoparticles – incubated in DMEM culture media (pH ~7.5) for 24 h – had hydrodynamic diameters ranging from 120 to 140 nm and surface zeta potential values ranging from +15 to +30 mV (Fig. 3A). All lipid-conjugated derivatives of parental polymers, i.e., NRC_6, PRC_6, PRC_{14}, ARC_8, NGC_4, PGC_{18} lipopolymers, also self-assembled to form nanoparticles with hydrodynamic diameters broadly ranging from 50 to 110 nm and surface zeta potential values ranging from +10 to +40 mV in the DMEM culture media (Fig. 3A). Also, the plot between zeta potential vs. total count of particles in Fig. 3B shows a single peak for the PRC_{14} polymer, which corresponds to a positive surface zeta potential value and therefore confirms the cationic nature of these drug carrier nanoparticles. For these parental polymers and lipopolymers, the cationic amine groups from aminoglycosides are likely exposed on the outer surface of the self-assembled nanoparticle and contribute to the positive zeta potential value.

Transmission electron microscopy (TEM) images of the negatively stained lipopolymer, PRC_{14}, confirmed the overall relatively uniform distribution of the nanoparticles (Fig. 3C); in this visualization, the particles appear light on a dark negatively-stained background. The diameter of PRC_{14} LPNs calculated from TEM microscopy (31.8 ± 4.3 nm) was smaller than the hydrodynamic diameter determined from the aqueous dispersions of the LPNs using dynamic light scattering (58.2 ± 3.1 nm) experiments, which is consistent with our previous observation[41]. It is likely that drying of the nanoparticle samples is partially responsible for the smaller diameters seen with TEM.

Polymer hydrophobicity values ($\log_{10}K_{(O/W)}$) were measured by calculating the logarithm of phase distribution coefficient ($K_{(O/W)}$), i.e., the ratio of the concentration of the drug carrier in the octanol phase to that in the aqueous (water) at equilibrium as described in Fig. 3D. Hydrophobicity, i.e., $\log_{10}K_{(O/W)}$ for parental polymers, was in the range of 1.0 to 1.3, which indicated moderately hydrophobic polymers (with a positive $\log_{10}K_{(O/W)}$ value). Upon conjugating the lipids onto the parental polymers, the hydrophobicity of the resulting lipopolymer nanoparticles increased to higher values (ranging from 1.4 to 1.7). It is anticipated that optimized hydrophobicity values of drug carriers can facilitate greater interactions with cells leading to increased delivery efficacies[49], although the impact on cytotoxicity is not fully understood.

Cytotoxicity testing of drug carrier library using primary rat hepatocyte culture models

a) Drug carrier hepatotoxicity analysis via imaging

We used primary cell cultures to evaluate the hepatotoxicity of aminoglycoside-derived lipopolymers, which were previously identified as promising drug carrier candidates[1,37,40,41]. We first used a rapid screening approach via 96-well cultures (Fig. S1; Supplementary Information) to establish the working concentration range of the nanoparticles for further detailed functional analyses. We then used two culture approaches, first with a top-gel and then a monolayer culture model (no-top-gel) in 12-well

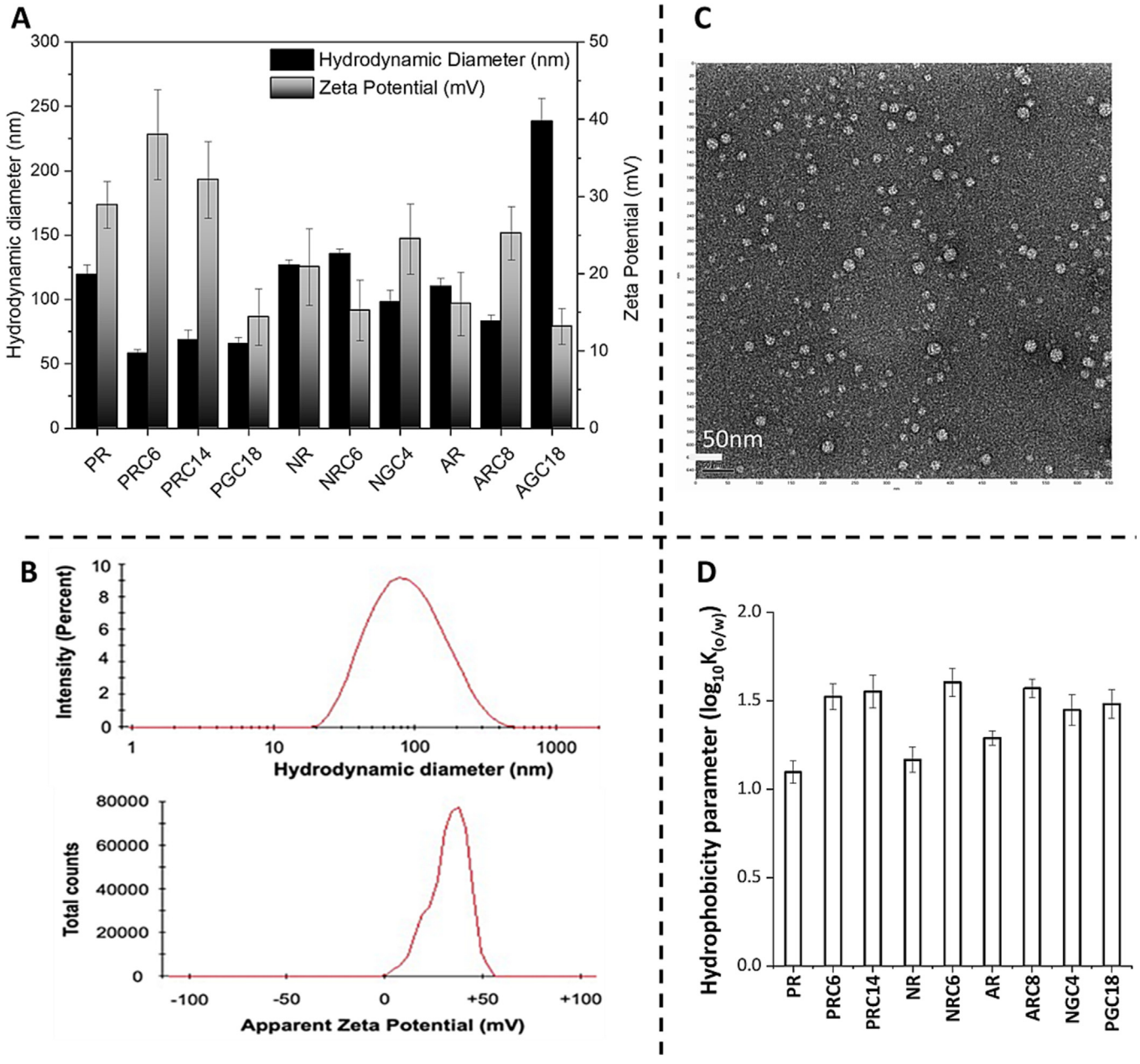

Fig. 3. Physicochemical characterization of aminoglycoside-derived drug carrier nanoparticles: **A**. Hydrodynamic diameter and the zeta potential values of drug carrier nanoparticles following incubation in DMEM media for 24 h at 37 °C, **B**. **(top)** Representative plot of hydrodynamic diameter (nm, logarithmic scale) with percent intensity on the y-axis and **(bottom)** representative plot of zeta potential vs. total counts for the PRC_{14} lipopolymer, **C.** Representative TEM image of PRC_6 lipopolymer nanoparticles, **D.** Hydrophobicity values of drug carrier library as determined using $\log_{10}K_{(O/W)}$. Additional details are provided in the supplementary information section.

plates to identify the most relevant approach to study the effects of the drug carrier library on primary rat hepatocyte viability and function.

Given the use of a library of nanoparticles with unknown toxicity ranges, we set out to determine the concentration ranges for testing the toxicity of the drug-carrier library (comprised of ten candidates). Briefly, we first screened a concentration range of 0–100 μg/mL for all particles with phase-contrast imaging (included in Fig. S1, Supplementary Information). These phase-contrast images did not indicate maximal toxicity, i.e. ~100 %, death, in this concentration range. We, thus, increased the concentration range to 200 μg/mL and conducted staining-based viability assays for this broader range (0–200 μg/mL, Fig. S1, Supplementary Information). These results indicated that we could observe significant toxicity for the hepatocytes at and above 100 μg/mL. Based on this screening, we used the 0 μg/mL to 200 μg/mL concentration range to measure IC_{50} toxicity values in the subsequent experiments we describe below.

We employed both top-gel (i.e., collagen sandwich) and no-top-gel approaches to assess their suitability to test the toxicity of

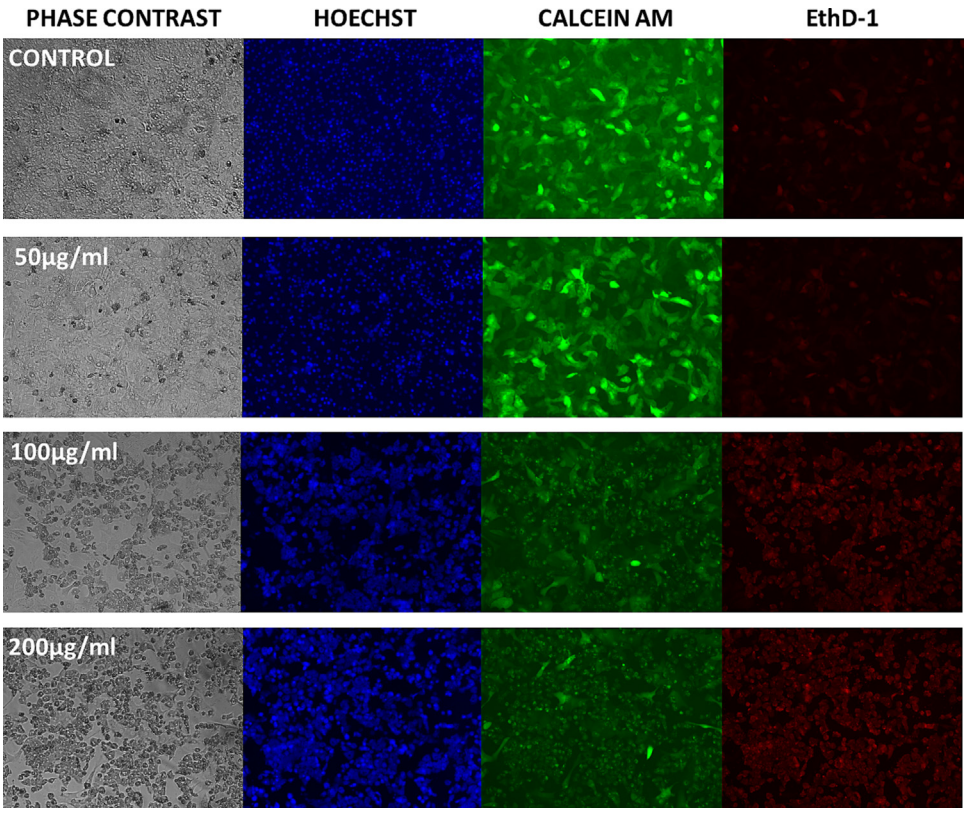

Fig. 4. Aminoglycoside-derived drug carrier nanoparticle toxicity in primary rat hepatocyte culture (no-top-gel, 12-well plate) for the representative nanoparticle (NR). Column 1: Phase contrast images at different doses. Column 2–4: Live & Dead staining images of cells at 72 h after collection of supernatant. Hoechst: Nuclear Stain (blue), Calcein-AM: Live stain (green), EthD-1: Nuclear Dead cell stain (red) at 10× magnification.

the drug carriers. Although the collagen sandwich model is generally considered to be superior for the long-term functionality of primary hepatocytes, the top collagen gel was a physical barrier to nanoparticle delivery in this study (Fig. S2; Supplementary Information) due to probable interaction of the carriers with collagen matrix. We thus carried out all subsequent toxicity assessments with the no-top gel hepatocyte culture where the total time for culture did not exceed 72 h. In this regard, if longer term cultures are needed to assess the chronic toxicity of LPN libraries, one might consider alternative approaches which do not limit the transport of LPNs into the cell. One such alternative is the ultrathin collagen coating (200 nm -1 µm thick) we had previously developed[50,51]. Nevertheless, this approach is labor intensive and requires automated liquid handlers for large number of experiments.

We observed significant effects of drug carrier exposure on primary rat hepatocytes in the no-top-gel model (representative polymer nanoparticle, NR shown in Fig. 4 and for the rest of the library from Figs. S4 to S13). Specifically, compared to the control group, increasing concentrations of LPN drug carriers led to highly deformed cell morphology and substantial deterioration in cell integrity. Fluorescence staining of the cells with Hoechst (nuclear stain; blue), Calcein-AM (live cytoplasmic stain; green), EthD-1 (dead cell, DNA binding stain; red) further confirmed some toxicity of the NR nanoparticles at concentrations above 50 µg/mL. Specifically, we observed substantially high staining in the red (EthD-1) channel, indicating a significant disruption of cell membrane integrity in cells exposed to the highest concentration of NR used (i.e., 100 and 200 µg/mL). We also observed a corresponding decrease in the green (Calcein-AM) channel, indicating a substantial disruption to the activity of intracellular esterases. Interestingly we also observed a more diffuse (not punctate) signal in the blue (Hoechst) channel in cells exposed to these highly toxic concentrations, which is a likely indicator of

Table 1
The half-maximal Inhibition Concentration (IC_{50}) of lipopolymer nanoparticles and parental aminoglycoside-derived polymers on primary rat hepatocytes. Values presented are mean ± standard error.

	IC_{50} (µg/mL)	
	Albumin	Urea
PR	102.7 ± 17.7	127.5 ± 9.1
PRC_6	98.0 ± 16.8	109.1 ± 2.6
PRC_{14}	81.8 ± 4.2	216.7 ± 23.8
PGC_{18}	154.7 ± 22.1	141.4 ± 0.8
NR	166.5 ± 8.3	125.3 ± 19.9
NRC_6	97.3 ± 29.8	150.0 ± 10.2
NGC_4	118.5 ± 7.9	136.2 ± 6.1
AR	99.0 ± 19.9	216.8 ± 22.2
ARC_8	104.6 ± 6.6	194.0 ± 23.2
AGC_{18}[a]	–	–

[a] This oversized particle did not show any toxicity in the concentration range evaluated.

nuclear injury as a result of treatment with the highest concentrations of the drug carrier nanoparticles.

b) Drug carrier hepatotoxicity analysis via functionality assays

The liver plays a vital role in eliminating xenobiotics, drugs or drug carriers from the body[52]. In any liver-like model, the secretion profiles of albumin and the urea are typical indicators of liver function. Albumin is a secreted plasma protein that facilitates the transport of hormones, enzymes, vitamins, fatty acids, and other essential substances, balances the pH, and maintains oncotic pressure[53]. Urea is synthesized by the liver as a result of the metabolism of nitrogen-containing compounds. Poor or impaired urea and albumin function can be used as indicators of hepatotoxicity[54]. Accordingly, to assess the hepatotoxicity of the drug carrier nanoparticle library in designed cell culture models, we monitored the change in albumin and urea secretion at different time points. The resulting IC_{50} values of drug carrier nanoparticles, based on urea and albumin assays, ranged from 81.8 μg/mL to 216.8 μg/mL (Table 1) as calculated from dose response curves shown in Fig. S3 (Supplementary Information).

Albumin concentrations for each drug carrier nanoparticle-treated hepatocyte culture group were measured via an ELISA assay for samples collected on the first and second days. The percent albumin secretion was calculated by normalizing each experimental group's secretion to that of the control group (untreated i.e. 0 μg/mL drug carrier) for each day as shown in Fig. 5-A. Statistical analysis showed significant changes in albumin secretion on day-1 for most of the nanoparticles which allowed us to calculate IC_{50} values from these day-1 results.

We observed statistically significant changes in the albumin secretion of drug carrier treated groups compared to the control (untreated) group for the following particles on day-1: PR, PRC_6, PRC_{14}, PGC_{18}, NR, NRC_6, NGC_4. The IC_{50} values for these particles were thus calculated from the day-1 viability results (Table 1). The administration of other nanoparticles, AR and $ARC_{8,}$ resulted in statistically significant albumin dose responses only on day-2. The administration of oversized (>200 nm) particle AGC_{18} did not result in any statistically significant changes

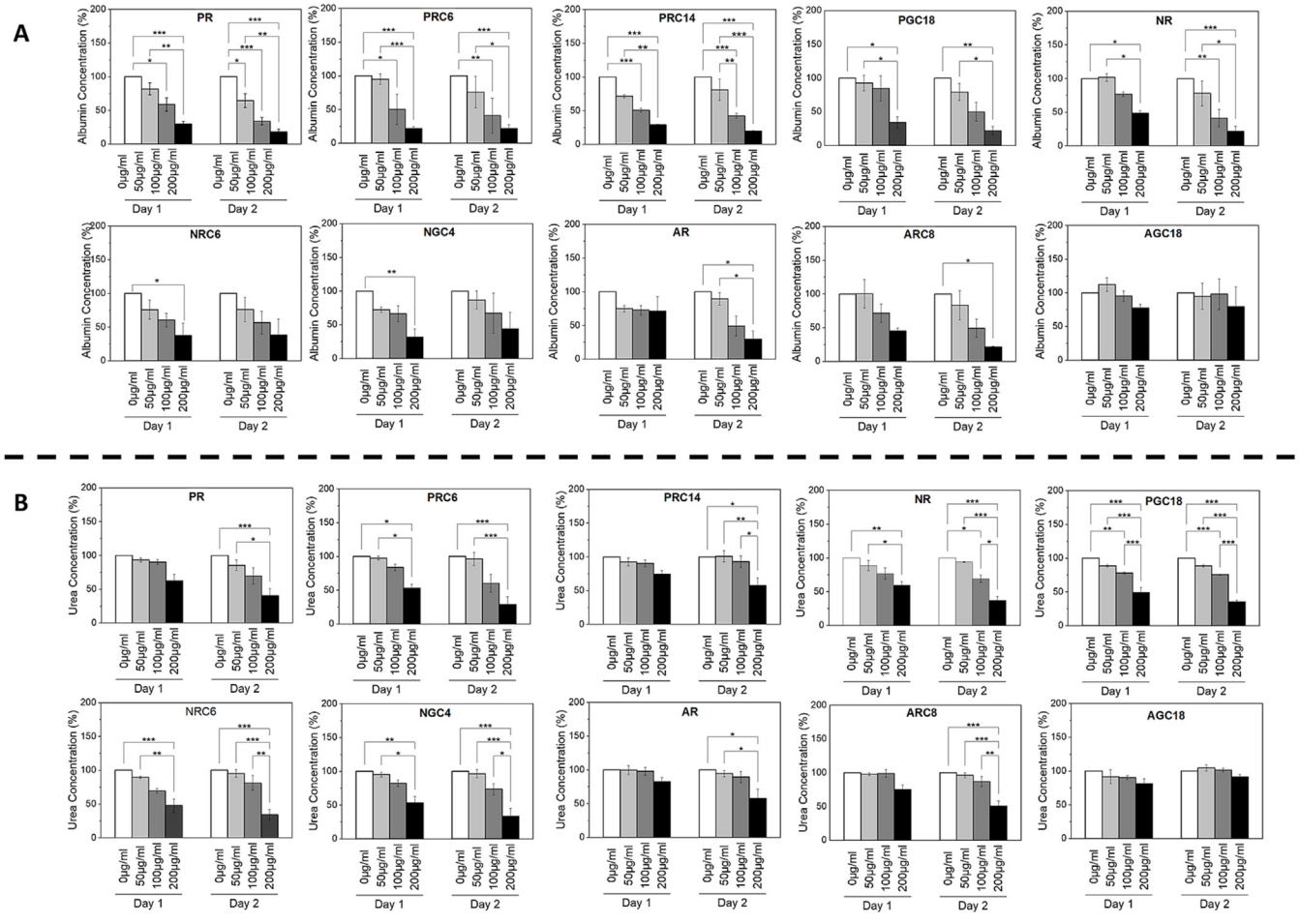

Fig. 5. Hepatocyte-specific functions i.e., secretion of albumin and urea following treatment with the drug carrier nanoparticle library: **A.** The percentage of albumin secretions normalized by control for 24 h (1st-day sample) and 48 h (2nd-day sample) after drug carrier administration **B.** The percentage of urea secretions normalized by control for 24 h and 48 h after drug-carrier administration. The values are the mean (% of albumin and urea concentration normalized with respect to the control group for each day of analysis) ± standard error from 3 independent cell culture tests with triplicates of each concentration group *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

during the experimental period. Accordingly, the IC_{50} values of AR and ARC_8 were calculated using the second-day data, and no IC_{50} value was calculated for AGC_{18}.

Nanoparticle size is an important determinant for hepatotoxicity. Accordingly in Fig. 6 we plot the IC_{50} values – derived from the albumin assay – and the hydrodynamic diameter of the LPNs. In general, we observed that increasing the diameter of the (LPNs) resulted in higher IC_{50} values, i.e., lower hepatotoxicity. While this trend was well established within the LPNs of the lipid-conjugated polymer family, two of the parental polymer LPNs did not follow this general trend. AGC_{18} demonstrated no toxicity at the concentrations evaluated (0–200 μg/mL; Table 1), likely due to its large diameter (>200 nm) that hinders significant interactions with cells[55,56]. We, therefore, hypothesize that hepatotoxicity of nanoparticle-based lipid-conjugated carriers depends on its hydrodynamic diameter. Nevertheless, particle size is not the only determinant of toxicity and chemistry and surface properties of such particles might also be important. Further research with a larger set of LPNs and chemistries will be critical in better understanding the effect of different particle parameters on the observed hepatotoxicity.

Secreted urea concentrations on the first- and second-day following treatment with the individual drug carriers are shown in Fig. 5-B. Statistical analyses showed that urea secretion results were statistically meaningful for calculating IC_{50} values for most of the nanoparticle library, except NR, NRC_6, NGC_4, and PGC_{18} only on day-2 compared to day-1 for albumin secretion. Like albumin, the statistical analysis of urea secretion did not indicate toxic effects for AGC_{18} (>200 nm) lipopolymer particle. In a previous study, Gokduman et.al, observed a similar delayed response for urea where they assessed the toxicity of iron oxide nanoparticles on primary rat hepatocyte cultures via both albumin and urea secretion[46]. They concluded that albumin secretion is both a more sensitive and earlier hepatotoxicity marker of the iron oxide nanoparticles. Overall, the current study indicates that the parental and lipopolymer nanoparticles used for delivery of

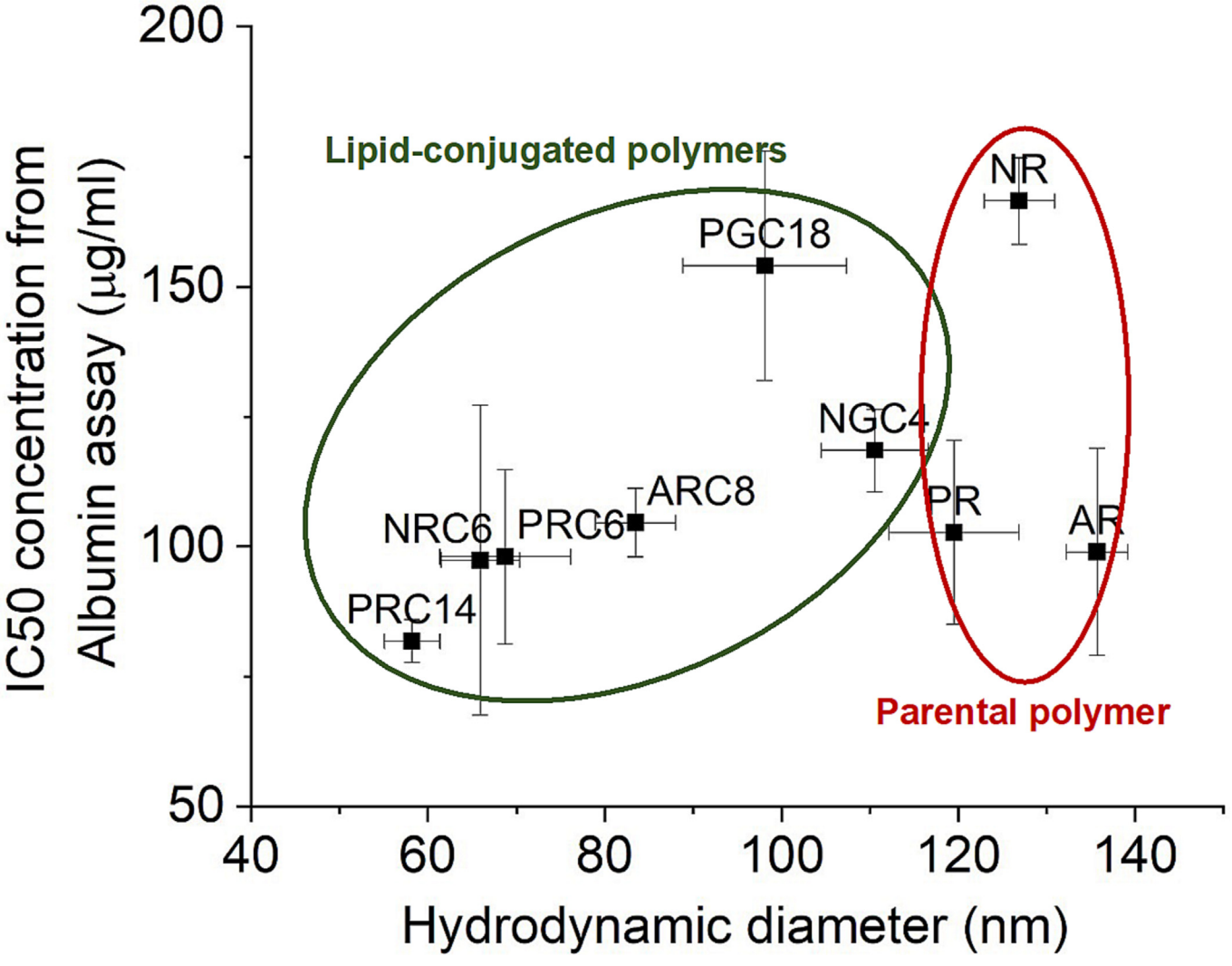

Fig. 6. Correlation diagram between the hydrodynamic diameter (nm) of drug carrier nanoparticles with corresponding IC_{50} concentration (μg/mL) evaluated from the albumin assay by assessing the toxicity with primary rat hepatocytes. In general, an increase in hydrodynamic diameter resulted in lower hepatotoxicity (higher IC_{50} value) for lipid-conjugated polymers or lipopolymer nanoparticles (LPNs; highlighted with green boundary).

small molecule drugs and nucleic acids have a relatively safe hepatoxicity profile and result in adverse events only at significantly high concentrations (≥~50 μg/mL).

Conclusions

The toxicity of nanoparticles can be a critical limitation to their use in biological applications and clinical translation as drug carriers. In this regard preclinical animal models are commonly used for efficacy and safety analyses before moving on to clinical testing in humans. Similarly, in vitro models of hepatotoxicity are commonly used before the animal studies and feature carcinoma-based cell lines, iPSC derived human hepatic cells and primary hepatic cells both in simple to use well culture models and the more novel 3D approaches[57,58]. While the choice of cell sources and tissue models are plentiful, here we chose rat primary hepatocytes in a 2D culture model due to their high metabolic recapitulation of the original liver tissues, ease of use, accessibility, affordability and potential predictivity of the preclinical animal studies that are commonly first performed in rodents. An extension to primary human and human iPSC derived hepatic cells will be considered in future work.

In this study, we investigated the suitability of different primary hepatocyte cultures for evaluating the cytotoxicity of recently developed aminoglycoside-derived polymer and lipopolymer nanoparticles (LPN), which were used as models of drug carriers[41]. These drug carrier nanoparticles are promising as carriers of nucleic acids and/or small molecules, but no hepatotoxicity data on these emerging carriers exist in the current literature. We, therefore, investigated different primary hepatocyte culture models to determine the hepatotoxicity of these drug carrier nanoparticles.

Our results indicate that hepatoxicity screening in a 96WP format is a useful approach to rapidly determine the working concentration range for novel nanoparticles under development as drug carriers. The study revealed that drug carrier nanoparticles were non-toxic to primary rat hepatocytes up to a <50 μg/mL concentration. These indicate that the LPN library is generally safe and well-tolerated, however, further testing in animals and that of the complete library will be needed to better establish the usefulness of this library for safe and efficacious delivery of therapeutic cargo. Albumin, as a hepatic biomarker, was a more sensitive and earlier indicator of toxicity, while urea excretion had delayed response as we have previously observed for iron-oxide nanoparticle hepatotoxicity. Taken together, the use of primary rat hepatocyte cultures is a robust preclinical approach for determining the hepatotoxicity of drug carriers before preclinical animal and clinical human studies.

Our future studies will involve an extension of these findings to larger libraries of drug carrier nanoparticles (with and without relevant cargo), leading to the development of structure-toxicity relationships, mechanisms of action studies – such as mitochondrial and other organelle injury and injury via the generation of reactive oxygen species – and validation of screening findings in vivo, leading to a robust pipeline for the accelerated discovery of drug carrier nanoparticles. In this context, it is likely that nano-antioxidants may provide benefit in lowering the toxicity of nanoparticle drug carriers by lowering free radical generation[59,60]. It is conceivable that these lipopolymer nanoparticles can simultaneously carry antioxidants that can lower their potential toxicity.

CRediT authorship contribution statement

Güneş Kibar: Conceptualization, Investigation, Methodology, Validation, Visualization, Formal analysis, Writing – original draft. **Subhadeep Dutta:** Conceptualization, Investigation, Methodology, Validation, Visualization, Formal analysis, Writing – review & editing. **Kaushal Rege:** Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing, Project administration. **O. Berk Usta:** Conceptualization, Resources, Supervision, Formal analysis, Funding acquisition, Writing – review & editing, Project administration.

Declaration of competing interest

Prof. Kaushal Rege is affiliated with Synergyan, LLC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2023.102651.

References

1. Goklany S, Lu P, Godeshala S, Hall A, Garrett-Mayer E, Voelkel-Johnson C, et al. Delivery of TRAIL-expressing plasmid DNA to cancer cells in vitro and in vivo using aminoglycoside-derived polymers. *J Mater Chem B* 2019;**7**:7014-25.
2. Zhao Y, Lee RJ, Liu L, Dong S, Zhang J, Zhang Y, et al. Multifunctional drug carrier based on PEI derivatives loaded with small interfering RNA for therapy of liver cancer. *Int J Pharm* 2019;**564**:214-24.
3. Yang J, Arya S, Lung P, Lin Q, Huang J, Li Q. Hybrid nanovaccine for the co-delivery of the mRNA antigen and adjuvant. *Nanoscale* 2019;**11**: 21782-9.
4. Tan X, Kim G, Lee D, Oh J, Kim M, Piao C, et al. A curcumin-loaded polymeric micelle as a carrier of a microRNA-21 antisense-oligonucleotide for enhanced anti-tumor effects in a glioblastoma animal model. *Biomater Sci* 2018;**6**:407-17.
5. Liang C, Li F, Wang L, Zhang Z-K, Wang C, He B, et al. Tumor cell-targeted delivery of CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA in osteosarcoma. *Biomaterials* 2017;**147**:68-85.
6. Bose RJ, Ravikumar R, Karuppagounder V, Bennet D, Rangasamy S, Thandavarayan RA. Lipid–polymer hybrid nanoparticle-mediated therapeutics delivery: advances and challenges. *Drug Discov Today* 2017; **22**:1258-65.
7. Yin Y, Hu B, Yuan X, Cai L, Gao H, Yang Q. Nanogel: a versatile nano-delivery system for biomedical applications. *Pharmaceutics* 2020;**12**:290.
8. Chyzy A, Tomczykowa M, Plonska-Brzezinska ME. Hydrogels as potential nano-, micro-and macro-scale systems for controlled drug delivery. *Materials* 2020;**13**:188.
9. Agrawal RD, Tatode AA, Rarokar NR, Umekar MJ. Polymeric micelle as a nanocarrier for delivery of therapeutic agents: a comprehensive review. *J Drug Deliv Ther* 2020;**10**:191-5.
10. Mahajan S, Patharkar A, Kuche K, Maheshwari R, Deb PK, Kalia K, et al. Functionalized carbon nanotubes as emerging delivery system for the treatment of cancer. *Int J Pharm* 2018;**548**:540-58.
11. Kazemzadeh H, Mozafari M. Fullerene-based delivery systems. *Drug Discov Today* 2019;**24**:898-905.

12. Amin MU, Ali S, Ali MY, Tariq I, Nasrullah U, Pinnapreddy SR, et al. Enhanced efficacy and drug delivery with lipid coated mesoporous silica nanoparticles in cancer therapy. *Eur J Pharm Biopharm* 2021;**165**:31-40.
13. Baeza A, Ruiz-Molina D, Vallet-Regí M. Recent advances in porous nanoparticles for drug delivery in antitumoral applications: inorganic nanoparticles and nanoscale metal-organic frameworks. *Expert Opin Drug Deliv* 2017;**14**:783-96.
14. Antimisiaris S, Marazioti A, Kannavou M, Natsaridis E, Gkartziou F, Kogkos G, et al. Overcoming barriers by local drug delivery with liposomes. *Adv Drug Del Rev* 2021;**174**:53-86.
15. Mangalgiri A, Shaikh A, Matole V, Ingale S. A brief review on niosome drug delivery system. *Research Journal of Pharmaceutical Dosage Forms and Technology* 2021;**13**:23-4.
16. Zacheo A, Bizzarro L, Blasi L, Piccirillo C, Cardone A, Gigli G, et al. Lipid-based nanovesicles for simultaneous intracellular delivery of hydrophobic, hydrophilic, and amphiphilic species. *Front Bioeng Biotechnol* 2020;**8**:690.
17. Yaghmur A, Mu H. Recent advances in drug delivery applications of cubosomes, hexosomes, and solid lipid nanoparticles. *Acta Pharm Sin B* 2021;**11**:871-85.
18. Wang H, Liu S, Jia L, Chu F, Zhou Y, He Z, et al. Nanostructured lipid carriers for MicroRNA delivery in tumor gene therapy. *Cancer Cell Int* 2018;**18**:1-6.
19. Trivedi S, Wadher K, Umekar M. Development of topical thymoquinone loaded polymer–lipid hybrid vesicular gel: in-vitro and ex-vivo evaluation. *J Liposome Res* 2021;**1–13**.
20. Persano F, Gigli G, Leporatti S. Lipid-polymer hybrid nanoparticles in cancer therapy: current overview and future directions. *Nano Express* 2021;**2**012006, https://doi.org/10.1088/2632-959X/abeb4b.
21. Surve DH, Jindal AB. Development of cationic isometamidium chloride loaded long-acting lipid nanoformulation: optimization, cellular uptake, pharmacokinetics, biodistribution, and immunohistochemical evaluation. *Eur J Pharm Sci* 2021;**167**106024.
22. Zainal-Abidin MH, Hayyan M, Ngoh GC, Wong WF. Doxorubicin loading on functional graphene as a promising nanocarrier using ternary deep eutectic solvent systems. *ACS omega* 2020;**5**:1656-68.
23. Yalcin TE, Ilbasmis-Tamer S, Takka S. Antitumor activity of gemcitabine hydrochloride loaded lipid polymer hybrid nanoparticles (LPHNs): in vitro and in vivo. *Int J Pharm* 2020;**580**119246.
24. Valencia-Serna J, Aliabadi HM, Manfrin A, Mohseni M, Jiang X, Uludag H. siRNA/lipopolymer nanoparticles to arrest growth of chronic myeloid leukemia cells in vitro and in vivo. *Eur J Pharm Biopharm* 2018;**130**:66-70.
25. Hossen S, Hossain MK, Basher MK, Mia M, Rahman M, Uddin MJ. Smart nanocarrier-based drug delivery systems for cancer therapy and toxicity studies: a review. *J Adv Res* 2019;**15**:1-18.
26. Monajati M, Tavakoli S, Abolmaali SS, Yousefi G, Tamaddon A. Effect of PEGylation on assembly morphology and cellular uptake of poly ethyleneimine-cholesterol conjugates for delivery of sorafenib tosylate in hepatocellular carcinoma. *BioImpacts* 2018;**8**:241.
27. Tang S, Li Y. Sorafenib-loaded ligand-functionalized polymer-lipid hybrid nanoparticles for enhanced therapeutic effect against liver cancer. *J Nanosci Nanotechnol* 2019;**19**:6866-71.
28. Wang J, Meng F, Kim B-K, Ke X, Yeo Y. In-vitro and in-vivo difference in gene delivery by lithocholic acid-polyethyleneimine conjugate. *Biomaterials* 2019;**217**119296.
29. Guguen-Guillouzo C, Corlu A, Guillouzo A. Stem cell-derived hepatocytes and their use in toxicology. *Toxicology* 2010;**270**:3-9.
30. LeCluyse EL, Bullock PL, Parkinson A. Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat hepatocytes. *Adv Drug Deliv Rev* 1996;**22**:133-86.
31. Ahmadian E, Babaei H, Mohajjel Nayebi A, Eftekhari A, Eghbal MA. Mechanistic approach for toxic effects of bupropion in primary rat hepatocytes. *Drug Res (Stuttg)* 2017;**67**:217-22.
32. Sivaraman A, Leach JK, Townsend S, Iida T, Hogan BJ, Stolz DB, et al. A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab* 2005;**6**:569-91.
33. Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J* 1989;**3**:174-7.
34. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol Prog* 1991;**7**:237-45.
35. Yang F, Medik Y, Li L, Tian X, Fu D, Brouwer KLR, et al. Nanoparticle drug delivery can reduce the hepatotoxicity of therapeutic cargo. *Small* 2020;**16**:1906360.
36. Samuelsson E, Shen H, Blanco E, Ferrari M, Wolfram J. Contribution of kupffer cells to liposome accumulation in the liver. *Colloids Surf B Biointerfaces* 2017;**158**:356-62.
37. Potta T, Zhen Z, Grandhi TSP, Christensen MD, Ramos J, Breneman CM, et al. Discovery of antibiotics-derived polymers for gene delivery using combinatorial synthesis and cheminformatics modeling. *Biomaterials* 2014;**35**:1977-88.
38. Gosnell H, Kasman LM, Potta T, Vu L, Garrett-Mayer E, Rege K, et al. Polymer-enhanced delivery increases adenoviral gene expression in an orthotopic model of bladder cancer. *J Control Release* 2014;**176**:35-43.
39. Miryala B, Zhen Z, Potta T, Breneman CM, Rege K. Parallel synthesis and quantitative structure–activity relationship (QSAR) modeling of aminoglycoside-derived lipopolymers for transgene expression. *ACS Biomater Sci Eng* 2015;**1**:656-68.
40. Miryala B, Godeshala S, Grandhi TSP, Christensen MD, Tian Y, Rege K. Aminoglycoside-derived amphiphilic nanoparticles for molecular delivery. *Colloids Surf B Biointerfaces* 2016;**146**:924-37.
41. Godeshala S, Miryala B, Dutta S, Christensen MD, Nandi P, Chiu P-L, et al. A library of aminoglycoside-derived lipopolymer nanoparticles for delivery of small molecules and nucleic acids. *J Mater Chem B* 2020;**8**:8558-72.
42. Zhen Z, Potta T, Lanzillo AN, Rege K, Breneman M. Development of a web-enabled SVR-based machine learning platform and its application on modeling transgene expression activity of aminoglycoside-derived polycations. *Comb Chem High Throughput Screen* 2017;**20**:41-55.
43. Christensen MD, Nitiyanandan R, Meraji S, Daer R, Godeshala S, Goklany S, et al. An inhibitor screen identifies histone-modifying enzymes as mediators of polymer-mediated transgene expression from plasmid DNA. *J Control Release* 2018;**286**:210-23.
44. Zhen Z, Potta T, Christensen MD, Narayanan E, Kanagal K, Breneman CM, et al. Accelerated materials discovery using chemical informatics investigation of polymer physicochemical properties and transgene expression efficacy. *ACS Biomater Sci Eng* 2018;**5**:654-69.
45. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J Cell Biol* 1992;**116**:1043-53, https://doi.org/10.1083/jcb.116.4.1043.
46. Gokduman K, Bestepe F, Li L, Yarmush ML, Usta OB. Dose-, treatment- and time-dependent toxicity of superparamagnetic iron oxide nanoparticles on primary rat hepatocytes. *Nanomedicine* 2018;**13**:1267-84.
47. Bale SS, Golberg I, Jindal R, McCarty WJ, Luitje M, Hegde M, et al. Long-term coculture strategies for primary hepatocytes and liver sinusoidal endothelial cells. *Tissue Eng Part C Methods* 2015;**21**:413-22.
48. Lee S, Lee DK. What is the proper way to apply the multiple comparison test? *Korean J Anesthesiol* 2018;**71**:353.
49. Miryala B, Zhen Z, Potta T, Breneman CM, Rege K. Parallel Synthesis and Quantitative Structure–Activity Relationship (QSAR) Modeling of Aminoglycoside-Derived Lipopolymers for Transgene Expression. *ACS Biomat Sci Eng* 2015;**1**:656-68.
50. McCarty WJ, Usta OB, Luitje M, Bale SS, Bhushan A, Hegde M, et al. A novel ultrathin collagen nanolayer assembly for 3-D microtissue engineering: layer-by-layer collagen deposition for long-term stable microfluidic hepatocyte culture. *Technology* 2014;**2**:67-74.
51. McCarty WJ, Prodanov L, Bale SS, Bhushan A, Jindal R, Yarmush ML, et al. Layer-by-layer collagen deposition in microfluidic devices for microtissue stabilization. *J VisExp* 2015e53078, https://doi.org/10.3791/53078-v.
52. Usta O, McCarty W, Bale S, Hegde M, Jindal R, Bhushan A, et al. Micro-engineered cell and tissue systems for drug screening and toxicology applications: evolution of in-vitro liver technologies. *Technology* 2015;**3**:1-26.

53. Moman RN, Gupta N, Varacallo M. Physiology. *Albumin*; 2017.
54. Yang X, Schnackenberg LK, Shi Q, Salminen WF. Hepatic toxicity biomarkers. *Biomarkers in Toxicology*. Elsevier; 2014. p. 241-59.
55. John JV, Jeong Y-I, Johnson RP, Chung C-W, Park H, Kang DH, et al. Folic acid-tethered poly (N-isopropylacrylamide)–phospholipid hybrid nanocarriers for targeted drug delivery. *J Mater Chem B* 2015;**3**:8268-78.
56. Zhang Y-N, Poon W, Tavares AJ, McGilvray ID, Chan WC. Nanoparticle–liver interactions: cellular uptake and hepatobiliary elimination. *J Control Release* 2016;**240**:332-48.
57. Soldatow VY, LeCluyse EL, Griffith LG, Rusyn I. In vitro models for liver toxicity testing. *Toxicol Res* 2013;**2**:23-39.
58. Tutty MA, Movia D, Prina-Mello A. Three-dimensional (3D) liver cell models-a tool for bridging the gap between animal studies and clinical trials when screening liver accumulation and toxicity of nanobiomaterials. *Drug Deliv Transl Res* 2022;**1–27**.
59. Omran B, Baek KH. Nanoantioxidants: pioneer types, advantages, limitations, and future insights. *Molecules* 2021;**26**PMC8624789.
60. Valgimigli L, Baschieri A, Amorati R. Antioxidant activity of nanomaterials. *J Mater Chem B* 2018;**6**:2036-51.