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Full Length Research Paper

Rapid and sensitive fluorometric analysis of novel galactosylated cationic liposome interaction with siRNA

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RNA interference (RNAi) is being harnessed for application in the gene knockdown approach to the treatment of disease conditions. Targeted delivery of small interfering RNA (siRNA) is however an important consideration for application of this technology. We report here on the preparation of two new hepatocyte-directed liposomes designed for this purpose, containing the cholesteryl cytofectins 3β[N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and 3β[N-(N', N', N'-trimethylammonium propane)-carbamoyl] cholesterol iodide (Chol-Q). Further we describe a simple yet sensitive fluorometric assay based on the displacement of siRNA-bound SYBR Green, to monitor the association of the duplex RNA with cationic liposomes under the conditions of high dilution encountered in transfection experiments.

Key words: siRNA, cationic liposomes, SYBR green, displacement assay.

INTRODUCTION

The discovery of the RNA interference (RNAi) pathway, in which small double stranded RNA molecules named small interfering RNAs (siRNAs) are incorporated into RNA-induced silencing complexes (RISC) that bind to cognate sequences in targeted mRNA molecules destined for degradation, has revolutionized the field of nucleic acid therapeutics. Although the efficacy of siRNA far exceeds that of their classical single-stranded antisense oligodeoxynucleotide counterparts, the polyanionic nature of the molecules renders them poorly permeable to the plasma cell membrane (Watts et al., 2008). Indeed, cellular uptake is an important factor in siRNA pharmacology (Fisher et al., 2007). Liposome vehiculation, which has been successfully employed to deliver vector DNA to mammalian cells, is being actively developed and adapted to improve cellular uptake of siRNAs (Keller, 2009). Furthermore, siRNA-liposome complexes (siRNA lipoplexes) have the design potential to be engineered for cell- or tissue-specific delivery by inclusion of an appropriate ligand into the liposome bilayer (Sato et al., 2007). Here we describe the formulation of two novel liver hepatocyte-targeted cationic liposomes. Formulations include the proven hepatotropic cholesteryl-β-D-galactopyranoside (CholβGal) (Singh et al., 2007) and the cholesteryl cytofectins 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and 3β[N-(N',N',N'-trimethylammonium propane)-carbamoyl] cholesterol iodide (Chol-Q) with dioleoylphosphatidyl ethanolamine (DOPE) as co-lipid. The β-D-galactopyranosyl moiety is recognized by the asialoorosomucoid receptor expressed on the plasma membrane of liver parenchymal cells (Bilder et al., 1995).

The assembly of defined siRNA lipoplexes from naked siRNA and cationic liposomes may be followed by an electrophoretic band shift assay using ethidium bromide or SYBR Green staining to locate siRNA on gels (Zhang et al., 2006; Buyens et al., 2008). We report here a sensitive fluorometric assay in which liposome binding of siRNA may be monitored directly in solution by siRNA-bound SYBR Green displacement. Furthermore we show a close correlation with results obtained by band shift assays on 2% agarose gels.

MATERIALS

SYBR Green II RNA gel stain (10 000x concentrate in DMSO) was from Cambrex Bio Science Rockland Inc. (Rockland, ME). Agarose (ultrapure, DNA grade) was purchased from BioRad (Richmond CA). Dioleoy-L-α-phosphatidyl ethanolamine was from SIGMA

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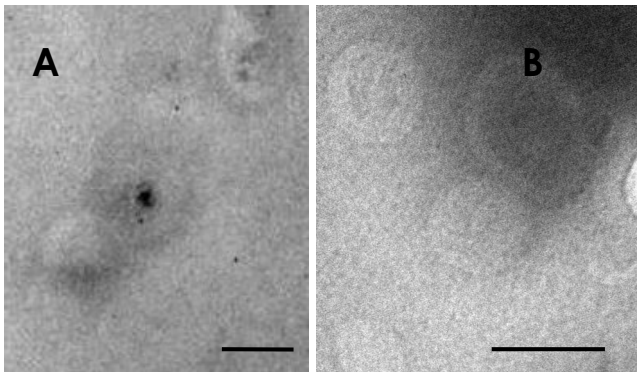

Figure 1. Transmission electron micrographs of targeted cationic liposomes. DOPE (2 µmole), CholβGal (0.5 µmole) and Chol-T (2 µmole) or Chol-Q (2 µmole) were deposited as a thin film in a test tube by rotary evaporation from chloroform: pyridine solution (12:1, v/v, 1.3 ml). Residual solvent was removed by overnight evacuation in a drying pistol. The film was rehydrated at 4°C for 14 h in a solution containing 20 mM HEPES, 150 mM NaCl (pH 7.5). The suspension was vortexed and sonicated in an Elma Transsonic bath-type sonicator for 5 min at 21°C. Lipo-T and Lipo-Q liposome preparations were routinely stored at 4°C and remained stable for several weeks. Liposome suspensions (1 µl) were applied to Formvar coated copper grids. After removal of excess liquid by blotting, grids were plunged into liquid ethane at -183°C. The vitrified samples were then viewed in a JEOL JEM-1010 transmission electron microscope at 100 kV. A Lipo-T (bar = 50 nm); B Lipo-Q (bar = 100 nm).

Chemical (St Louis, MI) and siGENOME non-targeting siRNA was obtained from Thermo Scientific Dharmacon Products (Lafayette, CO). 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was supplied by Merck (Darmstadt, Germany). Chol-T was prepared from cholesteryl chloroformate as described elsewhere (Singh et al., 2001) while Chol-Q was synthesized by methylation of Chol-T (Kisoon et al., 2002).

RESULTS AND DISCUSSION

Liposome preparation and characterization

Much interest is being expressed in the synthesis of novel cationic cholesteryl cytofectins for the manufacture of liposomes with the design capacity for in vivo delivery of siRNA (Islam et al., 2009; Carmona et al., 2009; Han et al., 2009). This class of molecule forms stable liposomes, when formulated with equimolar or near equimolar amounts of the neutral lipid DOPE. The Chol-T cytofectin contains a cholesteryl hydrophobic anchor, three carbon spacer and a dimethylamino head group which is protonated and therefore cationic at pH 7.5. The Chol-Q bears a close resemblance to Chol-T although importantly, it carries a quaternary trimethylammonium head group which is not pH sensitive. The DOPE co-lipid assumes an inverted hexagonal phase in an aqueous environment and when incorporated into membrane bilayers cause a degree of destabilization. This is a favourable property for nucleic acid delivery by liposomes as it promotes disruption of endosomal membranes following cellular uptake of lipoplexes by endocytosis. This permits the escape of the siRNA into the cytoplasm, thus avoiding lysosomal nuclease digestion. Furthermore, liposomes may be given a targeting aspect by attachment of cell or tissue-specific ligands (Shigeta et al., 2007). Here we investigate targeted liposomes formulated at a DOPE: cholesteryl cytofectin: cholesteryl-β-D-galactopyranosyl mole ratio of 4:4:1 and prepared by a method described elsewhere for the manufacture of N, N-dimethylaminopropylaminosuccinyl cholesterylformylhydrazide (MS09) containing liposomes (Singh et al., 2007). The hepatocyte-targeting component in the liposome formulation has been fixed at 11 % on a molar basis. In related studies Hashida and co-workers have incorporated into liposomes their Gal-C4-Chol hepatotropic ligand at a 5% level (Kawakami et al., 2001) while Maitani et al. (2001) have included a sterylglucoside at a 10% level. Thus relatively low levels are required to achieve the desired targeting without causing undue interference between the siRNA and the liposomal membrane cationic centres. Size distribution of the two new liposome vesicles Lipo-T and Lipo-Q, in HEPES buffered saline was found to be in the 80 - 120 nm range by TEM (Figure 1A and B). This is well within the estimated 200 nm diameter of liver sinusoidal fenestrations which must be entered for access to the hepatocytes.Images confirm that vesicles are essentially non-deformable and spherical in shape.

siRNA lipoplex formation and charge ratios

If synthetic siRNA molecules are to be successfully packaged into siRNA lipoplexes for in vivo cell specific delivery, the correct ratio of nucleic acid to cationic carrier must be chosen to ensure maximum protection of the siRNA from serum nuclease digestion. Currently this ratio is determined in a band shift assay. In essence, fixed amounts of siRNA are separately exposed to increasing amounts of the cationic liposomes. After brief incubation, reaction mixtures are subjected to electrophoresis on agarose or polyacrylamide gels. The liposomes are too large to enter the gel and remain in application wells. Liposome-associated siRNA would therefore also remain in the well. In this ‘band shift’ manner the minimum amount of liposomes required to fully bind a known amount of siRNA may be established. Figure 2A shows that at a siRNA: Lipo-T ratio of 1:24 (w/w) all the siRNA is liposome-bound and remains in the well after electrophoresis on 2% agarose, while this is achieved at a ratio of 1:28 for Lipo-Q (Figure 2B). This corresponds to a siRNA (-ve): cholesteryl cytofectin (+ve), charge ratio of 1:6 in both cases. Reaction mixtures are necessarily more concentrated than those prepared for cell culture and *in vivo* experiments and may therefore afford misleading results. We report here a rapid and sensitive method of establishing the required amount of liposome

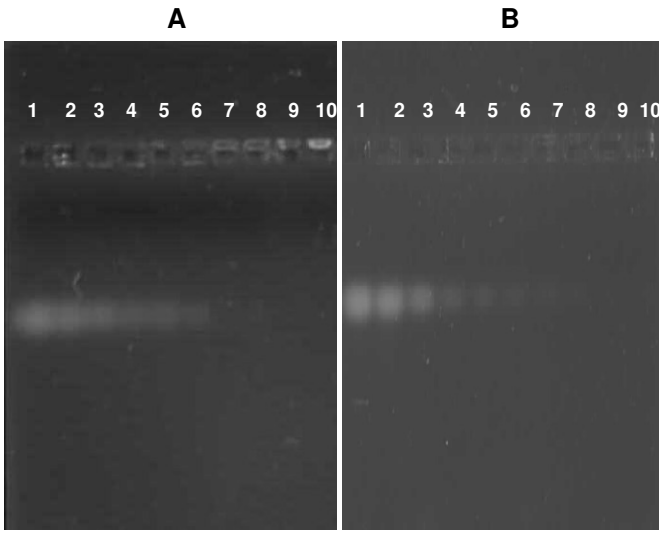

Figure 2. Gel retardation study of siRNA lipoplexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl containing siRNA (0.5 µg, 37 pmole) and increasing amounts of galactosylated cationic liposomes up to 16 µg were incubated for 30 minutes at 20°C before addition of gel loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in 2x gel buffer, 2 µl). Samples (8 µl) were subjected to electrophoresis at 50 V on 2% agarose gels in a buffer containing 36 mM Tris-HCl, 30 mM sodium phosphate, and 10 mM EDTA (pH 7.5) for 40 minutes. Gels were stained with 10 000x diluted SYBR Green stain and viewed under transillumination in a SYNGENE G-Box gel documentation and analysis system (Cambridge, UK). Lanes 2-10 contained liposomes in 1 µg increments (A Lipo-T, 7 - 15 µg; B Lipo-Q, 8 - 16 µg). Lane 1 in A and B contained siRNA alone.

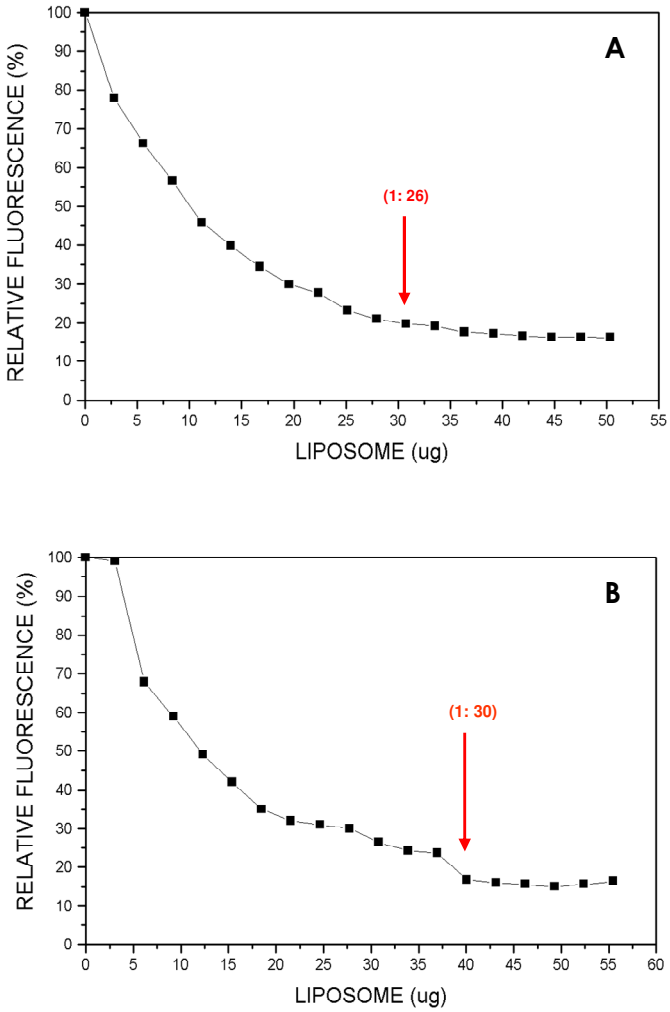

Figure 3. SYBR Green displacement assay. The fluorescence intensity of SYBR Green (10 000x diluted) in 20 mM HEPES, 150 mM NaCl, pH 7.5 (0.5 ml) was measured at an excitation wavelength of 497 nm and emission wavelength of 520 nm in a Shimadzu RF-551 spectrofluorometer (Japan). This was set at 0% relative fluorescence, whereupon 100 pmole siGENOME non-targeting siRNA (1.3 µg) was added and the relative fluorescence fixed at 100%. Targeted liposomes were then added in 2.5 µg aliquots in a stepwise manner to the SYBR Green-siRNA solution to a total of 55 µg and the relative fluorescence recorded at each stage.

under the more dilute incubation conditions employed in transfection experiments. The assay is based on SYBR Green displacement from as little as 100 pmole siRNA by increasing amounts of targeted cationic liposomes. The attendant reduction in SYBR Green fluorescence following dissociation from the nucleic acid is followed in a spectrofluorometer during the stepwise addition of the liposomes. Figure 3 clearly reveals that points are reached beyond which further additions of liposomes do not lead to further loss of fluorescence. These mark the points of maximum SYBR Green displacement, and complete liposome association of the siRNA. The end point ratios for Lipo-T and Lipo-Q were found to be 1:26 and 1:30 (w/w) respectively (Figure 3A and B), which is in good agreement with the values obtained by the band shift assays. Ethidium displacement assays devised to study plasmid DNA association with cationic liposomes, by comparison, employ DNA concentrations of 10 µg per ml or more (Ramezani et al., 2009; Singh et al., 2007). This is at least a 5 times higher nucleic acid concentration (on a w/w basis) than is being reported here for siRNA. The results show an excellent correlation between the band shift assay and the SYBR Green displacement assay. Moreover, the graphic presentation of dye displacement provides a clearer, unambiguous indication of the amount of liposome required to fully complex a siRNA cargo. Band shift assays typically require 8 or more separate incubation mixture in preparation for electrophoresis whereas the band shift assay reported here requires a fraction of the siRNA material, less SYBR Green and yields the desired result in a single reaction vessel in less than one hour.

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communication is dedicated to Professor AO Hawtrey on the occasion of his 80th birthday.

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