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Original Research

Recombinant Protein D from *Haemophilus influenzae* Induces Mouse Bactericidal Antibodies Against Typeable and Non-Typeable *Haemophilus influenzae*, which Partially Protect Infant Rats Against Serotype b Bacteraemia

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ABSTRACT

Aim
To evaluate the immunogenicity of a recombinant protein D from *Haemophilus Influenzae* (Hi) and the functional activities of the induced protein D antibodies in a mouse model.

Methods
Female Balb/c mice were immunised subcutaneously with recombinant protein D in the presence or absence of adjuvants and the serum immunoglobulin G (IgG) response to protein D was assessed by ELISA. The functional activity of the immune sera was evaluated *in vitro* using bactericidal assay against typeable Hi serotype b (Hib) and non-typeable Hi (NTHi) clinical isolates and *in vivo* using an infant rat bacteraemia model and a Hib clinical isolate.

Results
A dose-dependent IgG response was induced in mice immunised with the recombinant protein D and this response was further increased by the adjuvants used [CPG, $AlPO_4$ and $Al(OH)_3$], with the latter showing the greatest effect on the antibody response. Immune sera were very effective in bactericidal assay against several Hib and NTHi clinical isolates, with a higher serum bactericidal titre against the NTHi than against the Hib isolates. This is possibly due to the lower expression of protein D on the Hib isolates used in our study, compared to the NTHi isolates. In addition, anti-protein D antibodies were partially protective *in vivo* infant rat bacteraemia model against a challenge with Hib Eagan strain.

Conclusion
Our results suggest that recombinant protein D is a good vaccine candidate against Hi and should be given in combination with other vaccine candidates to ensure complete protection against Hib and NTHi.

Keywords
Haemophilus influenzae (Hi); *Haemophilus influenzae* serotype b bacteraemia; Rats.

INTRODUCTION

Haemophilus influenzae (Hi) is a gram-negative coccobacillus bacterial species which contain encapsulated and non-encapsulated strains. There are 6 encapsulated strains (a-f), classified on the basis of their antigenically-distinct capsular polysaccharides. The non-encapsulated strains, termed non-typeable (NTHi), do not possess a capsule and are genetically more diverse than the encapsulated strains.[1]

Both types of strains cause a variety of diseases in children and adults. Of the encapsulated strains, type b (Hib), is the most virulent and can cause bacterial meningitis and other invasive infections in children under the age of 4-years. The NTHi strains are, with *S.pneumoniae*, the leading cause of acute otitis media (AOM) in children. Together, they account for around 80% of bacterial otitis media cases.[2,3] NTHi has also been implicated in causing exacerbations in chronic obstructive pulmonary disease (COPD) which is currently the third leading cause of death worldwide and expected to be the leading cause of death by 2030.[4] As in otitis media (OM), of the bacteria isolated *S. pneumoniae*, *M. catarrhallis* and NTHi are the 3 most popular.[5]

There are currently a number of effective licenced vaccines against Hib.[6] These vaccines are comprised of the Hib capsular polysaccharide, polyribosyl-ribitol-phosphate, conjugated to various protein carriers (tetanus toxoid, non-toxic mutant of diphtheria toxin, or meningococcal outer membrane proteins) to overcome the weak immune response to capsular polysaccharide in children younger than 18-months of age. However, for NTHi, several vaccine candidates, mainly outer membrane proteins which are highly conserved between NTHi strains have been considered. These include outer membrane proteins (OMPs) such as P4,[7] P6,[8] lipooligosaccharides (LOS),[9] Adhesins 1 and 2[10] and protein D.[11] All of these OMPs have been shown to be immunogenic in previous studies. However, some OMPs, including Adhesins 1 and 2 (which are high molecular weight proteins that the bacteria use to bind to mucin in the respiratory tract), are able to be down regulated *via* phase variation to evade immune responses. The lack of constitutive expression of such proteins makes them poor vaccine candidates to be considered effective against all strains of Hi.[12] Of the OMPs, protein D is an attractive target for a vaccine because it is highly conserved between strains with limited genetic drift[13] and is expressed on all 127 known Hi strains, both encapsulated or not. The *hpd* gene which encodes protein D[13] varies by only a few nucleotides between all known strains of Hi. This low-level of variation is seen even during prolonged infections where the bacterium is under immunological pressure.[14] As no protein D mutations have been documented, it is likely that this protein serves an important function within the bacterium. Protein D has been prepared as lipidated and non-lipidated form and preclinical studies showed that protein D antibodies were able to induce 34-38% protection against NTHi otitis media in the chinchilla model.[15]

A ten-valent conjugate vaccine (Synflorix® from GlaxoSmithKline (GSK), London, UK) has been developed and licenced for use against pneumococcal invasive diseases. The vaccine uses protein D as a carrier for pneumococcal capsular polysaccharides of different serotypes. Eight serotypes are conjugated to protein D and the remaining two are conjugated to either diphtheria toxoid (DT) or tetanus toxoid (TT). Data from clinical trials with Synflorix® showed promising efficacy against NTHi as well as *S. pneumoniae*. The vaccine efficacy against episodes of otitis media caused by NTHi was reported to be 35%.[11]

As the NTHi strains lack capsular polysaccharide, immunisation with Hib conjugate vaccine cannot protect against infection with NTHi.[16,17] On the other hand, although protein D is expressed in all Hi strains, including Hib, its effectiveness as a vaccine candidate against Hib strains has not been evaluated.

The aim of this study is to investigate the potential of recombinant protein D as a vaccine candidate against both NTHi and Hib. The immunogenicity of recombinant protein D and the protective activity of the induced immune sera will be evaluated *in vitro* against Hib and NTHi clinical isolates, using the bactericidal assay and *in vivo* against Hib Eagan strain, using an infant rat Hib bacteraemia model.

MATERIALS AND METHODS

Expression, Purification and Characterisation of Recombinant Protein D

Expression and purification of protein D: The genomic DNA of Hib strain CMCC58547 (which is used for the production of Hib vaccine; source) was extracted to amplify the DNA encoding protein D (hpd) by PCR, using a pair of primers: (P1: 5'- AGCACATATGAGCAGCCATTCATCA-3' and P2: 5'-GAGGAAGCTTATTTTATTCCTTTTA-3').

Escherichia coli strain M15 and BL21 (DE3) (Novagen, WI, USA) were used for the plasmid construction and protein expression, which were cultured in luria broth (LB) medium at 37 °C. The PCR product was ligated with the pET30a (+) vector (Novagen, WI, USA) at restriction sites *Nde* I and *Hind* III. The constructed plasmid was designated pET30a(+)/hpd and transformed into *E.coli* BL21(DE3). The cloned DNA sequences were verified by sequencing analysis. Expression of the recombinant protein D was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to *E.coli* BL21(DE3) carrying the plasmid pET30a(+)/hpd at a final concentration of 0.2 mM. The expressed protein was purified using ion-exchange chromatography and hydrophobic chromatography in tandem with AKTA purifier 100 system (GE Healthcare, Chicago, IL, USA). Briefly, after induction with IPTG, the cells were collected by centrifugation and sonicated on ice bath. The inclusion bodies containing the recombinant protein were separated by centrifugation at 12,000 g for 15 min at 4 °C and solubilized in the binding buffer (6 M urea, 20 mM Tris at pH 9.5). The protein was loaded onto a Q Sepharose Fast Flow column (2.6 cm×20 cm; GE Healthcare, Chicago, IL, USA) and eluted with an increasing concentration of NaCl (0-1 M) in the binding buffer. The main eluate was collected and ammonium sulphate added at 0.5 M for the next step

of purification with Phenyl Sepharose 6 Fast Flow column (2.6 cm×20 cm, GE Healthcare, Chicago, IL, USA). The target protein was eluted with the buffer solution (50 mM PB, 6 M urea at pH 7.0) containing a decreasing concentration of ammonium sulphate (0.5-0 M). The recombinant protein D was refolded by gradually decreasing the concentration of urea to 0.5 M by dialysis for 48 h. The purity of the recombinant protein was estimated by SDS-PAGE and gel densitometry analysis, and protein concentration was determined by the Lowry method as described previously.[18]

Protein identification using LC-MS/MS analysis: Twenty (20) µg of the purified protein D was used for in-solution tryptic digestion according to the method described by Whiting et al.[19] Briefly, the sample was denatured, reduced and alkylated prior to overnight digestion with trypsin. The resulted digest was subjected to a U3000 direct nanosystem coupled with a nanoelectrospray and LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher, MA, USA) as described previously.[20] The technical triplicates of the sample were performed. Data analysis was carried out using PEAKS 8 (Bioinformatics Solutions Inc., Ontario, Canada) with precursor and fragment error tolerance as 10 ppm and 0.6 Da, respectively. Deamidation (NQ), carbamidomethylation (C), oxidation (M) and pyro-Glu (Q) were set as variable modifications. Three raw files were searched against a database containing the sequence of recombinant protein D (without signal peptide), and the Swiss-Prot all proteins as background.

Expression of protein D on Hib and NTHi: The relative level of protein D expression on Hib and NTHi isolates was evaluated using quantitative western blotting. In brief, 50 µg of total protein extract quantified by BCA assay (Thermoscientific, MA, USA) was separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry western transfer apparatus (Thermoscientific, MA, USA). The membrane was initially stained using pierce reversible protein stain (Thermoscientific, MA, USA) to be used for loading control. Non-specific binding sites on the membranes were blocked by incubation in 5% skimmed milk (Fluka®, Buchs, Switzerland) in TBST for 1 hat RT. After the blocking step, the blot was incubated with anti-protein D mouse serum followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Thermoscientific, MA, USA) for 1h at RT. The blot signal was then detected using an enhanced chemiluminescence (ECL) detection kit (Thermo scientific, MA, USA). Relative quantification of protein D expression was achieved through densitometry analysis of protein D bands normalized to total protein stain loading control.[21,22]

Endotoxin content: The endotoxin content of the purified protein D was determined using an in-house Limulus Amebocyte Lysate (LAL) assay. Protein D was reconstituted in 0.5 ml of Tris-HCl (0.25 M, pH 9) and mixed with LAL (Lonza) which reacts with bacterial endotoxin or lipopolysaccharide (LPS). The presence of endotoxin results in coagulation of the mixture which can be quantified relative to a reference LPS titrated in the same assay.

***Haemophilus influenzae* strains:** Four clinical isolates of NTHi, 3 from invasive disease cases (Haemophilus Reference Unit, Public Health England; PHE, UK) and one from OM cases (ATCC, LGC group Teddington, UK), were used in the study. In addition, three clinical isolates of Hib were also used in this study (Haemophilus Reference unit, PHE, UK).

Animals Immunisation

Female BALB/c mice (6-8-weeks: Charles River; 5-8 per group, as indicated for each experiment) were immunised subcutaneously on days 0 and 28 with various doses of protein D with or without adjuvant. Animals were bled on day 42 and sera collected and stored at -20 °C until used.

In vivo Protection Against Haemophilus influenzae Type b Challenge

Female and males (1:1 ratio) Sprague Dawley (Charles River, MA, USA) infant rats (3-5-days old; 10 per group) were injected intraperitoneally (IP) with 100 µl of immune serum diluted 1:1 or 1:25 with PBS or with 100 µl of normal rat serum as a control. After 24 h rats were challenged IP with 10^4 CFU of a clinical isolate of Hib (Eagan strain; Hib 1) grown to log phase in Muller Hinton broth. Blood was collected by cardiac puncture 24 h later and cultured on chocolate agar plates for 24 h to assess the presence of bacteraemia.

All animal studies were conducted according to the Animals (Scientific Procedures) Act 1986, under UK Home Office project licence PPL80/2634.

ELISA for Quantification of Anti-Protein D IgG and IgG Subclasses

Ninety six (96) well Nunc Maxisorbmicrotitre plates (Gibco, MD, USA) were coated with 100 µl/well of 2.5 µg/ml Protein D in carbonate buffer (pH 9.6) for 2 h at 37 °C and then incubated overnight at 4 °C. Next day, the plates were washed with PBS/0.05% $Tween_{20}$ (Sigma, MO, USA) and blocked with 100 µl/well of assay diluent [AD; PBS/1% BSA (Sigma, MO, USA), 0.3% $Tween_{20}$, 0.01 M EDTA (Fisher Scientific, MA, USA)] for 1 h at 37 °C. AD was then discarded and 11 serial two-fold dilutions of the immune sera in AD were prepared in the plate, starting at 1/50 dilution (in 100 µl volumes) and plates were incubated for 90 min at RT. Binding of protein D antibodies was detected with 100 µl/well of 1/500 dilution of biotin-conjugated goat anti-mouse IgG (Sigma, MA, USA) and plates incubated at RT for 90 min. This was followed by 1 h incubation at RT with 100 µl/well of streptavidin-horseradish peroxidase diluted 1/2000 (Sigma, MA, USA). One hundred (100) µl/well of substrate solution (Ortho-phenylenediamine/H_2O_2; Sigma, MA, USA) was added and plates incubated at RT in the dark for up to 30 min. The enzymatic reaction was stopped by adding 50 µl/well of 3 M HCl and the optical density was measured at 492 nm using a Multiskan MS plate reader (Labsystems, Helsinki, Finland; Thermo Life Sciences, MA, USA). Each serum sample was tested in duplicates on separate plates and data presented as mean endpoint titre for IgG (reciprocal of serum dilution giving OD>mean blank+2 SD). To detect the level of IgG1 and IgG2a anti-protein D antibodies, ELISA was performed as above, except

for the use of isotype/subclass specific detection antibodies: goat anti-mouse IgG2a-HRP (Invitrogen, CA, USA), or goat anti-mouse IgG1-HRP (Southern Biotech, AL, USA). Data for IgG1 and IgG2a anti-protein D are presented as relative concentration (µg/ml), calculated from standard curves generated from serially diluted, commercial mouse IgGI and IgG2a myeloma proteins of known concentration (Sigma, MO, USA), tested on plates coated with anti-IgG1 or anti-IgG2a instead of protein D, using an in-house parallel line bioassay programme (CombiStats™, Strasbourg, France) which relates the logarithm of the assay responses (OD) to the logarithm of the sera dilution, using at least 3 points on the linear portion of the titration curve.

Bactericidal Assay

Heat inactivated (55 °C for 30 min) anti-protein D immune sera were serially diluted in 40 µl volumes in the first 10 columns of a flat-bottom 96-well microtitre plate (Falcon, Mexico, USA), in assay buffer [Hanks balanced salt solution and 10% Foetal bovine serum (Gibco, MD, USA)]. Assay buffer was added to the last 2 columns which serve as negative control for the assay.

The bacteria to be tested were grown to log phase in Mueller-Hinton broth and then diluted in assay buffer to 1×10^4 CFU/ml before use in the assay. Twenty (20) µl of bacterial culture were added to each well, followed by 30 µl of 1:1 diluted baby rabbit complement (Mast Group, Ohio, USA), which was added to all wells, except the last column which serves as a negative control. The plate was incubated at 37 °C for 1 h in a shaking incubator (100 rpm). At the end of the incubation, 3×10 µl from each well were spotted onto pre-warmed chocolate agar plates which were tilted gently to allow the bacteria suspension to run along straight lines. The agar plates were incubated overnight at 37 °C and the number of colonies counted the next day. Each serum dilution was tested in triplicate wells and the results were presented as a bactericidal titre, which is the serum dilution that caused 50% killing of the bacteria.

STATISTICAL ANALYSIS

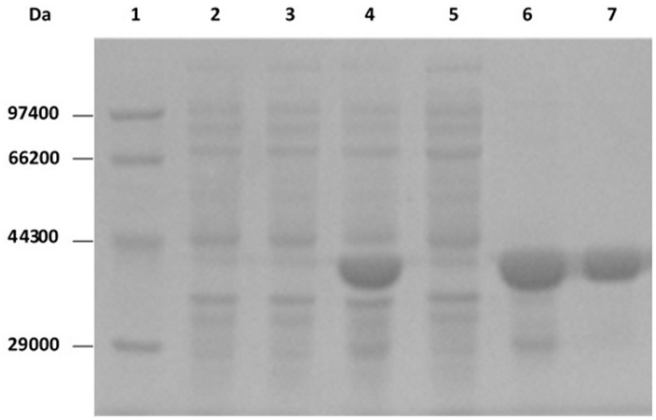
Comparison between the groups was done by the Mann Whitney t-wo sample rank sum test for the immunogenicity studies and by the student's *t*-test for the bactericidal assay. A Bonferroni correction was used to adjust the significance level for multiple comparisons with a control group.

RESULTS

Expression and Characterization of Recombinant Protein D

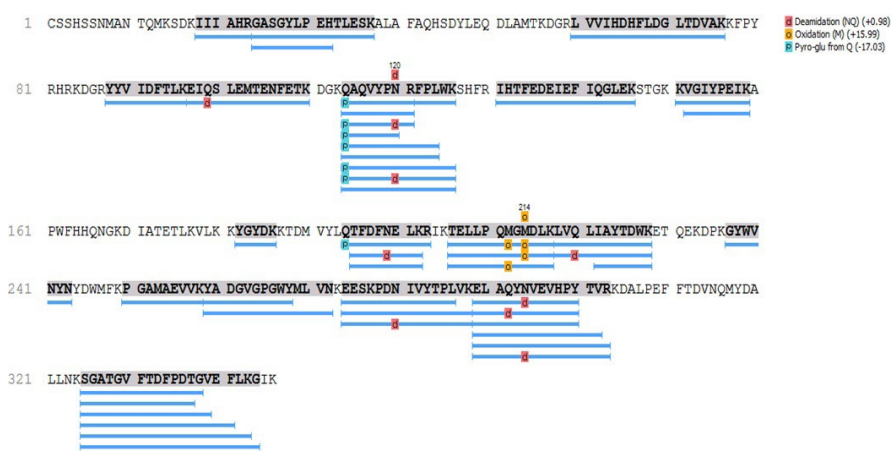
To generate recombinant protein D in *E.coli*, the respective gene was amplified from a Hib vaccine strain and cloned into a protein expression vector. The expression plasmid was constructed, and the DNA sequence confirmed by DNA sequencing. Following Isopropyl β- d-1-thiogalactopyranoside (IPTG) induction, the recombinant protein (346aa) without 18aa signal peptide and without affinity tag was successfully expressed in *E.coli* as inclusion bodies. The induced protein band corresponded to the predicted size of 40-kDa on SDS-PAGE (Figure 1), which accounted for 57% of the total cellular proteins analysed by using Image Master

Figure 1. *SDS-PAGE of Recombinant Protein D Isolated From E. coli Expression System*



Samples were electrophoresed on a 12% SDS-PAGE gel under reducing condition and stained by Coomassie blue. Lane 1, low range protein molecular weight marker (the molecular mass standards indicated in Da on left side); lane 2, lysate of E. coli cells carrying empty plasmid pET30a(+) after induction with IPTG; lane 3, lysate of E. coli cells carrying plasmid pET30a(+)/hpd without induction; lane 4, lysate of E. coli cells carrying plasmid pET30a(+)/hpd after induction with IPTG; lane 5, supernatant of E. coli cells carrying plasmid pET30a(+)/hpd after induction and sonication; lane 6, inclusion bodies of E. coli cells carrying plasmid pET30a(+)/hpd after induction and sonication; lane7, purified recombinant protein D.

Figure 2. *Identity of Protein D Specific Peptides by Mass Spectrophotometry*



VDS Software (GE Healthcare, IL, USA). The recombinant protein D was purified using ion-exchange chromatography and hydrophobic affinity chromatography methods, yielding a high-level of purity (>95%; Figure 1).

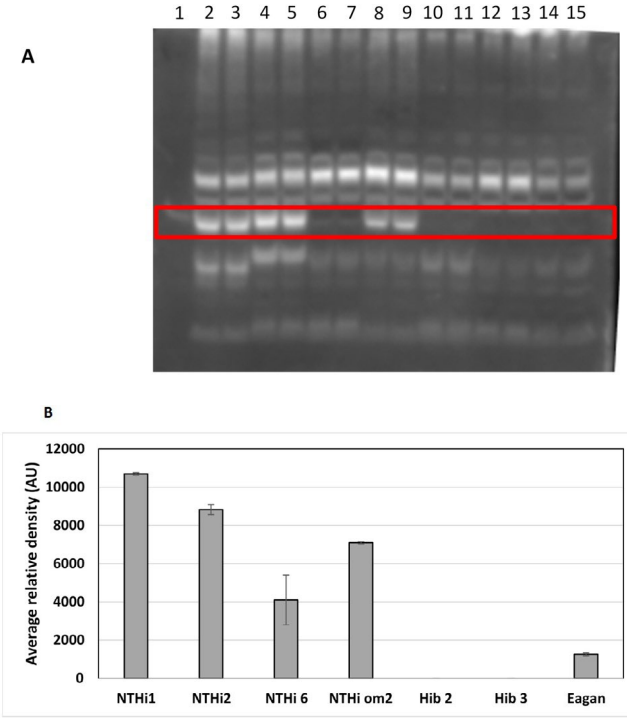
Identification of protein D using LC-MS/MS analysis indicated the presence of 35 unique peptides and an amino acid sequence coverage of 62% (Figure 2).

Relative Expression of Protein D on *Haemophilus influenzae* Strains

A quantitative western blot analysis was performed to enable a relative comparison of the level protein D expression on the different Hi strains used in this study. Upon analysis of the whole protein-normalised western blot signal, higher protein D expression levels were observed in the NTHi strains compared to Hib strains (Figure 3A, lanes 2-9). Protein D expressed by Hib strains 2.2 and 2.3 were below the detection limits of the assay performed and the Eagan strain expressed relatively much lower protein D levels (Figure 3A, lanes 10-15) compared to all 4 NTHi strains.

The endotoxin content of the recombinant protein, determined by the LAL assay, was very low (10 IU/mg of protein), suggesting its suitability for use in animal immunisation studies.

Figure 3. *Expression of Protein D in Various Haemophilus influenzae Strains*



(A) *Western blot analysis with anti-protein D sera performed on whole bacteria lysates. Recombinant protein D was used as marker (lane 1), NTHi 1(lane 2,3), NTHi 2 (lane 4,5), NTHi 6 (lane 6,7), NTHi OM2 (lane 8,9), Hib 2 (lane 10,11), Hib 3 (lane 12,13), Hib Eagan (lane 14,15).* ***(B)*** *Relative expression levels are presented as arbitrary units (AU) based on band density. All results were normalised to total protein stain.*

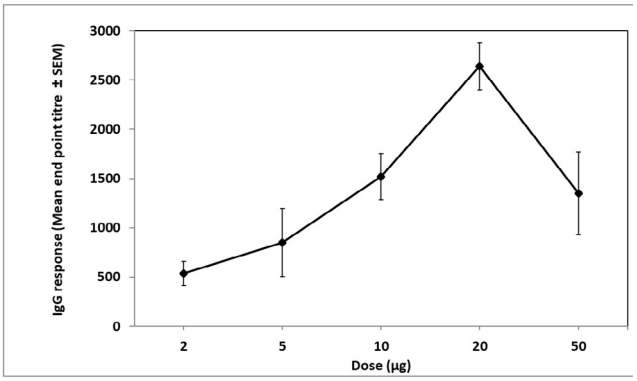
Expression of Protein D on the Different Haemophilus influenzae Strains

A quantitative western blot analysis was performed to enable a relative comparison of protein D levels among the Hi strains used in this study. Upon analysis of the whole protein normalised western blot signal, higher protein D expression levels were observed in the NTHi strains compared to Hib strains (Figure 3B). Protein D expressed by Hib strains 2 and 3 were below the detection limits of the assay performed and the Eagan strain expressed relatively lower protein D levels.

Immunogenicity of Protein D in a Mouse Model

Immunogenicity of various doses of protein D and kinetics of the immune response: Immunisation of mice with 2 µg of protein D on days 0 and 28 induced a weak IgG antibody response (Mean titre=538) which was further increased with increasing the dose to 5, 10 and 20 µg in a dose-dependent manner, reaching a maximum titre of 2,640 (Figure 4). Further increase in the immunisation dose to 50 µg yielded a slightly lower response (mean titre=1,348). Although the difference between 2 consecutive doses was not always significant due to the high variability in the response between individual animals, the response was linear in the range between 2 and 20 µg doses (R=0.968).

Figure 4. *Immunogenicity of Protein D*

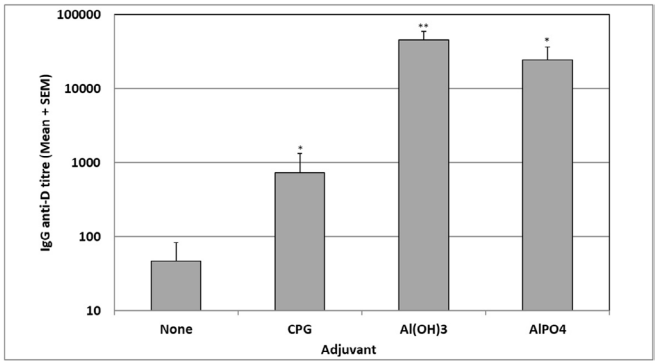


Groups of Balb/cmice (n=5) were immunised (subcutaneously) on days 0 and 28.Animals were terminally bled 2-weeks after the 2nd immunisation and sera tested for antibody response.The response is presented as mean end point titre for each group +SEM.

Effect of Various Adjuvants on Protein D Immunogenicity

Investigation of the effect of various adjuvants on the anti-D antibody response showed that all three adjuvant formulations: $Al(OH)_3$, CPG and $AlPO_4$ had significantly increased the anti-protein D IgG level when compared to the IgG level following immunisation with protein D on its own (Figure 5). However, the level of adjuvanticity varied for the different adjuvants, with CPG being the weakest adjuvant, inducing a 46 fold increase in the antibody response ($p<0.05$) followed by $AlPO_4$ (524 fold; $p<0.05$) and $Al(OH)_3$ (969 fold; $p<0.01$).

Figure 5. *Effect of Various Adjuvants on Protein D Immunogenicity*



Balb/c mice (n=8) were immunised subcutaneously on days 1 and 28 with protein D with or without adjuvant. Where adjuvant was used the dose was: 10μg for $Al(OH)_3$ and $AlPO_4$ and 5 μg for CPG. Mice were terminally bled 2-weeks after the last immunisation and sera were tested for anti- protein D IgG. Comparison between the groups was carried out using the Mann Whitney two samples rank sum test. indicates p<0.05 and ** indicates p<0.01, when compared to protein D alone.*

IgG Subclass Distribution

IgG subclass distribution in sera induced by immunising mice with protein D in the presence or absence of adjuvant was evaluated for IgG1 and IgG2a. Data displayed in Table 1 shows that IgG1 was more dominant than IgG2a, suggesting that the immune response is more skewed towards a Th2-type response. The $Al(OH)_3$ group had the highest levels of IgG1 (308.9 μg/ml) compared to IgG2a (0.038 μg/ml) with an >8,000 fold difference between the two subclasses. This was followed by the $AlPO_4$ group which had a 7,000 folds difference between the IgG1 (146.3 μg/ml) and IgG2a subclasses (0.021 μg/ml). The CPG and the no adjuvant groups had similar IgG1:IgG2a ratio (16) but with CPG group inducing a much higher response (0.48 and 0.029 μg/ml for IgG1 and IgG2a, respectively).

Table 1. *IgG1 and IgG2a Concentration in Sera of Mice Immunised with Protein D and Different Adjuvants*

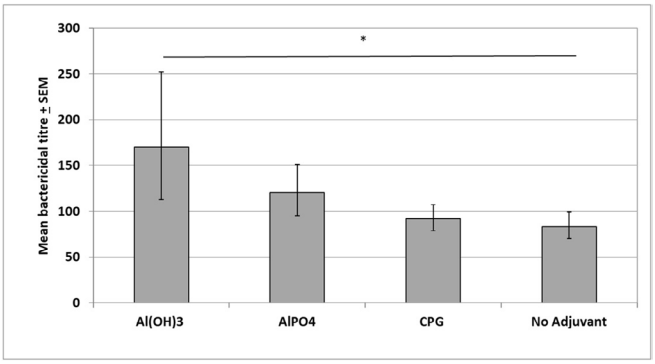
Adjuvant	Relative Concentration (μg/ml)		IgG1:IgG2a ratio
	IgG1	IgG2a	
No adjuvant	0.068	0.004	16
CPG	0.48	0.029	16
$Al(OH)_3$	308.85	0.038	8,127
$ALPO_4$	146.28	0.021	6,966

Sera samples generated after two immunisations with protein D in the presence or absence of adjuvant were analysed for anti-protein D IgG2a and IgG1 concentrations relative to a commercial mouse IgG1 and IgG2a myeloma protein reference standards.

Functional Activity of Protein D Antisera Against NTHi and Hib Clinical Isolates

To evaluate the functional activity of antibodies generated by recombinant protein D against NTHi, pooled sera samples from immunised animals were tested in serum bactericidal assays against a single NTHi clinical isolate (NTHi6, obtained from an invasive disease case). Immune sera of animals immunised with protein D, in the presence or absence of adjuvant, were bactericidal against the NTHi isolate used, with mean bactericidal titres ranging from 83-170 (Figure 6). Sera from mice immunised with $Al(OH)_3$-adjuvanted protein D were significantly more bactericidal than sera from mice immunised with protein D alone (mean bactericidal titr-

Figure 6. *Bactericidal Titres of Sera Samples, Induced by Immunisation with Protein D in the Presence or Absence of Different Adjuvants*



*Sera samples were pooled from each group of mice (n=8) and used to evaluate the bactericidal titre. Mean is an average of 3 repeats of the same assay done on separate days. Error bars are SEM. Data analysis was done by Student's t-test for independent samples to compare between the bactericidal titres of immune sera from animals immunised with differently adjuvanted protein D against NTHi strain 6.*p<0.05.*

Table 2. *Bactericidal Titres of Protein D Antisera Against Different NTHi and Hib Clinical Isolates*

Isolate	Number of Assays	Mean Bactericidal Titre+SEM	Source	Isolated from
NTHi 1	3	36.3+13.1	PHE	Invasive case (not-specified)
NTHi 2	6	79.5+18.5	PHE	Invasive case (not-specified)
NTHi 6	11	47.4+10.3	PHE	Invasive case (not-specified)
NTHi OM2	5	37.3+9.1	LGC	Otitis media
Hib1	5	24.6+2.3	PHE	Bacteraemia/Meningitis
Hib2	4	28.0+12.1	PHE	Epiglottitis
Hib Eagan	3	20.0+5.0	PHE	Meningitis

Comparison of the bactericidal titres of protein D antisera against a range of NTHi and Hib isolates. Each assay for each isolate was repeated at least 3 times. Data is presented as mean bactericidal titres +SEM. Data analysis was done by Student's t-test to compare between the serum bactericidal titres against the various NTHi or Hib isolates.

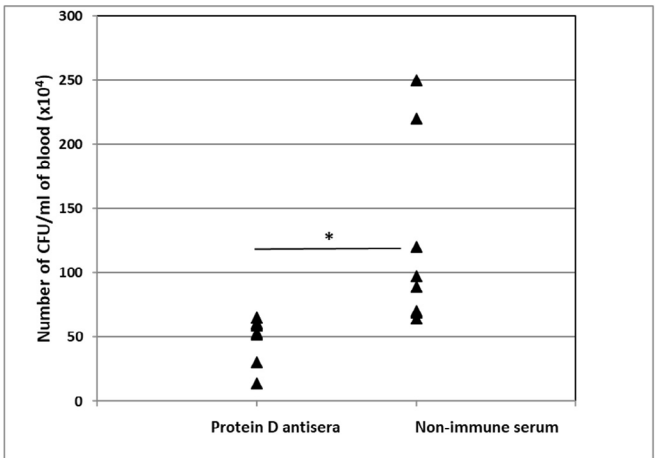
e=170 *vs* 83; $p<0.05$). $AlPO_4$ adjuvant slightly increased the bactericidal titre but this increase was not statistically significant (120 *vs* 83; $p>0.05$) and mice immunised with CPG-protein D or with protein D alone had lower and very similar serum bactericidal titres of 92 and 83, respectively. A pool of non-immune sera was used as a negative control in the assay and showed no, or very low, non-specific killing, indicating that the bacteria killing was mediated by protein D antibodies. To ensure that the bactericidal activity of protein D antisera covers a range of NTHi and also Hib strains, 3 more clinical isolates of NTHi obtained from invasive and non-invasive disease cases and 3 clinical Hib isolates from invasive disease cases were also evaluated for susceptibility in the bactericidal assay, using immune serum from mice immunised with $Al(OH)_3$–protein D (Table 2). Protein D antisera had bactericidal activity against all the NTHi isolates tested with no significant difference in the bactericidal titre against the different isolates ($p>0.05$). Mean bactericidal titres ranged from 37 for isolate OM2, to a maximum of 79.5 for isolate 2. No relationship was found between the original site of isolation of the bacteria and the bactericidal titre.

When Hib isolates were used as the targets in the killing assay, protein D antisera were found to be able to kill all Hib isolates to similar extents with bactericidal titres ranging from 20-29. The difference in bactericidal activity between the Hib isolates was not significant and was smaller than between the NTHi isolates. However, the mean bactericidal titres against Hib isolates were lower than those against NTHi isolates. Similar to NTHi isolates, there was no relationship between the original site of isolation of the Hib isolates and the bactericidal titre.

In vivo Functional Activity of Protein D Antisera Against Hib Bacteraemia

To test if protein D antibodies can kill Hib bacteria *in vivo*, infant rats were challenged with Hib Eagan strain, 24 h after adoptive transfer of immune sera from mice immunised with protein D-Al(OH)3. Infant rats were then bled after another 24 h and blood cultured on blood agar to measure Hib growth. The results of an experiment (Figure 7) showed that although the anti-D antibodies did not completely prevent Hib bacteraemia, it significantly reduced the mean CFU number following Hib challenge in infant rats that received protein D immune serum, compared to control animals that received non-immune serum (51 *vs* 122; $p<0.05$).

Figure 7. *Protection of Infant Rats Against Hib Challenge by Protein D Antibodies*



*Data analysis done by Student's t-test. * p<0.05.*

DISCUSSION

Recent data from the clinic has shown that high serum antibody titres to protein D was associated with reduced risk of developing AOM in children due to NTHi[23] and studies with pneumococcal vaccines using protein D as a carrier indicated a trend of protective efficacy against AOM caused by NTHi.[11,24] Moreover, Siggins et al[25] demonstrated that anti-protein D antibodies led to a reduction in bacterial load in a mouse model of NTHi-lung infection but did not prevent the infection, despite high-level of protein D serum antibodies.

However, efficacy of protein D against invasive diseases caused by encapsulated Hi such as Hib has not been previously investigated and earlier research studies focused mainly on its efficacy against NTHi-induced OM. In our study, we investigated the effectiveness of protein D antibodies against Hib clinical isolates and showed that, in addition to being effective in killing Hib isolates in the bactericidal assay, protein D-induced antibodies were also protective in our *in vivo* infant rat Hib bacteraemia model (causing a significant 60% reduction in CFU) and this was similar to the partial protection seen in the mouse model of NTHi-lung infection described by Siggins et al.[25] This suggests that full protection against Hib invasive diseases and NTHi by protein D might require the use of a more potent adjuvant[26] or the combination with other highly conserved outer membrane proteins of Hi that have demonstrated potential as vaccine candidates such as P6, OMP26 and recombinant fusion protein (protein E and Pilin A; PE-PilA).[26-28] In addition, it is also possible that protein D could have a synergistic effect when given with Hib conjugate vaccines to improve their efficacy. However, with the high efficacy of Hib conjugate vaccines used in the infant immunisation programmes, it is difficult to measure the additional effect of protein D on Hib disease in the clinic. It remains possible, however, that including protein D in the infant immunisation, as a component vaccine or as a carrier in Synflorix®, might enable reducing the number of doses of the expensive Hib conjugate vaccine.

In this study, we investigated the effect of aluminium based-adjuvants [$AlPO_4$ and $Al(OH)_3$] and CPG on the immunogenicity of protein D and showed that all adjuvants induced a significant increase in the level of anti-protein D IgG, with $Al(OH)_3$ inducing the highest fold increase (969) followed by $AlPO_4$(524) and CPG (46). It is not known how the different adjuvants potentiate the immune response, but possibly *via* formation of a depot at the site of immunisation and the slow release of the antigen, for the aluminium-based adjuvants[29] and by activating dendritic cells through TLR9 ligation by CPG.[30]

In our study, although the infant rats were not fully protected against Hib bacteraemia *in vivo*, there was a significant reduction in bacterial count in the blood (~60%) of passively immunised animals. This suggests that full protection against Hib invasive diseases might be achievable by protein D with the use a more potent adjuvant or by combining it with other highly conserved outer membrane proteins such as P6.

Although the incidence of invasive Hib diseases declined dramatically following the introduction of Hib conjugate vaccines, infection caused by other serotypes are increasing e.g. serotype a in Alaska[31,32] and serotype f in Brazil.[33] Similar phenomenon of serotype replacement has been seen after the introduction of pneumococcal vaccines where clinical cases caused by serotypes not covered by the vaccine have risen whilst overall cases have fallen.[34,35] Being highly conserved amongst all Hi strains, protein D is probably a good vaccine candidate that could prevent serotype replacement following the introduction of Hib conjugate vaccines and this should be investigated. Being a component of Synflorix vaccine, it would be interesting to know if Hi serotype replacement is lower in countries using Synflorix® vaccine compared to Prevenar, and the potential of protein D to target all serotypes of encapsulated Hi should be explored further.

CONCLUSION

In summary, our results showed that anti-protein D antibodies can effectively kill both Hib and NTHi strains, although not to the same degree, as it seems that the NTHi are more readily killed compared to Hib strains. We are not sure if this is due to the lower expression of protein D on the Hib strains compared to NTHi (as found in this study) or to reduced accessibility of the antibodies to bind to protein D on Hib due to the presence of the capsule and further studies are needed to explore this.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Model Liposomal Delivery System for Drugs and Vaccines

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ABSTRACT

Background
Liposomes have been used for drug delivery since their discovery 60-years-ago. The advantages they provide as carriers have been recognised and exploited to improve the delivery of numerous drugs and eliminate harmful side-effects. Liposomal delivery has been tested for anticancer drugs, anti-tuberculosis drugs, variety of vaccines, just to list a few.

Methods
We developed a series of liposomal formulations with the addition of cholesterol and polyethylene glycol. The uptake of these formulations by human epithelial prostate cancer (PC-3) cells and mouse macrophages was examined and analysed by flow cytometry and confocal microscopy.

Results
Among the liposomes tested, small anionic liposome vesicles (≤200 nm) prepared with egg phosphatidylglycerol as the main lipid were most effectively taken up by PC-3 cells and macrophages.

Conclusion
We produced a liposome formulation that can be used as a model system for the delivery of drugs and vaccines.

Keywords
Liposomes; Drug delivery; Vaccines delivery; Egg phosphatidylglycerol; Polyethylene glycol.

INTRODUCTION

Since their discovery by Alec Bangham and colleagues in the 1960s, liposomes have been one of the most investigated forms of nano carriers.[1] They were first described as lamellae of swollen or enlarged lipids that could act as a model membrane system.[2] Liposomes are spherically shaped amphiphilic lipid bilayers ranging in size from 20 nm to 10 μm.[3,4] Their structure is strongly correlated with their unique ability to physically entrap a variety of compounds, including proteins, nucleotides, plasmids, macromolecules and ribonucleic acid/deoxyribonucleic acid (RNA/DNA). Hydrophobic compounds can be embedded in the phospholipid bilayer, whereas hydrophilic compounds are entrapped within the aqueous core of the lipid vesicles.[5] Liposomes can increase drug uptake and reduce drug toxicity, while prolonging biological half-life.[6] Consequently, liposomes can significantly increase the therapeutic index of the drugs they deliver.[6,7]

Liposome vesicles can be prepared with synthetic or natural lipids, such as distearoyl phosphatidyl choline (DSPC), dioleoyl phosphatidyl ethanolamine (DOPE) orphosphatidylglycerol (PG). The inclusion of lipids allows liposomes to be biocompatible, non-toxic, flexible, non-immunogenic and biodegradable.[8] The physicochemical properties of liposome vesicles depend on the phospholipid (and other components) used in the formulation, and can influence the vesicle's surface charge, size, permeability and stability.[9]

Classical liposomes, better known as the first generation of liposome vesicles, were developed to encapsulate compounds for non-specific uptake through endocytosis, adsorption or lipid exchange once administered in a host.[4,10] However, these vesicles were found to be rapidly eliminated by the reticuloendothelial system (RES), greatly reducing their therapeutic efficacy.[11] Coating vesicles with polyethylene glycol (PEG) is often done to increase the amount of time that the vesicles spent circulating through the blood (i.e. stealth shielding).[12,13] PEGylation of liposomes results in the formation of hydrophilic barriers on the surface of the vesicles, which decreases the interaction of the vesicles with RES and increases blood circulation half-life.[11,14] The addition of cholesterol to formulations has also been employed to enhance transmembrane permeability and rigidity.[5] Adding cholesterol to the liposome membrane can lead to reduced binding of sodium ions to the membrane. This reduces the interaction between vesicles and serum opsonins, and increases the circulation time of the liposomes in the blood stream.[15,16]

Liposomes are widely used to improve cellular uptake of variety of drugs and antigens. For example, a vaccine against tuberculosis (TB), called bacille calmette-guerin (BCG), was developed and widely distributed more than 100-years-ago. BCG is effective in protecting vaccinated children against acute forms of the disease, but it has shown limited and variable protection in adults, with the protective efficacy ranging from 0% to 77%.[17,18] A new TB vaccine and/or more effective treatments are urgently required to fight this disease. TB is caused by mycobacterium tuberculosis (MTB), which infect macrophages. Encapsulation of the anti-TB vaccines or drugs within liposome vesicles can be applied to improve their delivery to the macrophages. Similarly, a variety of strategies has been examined to facilitate uptake of anticancer agents by tumour cells.[19,20] As prostate cancer is still the most common cancer diagnosed in men,[21] drug delivery systems targeting this cancer have been intensively investigated.[22,23] Thus, we developed three liposome formulations to better understand the characteristics needed to improve the uptake by human epithelial prostate cancer (PC-3) cells and mouse macrophages.

MATERIALS AND METHODS

The lipids used in the liposome formulations: DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), egg PG (L-α-phopshtidyl-DL-glycerol (egg, chicken)), PEG (3000), PE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000] (ammonium salt)), DOPE (1, 2-Dioleoyl-sn-glycero-3-phosphoethanolamine)and cholesterol, were obtained from Avanti Polar Lipids, Inc (Alabaster, Alabama, USA). All chemicals and solvents used were of analytical grade or equivalent. Chloroform, anhydrous chloroform and methanol were supplied by Merck KgaA (Darmstadt, Germany). Buffer HEPES glucose (BHG), phosphate-buffered saline (PBS), L929 cell-conditioned medium (LCM), Roswell Park Memorial Institute (RPMI) medium, foetal bovine serum (FBS), TrypLExpress, trypanblue stain (0.4%) and Opti-MEM/GlutaMax, Dulbecco's Modified Eagle's Medium (DMEM), Newborn Calf Serum (NBCS) and Ethylene diaminetetra acetic acid (EDTA) were acquired from Gibco by Life Technologies. Paraformaldehyde (PFA) was obtained from Scharlau. Staining Vybrant™ DIL cell-labelling solution was obtained from Invitrogen™, and Hoechst 33342 solution was supplied by ThermoScientific™.

Extrusion of the liposome formulations was carried out using an Avanti polar lipids mini extruder, and the formulations were characterised using a dynamic light scattering (DLS) instrument by Malvern Instruments, Malvern, United Kingdom. Cell uptake was analysed using a BD Accuri™ C6 flow cytometer and upright ZEISS LSM 510 META confocal microscope. PC-3 cells were obtained from the American Type Culture Collection (ATCC, United States). The complete media used includes phenol free iscove's modified dulbecco's medium (IMDM) GlutaMax™ medium (supplemented with 10% fetal bovine serum (FBS), 50 µM 2-mercaptoethanol and 100 U/mL penicillin, and 100 µg/mL streptomycin).

Preparation of Liposomal Formulations

Unilamellar vesicles composed of the selected lipids were prepared through thin-film hydration, following an established procedure.[24] Briefly, a mixture of the selected phospholipids (Table 1) were dissolved in chloroform, then dried using nitrogen gas to form a thin lipid film. The film was further dried in a lyophilliser for 14 hours. The homogenous film was hydrated with BHG (pH 7.2) solution filtered through a 0.22 µm filter for sterilisation, then extruded using an extruder device through a 200 nm polycarbonate membrane at room temperature to obtain homogenous-sized vesicles.

Table 1. *Composition of the Liposome Formulations Tested*

Formulation	Components	Molar Ratio
F1	DSPC:Cholesterol	7:2
F2	Egg PG:Cholesterol	9:1
F3	DOPE:PEG(3000)PE	50:1

Characterisation of Liposomal Formulations

Liposome vesicles (Table 1) were characterised for particle size (nm) and surface charge (zeta potential, mV) using DLS. Particle size dispersion distribution was quantified in terms of polydispersity index (PDI). Disposable cuvettes were used for the measurements at 25 °C and 173° light scattering.

Cell Lines

Human epithelial prostate cancer cells (PC-3 cells) were derived

from the metastatic site (bone; grade IV, adenocarcinoma) of a 62-year-old male.[25] PC-3 cells are commonly used for research in drug development, as they provide a suitable transfection host.[25] Macrophage cells were differentiated from the bone marrow of C57BL/6 mice aged between 2 and 5-weeks.

Flow Cytometry for Cell Uptake Analysis

PC-3 cells: PC-3 cells were cultured on a 48-well plate for 24-hours and counted with the aid of trypan blue. DIL dye (2 μL/mL) was added to the liposome vesicles. Cells were then plated at a density of 4×10^5 cells/well in 24 wells of a 48-well plate and incubated for one day. 20 μL of the liposome formulations (F1-F3) was added to the designated wells and 200 μL of RPMI medium was added to each well and the cells were incubated for 3-hours. After incubation, the cells were treated with FACS (x3) and pharmaceutical benefits scheme (PBS) (x1) and then with TrypLE for 10-minutes and FACS buffer was added. Flow cytometry was performed using a red laser (640 nm) to detect the DIL dye (excitation: 549 nm; emission: 565 nm) present in the cells.

Macrophages: Bone marrow was seeded in 15 cm dishes in DMEM supplemented with 20% LCM. The bone marrow-derived-macrophages were harvested six-days later and 0.5×10^6 cells were seeded in sterile 5 mL polystyrene FACS tubes in 1 mL of DMEM supplemented with 10% LCM. The tubes were incubated overnight at 37 °C with 5% CO_2. The next day, a pipette was used to reduce the culture medium to 100 μL and the cells were returned to the incubator for 1-hour. 20 μL of liposome formulation (F1-F3) was added to the designated wells, and the cells were incubated at 37 °C with 5% CO_2 for 1-hour. The tubes were placed on ice and 3 mL of ice-cold sterile 3% NBCS in PBS with 0.5 mM EDTA was added to each tube. Cells were centrifuged at 176 xg and 4 °C for 5-minutes, then the supernatant was removed. 3% NBCS in PBS with 0.5 mM EDT (3 mL) (x2) was added to the cells and the supernatant was removed each time. The cells were then re-suspended in 3% NBCS in PBS with 0.5 mM EDTA to achieve a total volume of 250 μL. Flow cytometry (BD FACSCanto™ or Beckman Coulter Gallios and analyzed using FlowJo software; TreeStar, Inc., San Carlos, CA, USA) was performed using a red laser (640 nm) to detect the DIL dye (excitation: 549 nm; emission: 565 nm) associated with the cells.

Confocal Microscopy for Cell Uptake Analysis

PC-3 cells: PC-3 cells were prepared in complete media and seeded on glass coverslips in 12-well plates (1.6×10^5 cells/well). The cells were incubated for 24-hours at 37 °C to encourage adherence. The liposome vesicles were stained by the addition of DIL dye (2 μL/mL) and the nucleus of the cells was stained by Hoechst (ThermoScientific™). The cells were washed with PBS buffer (x1) and Opti-MEM/GlutaMax™ (x1). Then, 20 μL of each liposome formulation (F1-F3) was added to each well and the plates were incubated for three hours. The cells were treated with PBS (x3) and then with 300 μL of 4% paraformaldehyde (PFA) at room temperature for 15-minutes. The PFA was removed, and the cells were washed with PBS buffer (x3). The cover slips were removed and mounted on slides for viewing under confocal microscopy using a green filter (500-570 nm) to detect the 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyl indocarbocyanine perchlorate (DIL) dye (excitation: 549 nm; emission: 565 nm) present in the cells. The assay was carried out in triplicate.

Macrophages: Bone marrow-derived-macrophages were prepared in complete media and seeded in 24-well plates (2.5×10^5 cells/well) containing coverslips. The cells were incubated for 24-hours at 37 °C and 5% CO_2. The liposome vesicles were stained by the addition of DIL dye (2 μL/mL) and the nucleus of the cells was stained by Hoechst (ThermoScientific™). The next day, the medium was removed, and 20 μL of the liposome formulations (F1-F3) were added to cells and incubated for four-hours. The medium was again removed, and the cells were washed with PBS (x2). The cells were then fixed with 300 μL of 4% PFA at room temperature for 15-minutes. The PFA was removed, the cells were washed with PBS buffer (x3), and 300 μL of FACS blocking buffer was added for one hour at room temperature. Cells were viewed under confocal microscopy (ZEISS LSM 510 META) using a green filter (500-570 nm) to detect the DIL dye (excitation: 549 nm; emission: 565 nm) present in the cells. The assay was carried out in triplicate.

RESULTS

Characterisation of the Liposome Vesicles

Liposome formulations F1-F3 were prepared using DSPC, egg PG and DOPE, respectively, as their main lipid components with the addition of cholesterol and PEG (3000) PE. The vesicles were characterised by their mean particle size, PDI and surface charge, as shown in Table 2. All formulations had an average vesicle size between 150 nm and 250 nm. F1 and F2 had PDI values of less than 0.15, suggesting the formation of homogenous vesicles. F3 had a higher PDI value (0.7), indicating high polydispersity within the sample. F1 was neutral, F2 was negatively charged and F3 was positively charged.

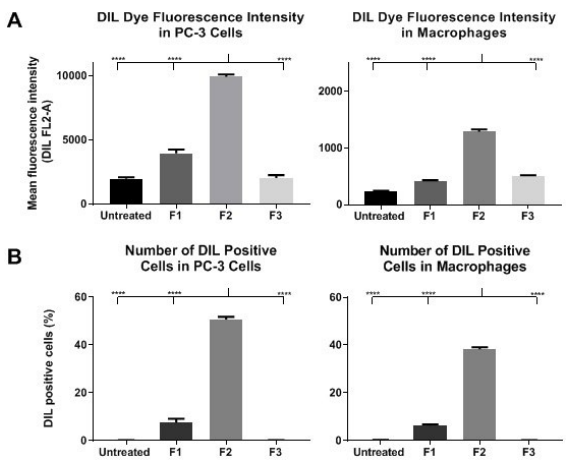
Table 2. *The Average Particle Size, Polydispersity Index (PDI) Value and Surface Zeta Potential of Three Liposome Formulations Tested for Cell Uptake. The Results are Expressed as the Mean±Standard Deviation (n=5)*

Formulation	Components	Particle Size (nm)	PDI Value	Surface Charge (mV)
F1	DSPC:Cholesterol	243±6	0.04±0.03	-4.0±2.4
F2	Egg PG:Cholesterol	165±2	0.14±0.02	-47.2±0.4
F3	DOPE:PEG(3000)PE	159±7	0.70±0.04	20.8±0.8

Uptake of Liposome Vesicles by PC-3 Cells and Macrophages

The formulations were tested for their ability to be taken up by PC-3 cells and macrophages (Figure 1) using flow cytometry. PC-3 cells treated with F2 showed the highest fluorescence intensity, followed by the cells treated with F1. The cells treated with F3, which contained DOPE as the main lipid, had similar fluorescence intensity as the untreated cells. F2 was also taken up most effectively by macrophages. In contrast, the macrophages treated with F1 and F3 had low DIL dye fluorescence intensity, comparable to untreated cells. Overall, F2, which was prepared using egg PG, was taken up most effectively by both cell types.

Figure 1. *Uptake of Liposome by PC-3 and Macrophage Cells Quantified*



*(A) DIL intensity and (B) the number of DIL positive cells as detected by flow cytometry. Each assay was carried out in triplicate and 10,000 events were collected for each measurement. Error bars represent standard deviation. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc multiple comparison test (****, p<0.0001).*

Uptake of Liposome Vesicles in PC-3 Cells and Macrophages Visualised through Confocal Microscopy

Formulation uptake in PC-3 cells and macrophages was also assessed using confocal microscopy (Figures 2 and 3) as a non-quantitative assay to visualise cell uptake. F1 and F2 were effectively taken up by both PC-3 cells and macrophages.

Figure 2. *Microscopy Images of the Cellular Uptake of F1–F3 in PC-3 Cells and with 40x Magnification and the Use of a Green Filter (500 to 570 nm) (excitation: 549 nm; emission: 565 nm). The Nucleus of the Cells was Stained Blue Using Hoechst Solution and the Liposome Vesicles were Stained Red with DIL Dye. The Assay was Carried Out in Triplicate*

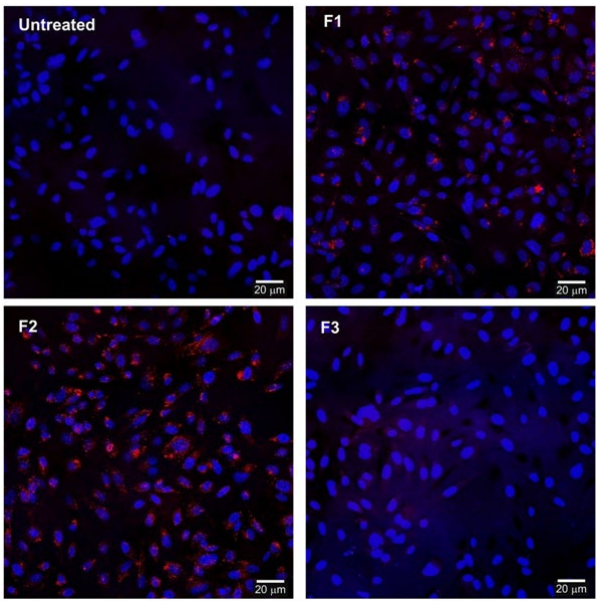
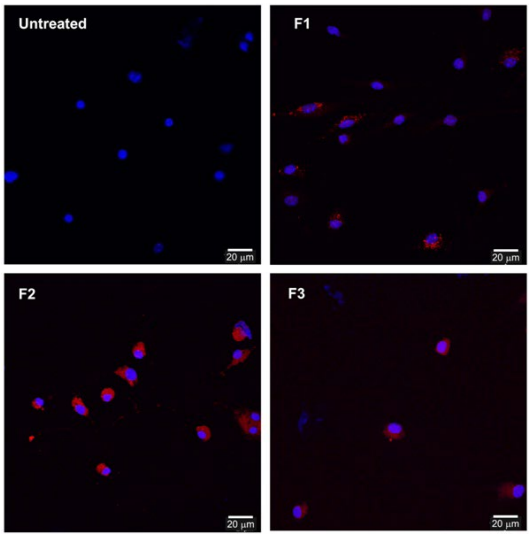


Figure 3. *Confocal Microscopy Images of the Cellular Uptake of F1- F3 in Macrophages with 40x Magnification and the Use of a Green Filter (500 to 570 nm) (excitation: 549 nm; emission: 565 nm). The Nucleus of the Cells was Stained Blue Using Hoechst Solution and the Liposome Vesicles were Stained Red with DIL Dye. The Assay was Carried out in Triplicate*



DISCUSSION

The three liposome formulations were designed to bear positive, negative and neutral charges. The liposome formulations were characterised and analysed to determine their ability to be taken up by two different cells lines: PC-3 cells, which are a commonly used prostate cancer cell line, and macrophages, as representative cells used for targeting delivery of drugs (e.g. anti-TB). Macrophages are antigen-presenting cells (APCs) and therefore are also crucial for vaccine delivery.[26,27] Liposomes can deliver their cargo directly to the cytoplasm of APCs and therefore allow antigen processing and loading on major histocompatibility complex-I (MHC-I). These antigen fragments, known as CD8 epitopes, presented by MHC-I activate cytotoxic T-lymphocytes and cellular immunity in general. Cellular immunity is responsible for immune defence against intracellular pathogens such as viruses and Mtb.[28-30] Consequently, liposomes have been widely used for vaccine delivery including subunit vaccines against TB.[31-34] Liposomes have also been proposed as a secure drug delivery platform for the treatment of cancers, including prostate cancer.[35-37]

The uptake of liposome formulations F1-F3 (Table 1) was determined by flow cytometry and confocal microscopy. F2 prepared with egg PG as the main lipid had the greatest cell uptake in human PC-3 cells and mouse macrophages. Surprisingly, the smaller, positively charged liposomes (F3) were taken up less effectively. However, this is likely explained by the positively charge liposomes being shielded by PEG, which generally reduces cell (including macrophage) uptake.[35] The addition of PEG to liposome vesicles results in a thick coating of hydrophilic PEG on the surface of the vesicles.[38,39] This 'shield' reduces cell adhesion and inhibits uptake by macrophages.[40,41] Moreover, it has also been shown that small, negatively charged liposomes have a higher

internalisation capacity in mononuclear phagocytes, in comparison to larger, positively charged liposome vesicles.[42,43] While F3 had the same size as F2 (according to DLS), the high PDI of F3 is notable, as it suggests the presence of large aggregates.

Different lipids were used in the liposome formulations, which could have affected vesicle uptake. The high uptake of F2 could be related to the presence of egg PG. Liposomes are taken up by macrophages through a receptor-mediated process.[44,45] Specifically, it was observed that alveolar macrophages have receptors that have higher affinities for liposome vesicles prepared with PG in comparison to phosphatidylcholine (PC).[46,47] Therefore, the higher uptake of PG vesicles (F2) compared to PC vesicles (F1) by mouse macrophages in this study is consistent with previous reports.

CONCLUSION

Liposomes have been utilised for the delivery of many biologically active compounds to improve their cellular uptake and reduce toxicity. Liposomal delivery is known to increase the therapeutic index by increasing drug biocompatibility, decreasing drug toxicity and reducing drug exposure to healthy cells. However, many challenges still exist in developing optimised liposome delivery systems for clinical use. Therefore, this study aimed to develop a series of liposome delivery systems for drugs and vaccines. We determined that small, negatively charged liposomes, prepared with egg PG, had high internalisation potential in prostate cancer cell line and macrophages. Further analysis and characterisation of liposomes bearing a drug or an antigen is required to determine the real efficacy of the developed formulations.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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