RESEARCH REPORT

Post-coupling strategy enables true receptor-targeted nanoparticles

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ABSTRACT

A key goal of our research is the targeted delivery of functional biopharmaceutical agents of interest, such as small interfering RNA (siRNA), to selected cells by means of receptor-mediated nanoparticle technologies. Recently, we described how pH-triggered, PEGylated siRNA-nanoparticles (pH triggered siRNA-**ABC** nanoparticles) were able to mediate the passive targeting of siRNA to liver cells *in vivo*. In addition, PEGylated siRNA nanoparticles enabled for long-term circulation (LTC siRNA-**ABC** nanoparticles, LEsiRNA nanoparticles) were shown to do the same to tumour cells *in vivo*. Further gains in the efficiency of siRNA delivery are expected to require active targeting with nanoparticles targeted for delivery and cellular uptake by means of attached biological ligands. Here we report on the development of a new synthetic chemistry and a bioconjugation methodology that allows for the controlled formulation of PEGylated nanoparticles which surface-present integrin-targeting peptides unambiguously and so enable integrin receptor-mediated cellular uptake. Furthermore, we present delivery data that provide a clear preliminary demonstration of physical principles that we propose should underpin successful, *bonefide* receptor-mediated targeted delivery of therapeutic and/or imaging agents to cells.

KEYWORDS: Integrin ligand, integrin receptor, nanoparticles, delivery, cellular uptake, siRNA, RNAi

INTRODUCTION

The specific targeting of nanoparticles to target cells in an organ of interest has been a long-term goal of many laboratories interested in using nanoparticles to mediate the functional delivery of biopharmaceutical agents (including nucleic acids) to defined target cells. One of the main problems in using targeting ligands in conjunction with nanoparticles is the competition between specific-binding mediated cell entry and non-specific enhanced cell uptake mechanisms (Andreu et al, 2008). Previously, we have observed that non-specific effects can be completely overwhelming (Waterhouse et al, 2005), an observation that has led to a series of further studies (Wang et al, 2009) intended to determine and characterize those factors that might influence non-specific enhanced cell uptake mechanisms such as nanoparticle size and heterogeneity, surface hydrophilicity and charge, not to forget other factors such as ligand conformation, points of attachment and surface density.

Recently, we described how pH-triggered, PEGylated siRNA nanoparticles (also known as pH-triggered siRNA-**ABC** nanoparticles according to our recently described **ABCD** nanoparticle structural paradigm (Kostarelos and Miller, 2005; Thanou et al, 2007; Miller, 2008a; Miller, 2008b). These nanoparticles with a surface-covering of 5mol% polyethylene glycol 2000 (PEG^{2000}) were able to mediate the passive targeting of siRNA to liver cells *in vivo*, thereby providing proof of concept for an effective RNAi therapeutic approach to the treatment of hepatitis B virus infections (siFECTplus^{TM} nanoparticles) (Carmona et al, 2009). In addition, PEGylated siRNA nanoparticles enabled for long-term circulation (LTC siRNA-**ABC** nanoparticles; LEsiRNA nanoparticles) were shown to do the same to tumour cells *in vivo*, thereby providing proof of concept for an effective RNAi therapeutic approach to cancer treatment (Kenny et al, 2011). In the case of the pH-triggered, PEGylated siRNA nanoparticles, the PEG coating required for the biological stabilization of these nanoparticles was introduced by a purpose-designed post-coupling methodology.

Here, we report how this methodology has now been adapted to introduce integrin-targeting peptide motifs onto liposome and nanoparticle surfaces and so enable integrin receptor-mediated cellular uptake of agents to cells. There have been some spectacular data obtained in recent years using $\alpha_v\beta_6$ integrin-receptor binding motifs for receptor-mediated delivery of different imaging agents to cells *in vivo* (DiCara et al, 2007; Hausner et al, 2007; DiCara et al, 2008; Hausner et al, 2009). We have described previously the use of integrin-receptor targeting peptides to promote functional gene delivery to cells (Harbottle et al, 1998; Cooper et al, 1999). Here, we now report on the use of an alternative cyclic pentapeptide construct that makes use of a single D-tyrosyl amino acid residue to present the arginine-glycine-aspartate (RGD) amino acid residue triad in an "active" conformation capable of mediating intracellular delivery through $\alpha_v\beta_{3/5}$ receptor binding and internalization (Chen et al, 2004). Arginine-glycine-glutamate (RGE) controls are also reported in order to provide the means to demonstrate pure integrin-receptor mediated cellular uptake by RGD presenting imaging nanoparticles.

MATERIALS AND METHODS

Lipids and synthetic chemistry

Dioleoyl L-α-phosphatidylethanolamine (DOPE) **1**, dimyristoyl L-α-phosphatidylcholine (DMPC) **2** (Sigma), DOPE-Lissamine-Rhodamine B (DOPE-Rhoda) **3** were obtained from Avanti Polar Lipids (USA) (Figure 1). General synthetic procedures were performed as described previously (Carmona et al, 2009; Mével et al, 2010). Syntheses of N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) **4** and cholesteryl-aminoxy (CA) lipid **5** were performed as described previously (Keller et al, 2003; Oliver et al, 2004; Carmona et al, 2009) (Figure 1). The synthesis of other compounds necessary for our experiments were performed as described (Scheme 1). HPLC purification of final products required the use of Vydac C-4 reversed phase preparative column with 1ml/min flow rate: Mobile phases as follows used trifluoroacetic acid (TFA); **A**: H_2O (0.1%, v/v, TFA); **B**: MeCN (0.1%, v/v, TFA); **C**: MeOH (0.1%, v/v, TFA). Program set at: 0-15.0min (100%, **A**), 15.1-25.0min (0-100%, **B**), 25.1-45min (100%, **C**), 45.1-55min (100%, **A**). Elution parameters: RGD-PEG^{1000}-CHO **13a** R_t 15.2min, RGE-PEG^{1000}-CHO **13b** R_t 14.8min, PrNHCO-PEG^{1000}-CHO **14** R_t 15.8min, CA lipid **5** R_t 23.8min, RGD-PEG^{1000}-CA **15a** product R_t 24.1min, RGE-PEG^{1000}-CA **15b** product R_t 23.8min, and PrNHCO-PEG^{1000}-CA **16** product R_t 26.3min; mass spectrometry: m/z (ESI) 1878 ($M\text{-}H^-$, RGD-PEG^{1000}-CHO **13a**), 1917 (MNa^+, RGE-PEG^{1000}-CHO **13b**), 1326 (MH^+, PrNHCO-PEG^{1000}-CHO **14**), 2406 (MH^+, RGD-PEG^{1000}-CA **15a**), 2443 (MNa^+, RGE-PEG^{1000}-CA **15b**), 1869 (MNa^+, PrNHCO-PEG^{1000}-**CA 16**) (Figure 1).

Liposome and nanoparticle formulations

CL1 and **CL2** were prepared in the following way (Table 1). Designated lipids were dissolved in chloroform at 5mg/ml and then appropriate aliquots were combined in a presilanized round-bottom flask (5ml). In each case, the organic solvent was evaporated to dryness to form an even thin lipid film that was further purged with a stream of argon gas to remove residual traces of the organic solvent. The lipid film was hydrated using double distilled H_2O (ddH_2O) to give a multilamellar liposome suspension that was subsequently subjected to sonication in a water bath at 40ºC for 30min (sonomatic® water bath, Langford Ultrasonics, 33 kHz ultrasound frequency). Post-sonication, small unilamellar vesicles (50-80nm) (**B**-component, **CL1** or **CL2**) were diluted to 1.5mg/ml and incubated at room temperature for 15min. Thereafter, appropriate aliquots of α-terminally modified-PEG^{1000}-CHO **13a**, **13b** or **14** (1mg/ml in water, **CD** molecules) were introduced for post-coupling such that their final composition was between 1 and 10mol% of total lipid. The pH of solution was monitored by pH boy (Camlab Ltd, Cambridgeshire, UK) and adjusted to pH 4 if required by addition of small aliquots of aqueous solutions of NaOH (0.99M) or HCl (0.99M). On completion of the reaction (approximately 16-24hr) (as judged by HPLC analysis), the solution pH was accurately readjusted to pH 7 resulting in 10mol% PEGylated **BCD1** and 1-5mol% PEGylated **BCD2** nanoparticles, respectively, ready for use. 1-5mol% PEGylated **ABCD2** nanoparticles were prepared by combining 1-5mol% PEGylated **BCD2** nanoparticles with various volumes of aqueous 4mM HEPES, pH 7.0-7.4, siRNA solution (50μM **A**-component) under heavy vortex conditions (final [siRNA] typically 100μg/ml; 7μM) using a lipid:siRNA ratio of 12:1 (w/w) that correlates with a lipid-nucleic acid N/P charge ratio of approximately 2.

FACS analysis

These experiments were performed on a FACS Calibur instrument (BD Biosciences). In each set of indicated experiments 1, 2 or 5mol% PEGylated **ABCD2** nanoparticles (140±60nm) were prepared with siRNA ([siRNA] 80pmoles/well) and uptake studies performed with HUVEC cells previously grown to 80% confluent at 37ºC, 10% CO_2.

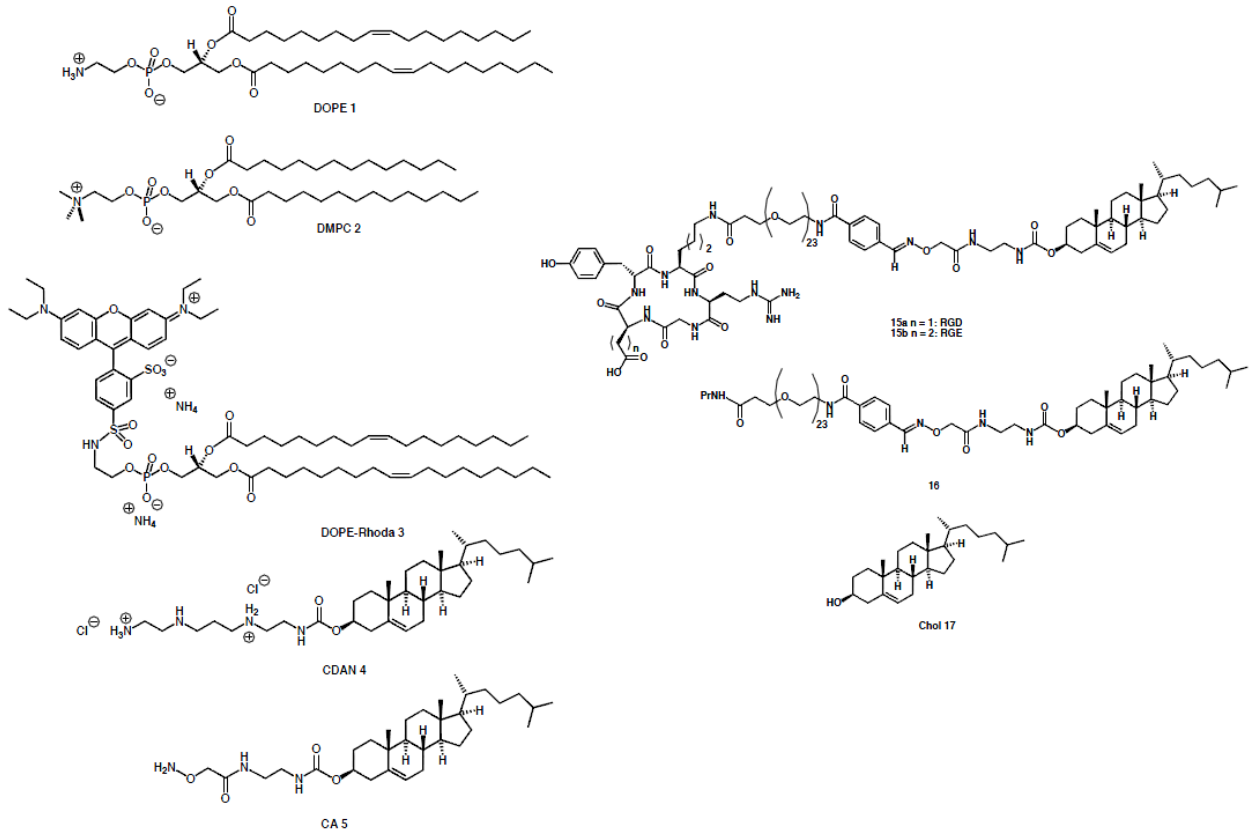
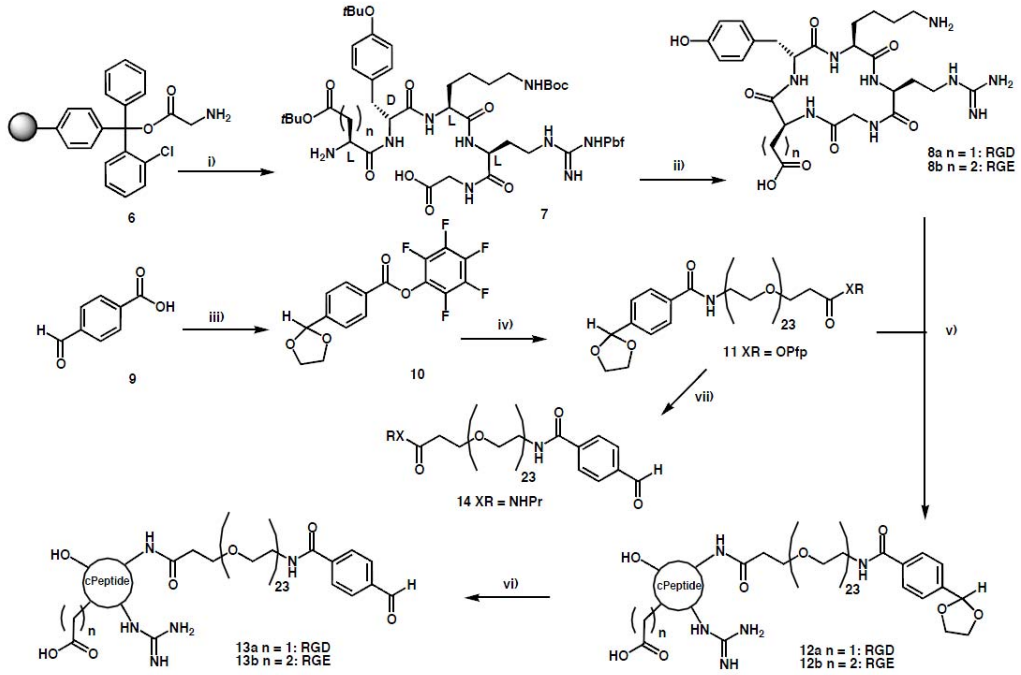


Figure 1. Main lipids used and oxime lipid conjugates formed during the reported investigations. Main lipids were used to prepare liposomes **CL1** and **CL2** and hence 10mol% PEGylated **BCD1** and 1-5mol% PEGylated **BCD2** nanoparticles.



Scheme 1. Syntheses of α-terminally modified-PEG^{1000}-CHOs: i) a) Side chain protected, Fluorenylmethyloxycarbonyl (Fmoc) amino acid residue *N*-terminal coupling of L-Arg(*N*Pbf), L-Lys(εBoc), D-Tyr(*Ot*Bu) and finally L-Asp(*Ot*Bu) [or L-Glu(*Ot*Bu)] to glycyl-2-chlorotrityl resin **6** using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) with di-isopropylethylamine (DIPEA) to mediate residue coupling and 2% (v/v) each of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and piperidine in dimethylformamide (DMF) for Fmoc deprotection between rounds of peptide bond formation: b) resin release with 20%

(v/v) each of acetic acid and trifluoroethanol (TFE) in CH_2Cl_2, 31-78%; ii) a) 3eqv diphenylphosphoryl azide (DPPA) in dry DMF, 5 eqv $NaHCO_3$, 0°C – ambient temperature, pH 8.5, 74-80%: b) 2.5% (v/v) each H_2O and triisopropylsilane (TIS) in trifluoroacetic acid (TFA), 80-100%; iii) a) HO-C_2H_4-OH, Al_2O_3, CCl_4, reflux, 27%: b) pentafluorophenol (PfpOH), *N,N'*-Dicyclohexylcarbodiimide (DCC), EtOAc, 71%; iv) a) amino-PEG^{1000}-propionic acid, 4 eqv triethylamine (TEA), $CHCl_3$, reflux, 84%: b) 1.2 eqv PfpOH, 1.2 eqv DCC, EtOAc, 76%; v) 0.2M Na_2HPO_4, 0.1M NaOH, 69-100%; vi) 10% (v/v) TFA in H_2O, 100%; vii) a) propylamine ($PrNH_2$), 4 eqv TEA, $CHCl_3$, reflux, 100%: b) 10% (v/v) TFA in H_2O, 51% (v/v).

Table 1. Lipid compositions of two liposome formulations prepared in double distilled water with final diameters of 50-80nm. For lipid structures see Figure 1.

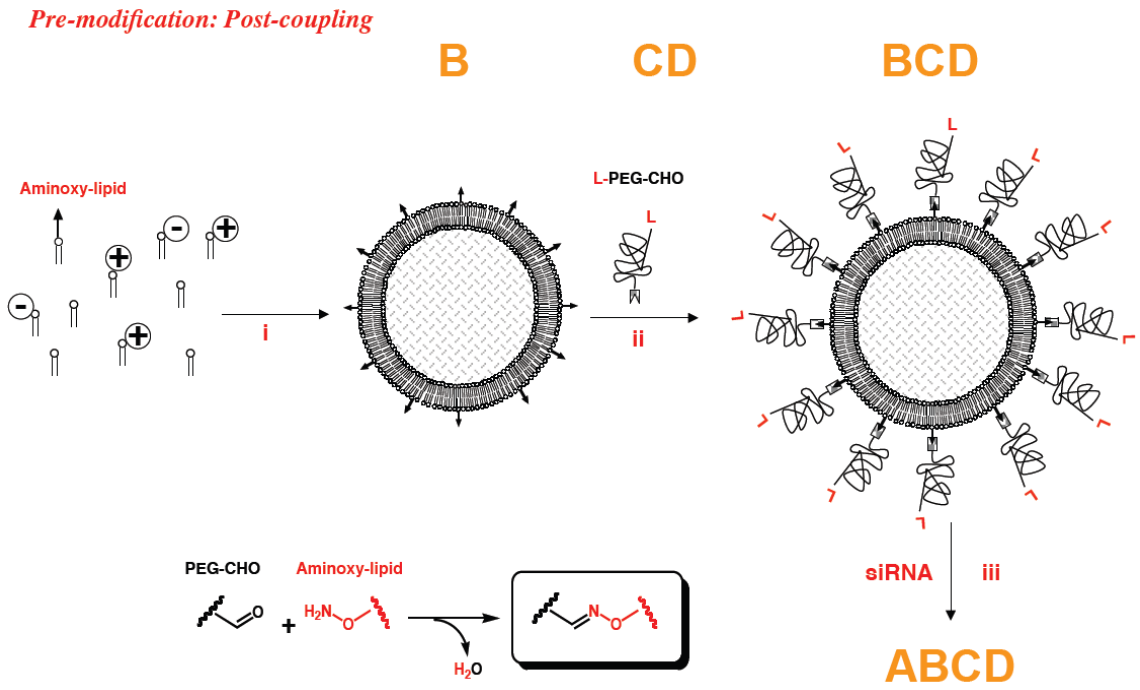
Molar % of Lipid	**CL1**	**CL2**
DOPE **1**	-	60
DMPC **2**	59	-
DOPE-Rhoda **3**	1	1
CDAN **4**	-	20
CA **5**	20	10
Chol **17**	20	9

RESULTS

Post-coupling represents an optimal way to attach a biological targeting ligand of interest to a nanoparticle surface with controlled mol% attachment and ligand orientation (Scheme 2). Our approach to the development of a post-coupling methodology has been to learn from the chemistry employed to create pH-triggered, PEGylated siRNA nanoparticles (as mentioned in the Introduction) in order to design-hybrid targeting ligands (L) that comprise a peptide targeting moiety conjugated to a PEG^{1000} extension chain enabled for postcoupling to a nanoparticle surface (Scheme 2). RGD peptides and corresponding RGE control peptides were prepared as follows (Scheme 1). Starting with glycyl-2-chlorotrityl solid-phase peptide resin **6**, the desired pentapeptide RGD-targeting moiety was prepared in an open chain, protected form **7** by solid-peptide synthesis, then subject to cyclization and deprotection to give cyclic pentapeptide **8a**. A control cyclic-RGE pentapeptide **8b** (with aspartate [D] replaced by glutamate [E]) was prepared in an equivalent manner. Thereafter, the aldehyde functionality of 4-carboxybenzaldehyde **9** was acetal protected and the carboxy function was activated for coupling by means of pentafluorophenol (PfpOH) esterification giving **10**. Reagent **10** was then coupled to amino-PEG^{1000}-propionic acid and the free carboxylate activated once again for coupling by means of PfpOH esterification giving heterofunctional PEG^{1000} moiety **11**. The coupling of **11** to **8a** or **8b** resulted in RGD acetal **12a** and RGE acetal **12b** respectively. Routine acetal deprotection yielded the desired products RGD-PEG^{1000}-CHO **13a** and control RGE-PEG^{1000}-CHO **13b** (Scheme 1). An alternative control aldehyde was obtained through combination of heterofunctional PEG^{1000} moiety **11** with propylamine followed by acetal deprotection resulting in the non-peptide control product PrNHCO-PEG^{1000}-CHO **14** (Scheme 1). All three α-terminally modified-PEG^{1000}-CHO compounds prepared as described above were used in subsequent experiments described below.

Using all three α-terminally modified-PEG^{1000}-CHO compounds in turn, coupling reactions were performed individually with neutral cholesteryl-aminoxy lipid (CA) **5** (Figure 1) and then with neutral liposomes (equivalent in composition to **CL1**, Table 1). Reactions were monitored by HPLC and mass spectrometry in water at pH4 and shown to reach completion after 16-24h stirring at ambient temperature giving **15a**, **15b**, or **16** as appropriate. Liposome-coupling experiments were then performed under similar conditions in order to couple RGD-PEG^{1000}-CHO **13a** (or control aldehydes RGE-PEG^{1000}-CHO **13b** and PrNHCO-PEG^{1000}-CHO **14**) to alternative liposome formulations **CL1** or **CL2** (Table 1, Figure 1), making use of CA lipid **5** once again in order to present the aminoxy functional groups required for conjugation of liposome surfaces with each α-terminally modified-PEG^{1000}-CHO compound in turn (Scheme 2). Upon conclusion of these liposome-coupling reactions, pH was raised to 7 in order to stabilize the resulting oxime bonds and the resulting PEGylated liposomes. Resulting ligand-PEGylated liposomes (approx 100nm in diameter) were described as belonging to either **BCD1** or **BCD2** nanoparticle families depending upon whether the parent liposome formulation used in preparation was **CL1** or **CL2**, respectively.

Initially **BCD1** family members RGD-**BCD1**, RGE-**BCD1** and PrNHCO-**BCD1** were prepared with 1mol% of DOPE-



Scheme 2. Schematic illustration of nanoparticle formulations involving the inclusion of an aminoxy coupling lipid by a formulation procedure known as premodification followed by the post coupling of α-terminally ligand (L)-modified PEG aldehydes (L-PEG-CHO).

Rhoda **3** for fluorescence microscopy cell uptake studies. The results demonstrate that only 10mol% PEGylated RGD-**BCD1** nanoparticles were capable of delivering measurable and observable levels of fluorescent-label to integrin-rich human umbilical vein endothelial cells (HUVEC) (Figure 2A). These data are completely consistent with receptor specific-binding mediated cell entry. The discrimination between RGD presenting and control nanoparticles is near total. This is very interesting in view of the physical properties of **BCD1** nanoparticles, namely approx 100nm in diameter, 10mol% PEGylation with PEG^{1000}, and a ζ-potential of essentially zero mV. The lack of charge originates from the fact that the majority lipid components needed to prepare **CL1** liposomes were neutral while the minority CA lipid **5** component possesses an aminoxy functional group unprotonated at pH 7 due to its pKa value of about 4.5 (compared with the pK_a values of more typical amine functional groups that are found around 10) (Castro, 1990). We would argue that such nanoparticle physical properties could be an ideal starting point from which to optimize receptor specific cellular uptake of nanoparticles relative to non-specific enhanced cell uptake.

Subsequently, **BCD2** family members RGD-**BCD2**, RGE-**BCD2** and PrNHCO-**BCD2** were prepared with 1.0 mol% of DOPE-Rhoda **3** and up to 5mol% of each α-terminally modified-PEG^{1000}-CHO for fluorescence-activated cell sorting (FACS) studies. In addition these cationic liposome systems were further combined with a non-functional nucleic acid (small interfering RNA [siRNA]) (**A** component) in order to neutralize the positive charge and convert the **BCD2** family members in to a varieties of corresponding low charge **ABCD2** nanoparticles with lipid:siRNA N/P charge ratios of approximately 2. The physical properties of these nanoparticles were diameters of approximately 140nm, up to 5mol% PEGylation with PEG^{1000}, and a ζ-potential of less than 10mV. In these cases, FACS studies were performed with low charge 1-, 2- and 5mol% PEGylated RGD-**ABCD2**, RGE-**ABCD2** and PrNHCO-**ABCD2** nanoparticles. In each case, the discrimination between RGD presenting and control nanoparticles was still clearly present and was found to increase as a function of the mol% PEGylation coverage with PEG^{1000} (Figure 2B). The fact that the discrimination was not as substantial as observed above (Figure 2A) appears to be due to the lower mol% levels of PEGylation coverage and the presence of residual nanoparticle charge. Therefore, control of both PEGylation levels (plus other PEG-structure related criteria) and residual charges are essential in order to optimize receptor mediated cell uptake in preference to non-specific enhanced cell uptake mechanisms.

DISCUSSION

Biological receptor mediated functional delivery of imaging agents is well established (DiCara et al, 2007; DiCara et al, 2008; Hausner et al. 2007; Hausner et al, 2009), even of genes (Harbottle et al, 1998; Cooper et al, 1999). However, receptor-mediated delivery cannot be taken as a given following the covalent attachment of a biological targeting ligand to a nanoparticle (Waterhouse et al, 2005; Andreu et al, 2008). Therefore, in our view, receptor-mediated delivery phenomena should never be treated with the casual assumption that receptor-mediation is inevitable once an appropriate receptor-specific ligand has been mounted on a nanoparticle surface. Any given receptor-specific ligand should in fact be subject to a standard set of trial experiments

to ensure that the biophysical properties of a nanoparticle platform and the presentation of the receptor-specific ligand are optimized for *bonefide* receptor-mediated delivery of the corresponding nanoparticle. In this paper we describe just such a set of trial experiments (Figure 2) with sufficient controls to demonstrate clearly that specific receptor-mediated uptake by cells has been enabled over background. Accordingly, we would like to propose that both studies involving first the delivery of an imaging agent and then second a potential agent of pharmaceutical interest (API), should be standard assays to perform and demonstrate before all else that a nanoparticle-attached ligand is truly a receptor-specific ligand in the context of the nanoparticle platform to which the ligand is covalently attached. If this is not shown, then nanoparticle biophysical properties, ligand attachment and orientation, and mol% ligand presentation should all be systematically altered until specific receptor-mediated cell uptake can be observed substantially over and above any non-specific enhanced cell uptake background (Kamaly et al, 2009; Kamaly et al, 2010).

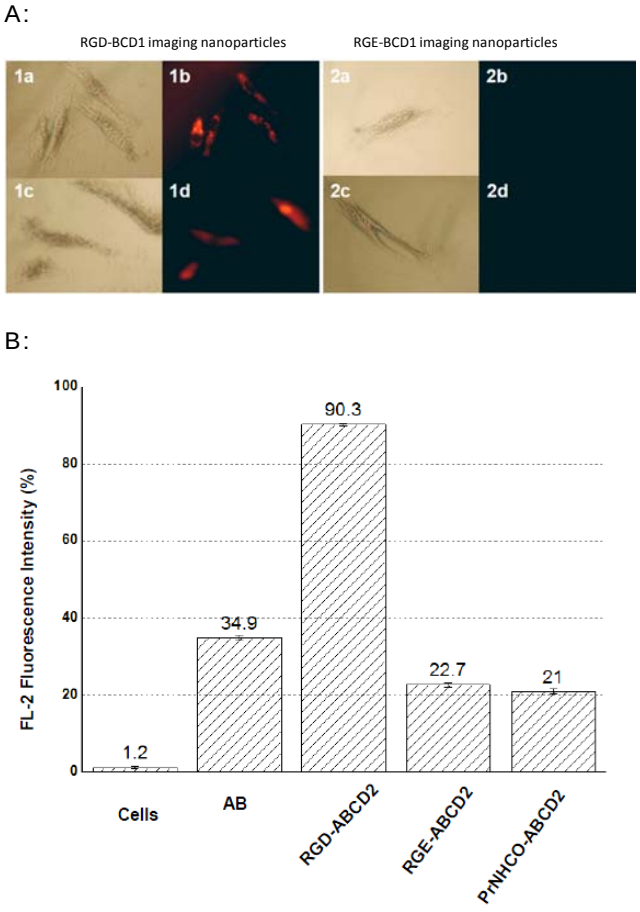


Figure 2. A. Fluorescence microscopy of HUVEC cells, previously grown to 80% confluent at 37°C with 10% (v/v) CO_2, treated with 10mol% PEGylated **BCD1** nanoparticles prepared from RGD-PEG^{1000}-CHO **13a** or RGE-PEG^{1000}-CHO **13b**. Cells were in contact with nanoparticles for at least 4hr prior to mounting and observation of rhodamine fluorescence under a NiKon 600 Fluorescence microscope. **B.** Fluorescence activated cell-sorting (FACS) analysis involving HUVEC cells. Cell uptake was determined after 4hr using rhodamine fluorescence and detected using the FL-2 channel. Data were plotted as a function of theoretical maximum of cell uptake. Data shown come from an experiment with 5mol% PEGylated **ABCD2** nanoparticles. Other similar experiments were conducted with 1 and 2mol% PEGylated **ABCD2** nanoparticles. **AB** corresponds with simple siRNA-lipoplex control nanoparticles, formulated from **CL2** cationic liposomes and an equivalent final [siRNA].

In our case here, we can say with confidence that our post-coupling chemistry and methodology for the attachment of integrin-targeting RGD ligands has resulted in the successful formation of integrin-targeted imaging nanoparticles that can also mediate integrin-specific delivery of an API such as siRNA to $\alpha_v\beta_{3/5}$ integrin-receptor presenting cells. Therefore, this post-coupling chemistry and premodification-postcoupling methodology could be applicable to other nanoparticle platforms with equal success provided that the biophysical properties of the nanoparticle platform also conform to the following biophysical parameters:

- Nanoparticle dimensions of approx 100nm in diameter
- Nanoparticle ζ-potential values that converge on neutral (0 mV)
- Nanoparticle ligand surface coverage of approx 2 mol% (or higher)

Further research with this and other nanoparticle systems will now be needed to demonstrate if these three nanoparticle "rules" for receptor-mediated cell entry are indeed general rules or simply guidelines for receptor-mediated cellular uptake of nanoparticle systems by corresponding receptor expressing cells *in vitro*, *ex vivo* and/or *in vivo*.

CONCLUSIONS

The data described here represent the completion of a first study involving the preparation of ligand-mounted PEGylated nanoparticles constructed by a bespoke pre-modification postcoupling methodology. Data suggest for the first time in our hands that this methodology may be used to ensure that receptor mediated cell uptake of attached nanoparticles can be "engineered" to dominate non-specific enhanced cell uptake mechanisms.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

PEG; polyethylene glycol
CDAN; N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine
CA; cholesteryl-aminoxy lipid
DOPE; dioleoyl L-α-phosphatidylethanolamine

DMPC; dimyristoyl L-α-phosphatidylcholine
DOPE-Rhoda; Lissamine-rhodamine B-DOPE
HBTU; 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
DIPEA; di-isopropylethylamine
DBU; 1,8-diazabicyclo[5.4.0]undec-7-ene
DMF; dimethylformamide
TFE; trifluoroethanol
DPPA; diphenylphosphoryl azide
TIS; triisopropylsilane
TFA; trifluoroacetic acid
PfpOH; pentafluorophenol
DCC; *N*,*N*′-Dicyclohexylcarbodiimide
TEA; triethylamine

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