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Effects of arginine-based surface modifications of liposomes for drug delivery in Caco-2 colon carcinoma cells

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Highlights

- Liposomes modified with arginine derivatives to target colon cancer cells
- Encapsulated doxorubicin in many modified liposomes exhibited improved toxicity
- Statistical correlations provide design rules for efficient cancer therapy

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Abstract

Liposomal encapsulation of chemotherapeutics improves circulation time and decreases off-target effects through the enhanced permeability and retention (EPR) effect. Improving the efficacy of these drug carriers through surface modification could benefit patients. A library of arginine derivatives was conjugated to liposomes through carbodiimide chemistry. Both unmodified and modified liposomes were loaded with doxorubicin and exposed to Caco-2 colon carcinoma cells to measure the half maximal inhibitory concentration (IC_{50}). Most of the modifications improved the toxicity of doxorubicin. Principal component analysis (PCA) was used to uncover correlations between physicochemical properties (a measure of lipophilicity (log *P*), partition coefficient (log *D*), number of hydrogen bond donors, number of hydrogen bond acceptors, freely rotating bonds, surface tension, polarization surface area, and isoelectric point) and the IC_{50} of encapsulated doxorubicin. Generalized rules for improved toxicity were also developed, which stated that improved drug carriers should have at least 4 hydrogen bond donors, between 4 and 6 freely rotating bonds, an isoelectric point above 5.5, and a log *P* between -2 and -1. Using these relationships along with previously obtained correlations for macrophages, selective targeting and the understanding of how to rationally design such drug carriers can be improved.

Keywords: liposomes; Caco-2; arginine derivatives; drug delivery

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1. Introduction

Many cell-penetrating peptides (CPP) are rich in arginine residues, including HIV-1 Tat peptides and penetratin.[1,2] The exact internalization mechanism of arginine-rich peptides is controversial, with many suggesting that macropinocytosis is responsible for uptake,[3,4] while others suggest that non-endocytic pathways, such as direct membrane translocation, are involved.[5,6] One of the first steps in internalizing these peptides involves CPPs binding with the negatively charged polysaccharides and lipids comprising the cell wall. The positive charge of CPPs is one component facilitating internalization, however, the functional guanidine group of arginine is also a key component. Peptides containing nine L- or D-arginine residues are much more efficient at entering cells than equivalent length lysine, histidine, or ornithine peptides,[7] demonstrating that charge alone is insufficient to promote internalization. Additionally, replacing lysine residues with arginine in Tat results in more effective internalization.[8] CPPs have been used *in vivo* to treat diseases through tethering the CPP to a therapeutic protein, such as Tat-Bcl-xL protein (B-cell lymphoma-extra large) to inhibit apoptosis for ischemia.[9] Penetratin and Tat have also been tethered to liposomes to alter doxorubicin efficiency.[10] Tat-liposomes had increased intracellular accumulation in A431 squamous carcinoma cells, while penetratin-liposomes had a more modest increase in intracellular accumulation. Even though Tat increased uptake of the liposomes, upon loading with doxorubicin, cytotoxicity was not improved.[10]

Previously, we have modified liposomes with arginine-like derivatives to improve selective targeting to macrophage subpopulations.[11] Macrophages exhibit a spectrum of phenotypes, the extremes of which are classically activated M1 macrophages and alternatively

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activated M2 macrophages. Tumor associated macrophages (TAMs) are typically phenotypically M2 macrophages.[12–14] These cells release growth factors and pro-angiogenic factors that enable metastasis and are correlated with poor prognosis in cancer patients.[15,16] Due to the anti-tumor activity of M1 macrophages, our goal was to selectively target M2 macrophages through chemical modifications of liposomes carrying doxorubicin. Principal component analysis (PCA) was used to examine the relationships between the physicochemical properties of the liposome modifiers and the half maximal inhibitory concentration (IC_{50}) for classically activated M1, alternatively activated M2, and naïve M0 macrophages. Lipinski's rule of five is a rule-of-thumb used to predict druglikedness. This rule states that "poor absorption or permeation are more likely when: there are more than 5 H-bond donors (expressed as the sum of OHs and NHs); the molecular weight is over 500; the log *P* is over 5; there are more than 10 H-bond acceptors (expressed as the sum of Ns and Os); compound classes that are substrates for biological transporters are exceptions to the rule." [17] We used the physicochemical properties from this rule and its variants to examine correlations between materials properties and drug delivery. M2 cells were found to be dependent on the zeta potential of the liposomes and the lipophilicity (log *P*) of the modifier, while M1 and M0 cells were correlated with hydrogen bond donors and the number of freely rotating bonds.

One of the next steps in rational design of drug carriers is to ascertain how other cells respond to surface modifications of liposomes. Tumors taken from cancer patients and animal experiments have shown changes in branching of N-linked oligosaccharides.[18] These changes have resulted in increased lectin leucoagglutinin binding for breast and colon cancers.[19] In fact, aberrant glycosylation is a hallmark for the transition from healthy to malignant tissue.[20] Since

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the first interaction between liposomes and cells will be with the glycan layer, which is composed of the glycocalyx and mucin for epithelial cells in the gastrointestinal tract,[21] altering the surface composition of the liposome may result in altered killing capacity of the drug carriers.

Caco-2 cells (a human cell line of heterogenous colorectal adenocarcinoma) overexpress P-glycoprotein (P-gp), which is an energy-dependent drug efflux pump that leads to resistance to chemotherapeutics[22] by actively pumping doxorubicin in the basolateral-to-apical direction.[23,24] Previously, polyether-modified poly(acrylic acid) block copolymers were used to enhance doxorubicin delivery by exploiting the high charge density and surface activity of the polymer. Increased accumulation of doxorubicin in Caco-2 cells was mediated using these polymers.[23] The overexpression of P-gp presents a challenge in delivering chemotherapeutics to these cells and likely requires a different rational design of the drug carriers than targeting subpopulations of macrophages.

Doxil® was the first FDA approved nanomedicine and is a liposomal formulation of doxorubicin for chemotherapeutic treatment of a variety of cancers. One of the major complications of doxorubicin is cardiomyopathy, with mortality at ~50%.[25] This risk is significantly diminished using liposomal doxorubicin, while drug efficacy remains similar to free doxorubicin.[26] Liposomal doxorubicin has also been observed to Doxil® significantly reduces these off-target effects and improves the circulation half-life to 90 h.[27] The improved circulation time allows Doxil® to target tumors through the EPR effect, which is a passive targeting method.[27] One method to improve targeting of liposomal doxorubicin, thereby reducing off-target effects such as cardiomyopathy, is to introduce active targeting. Several FDA approved

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antibodies targeting specific receptors overexpressed in tumors are clinically in use.[28] While antibodies are efficient at targeting tumors, they suffer from high cost, limited shelf life, and the potential to elicit an immune response upon repeated dosing.[29] A potential alternative to antibodies is using small molecules. Identification of "hit" molecules is typically done through high-throughput screening of libraries of compounds. Weissleder and coworkers developed a chemically diverse library focusing on primary amines, alcohols, carboxylic acids, thiols, and anhydrides to target pancreatic cancer.[30] This approach has also been used to target TAMs[11,31,32] and to develop delivery materials for gene delivery.[33–35] Our goal is to examine the physicochemical properties that enhance delivery to Caco-2 cells to improve rational design, thus decreasing the size of a potential library used in screening.

Rational design of drugs and drug carriers has been a focus of biomedical research for decades. Recent advances have been made in developing lipids for siRNA delivery in that the lipids must be cationic to disrupt the endosome, enabling cytosolic delivery of the payload.[36,37] The size, shape, and surface charge of nanoparticles influence biodistribution;[38] however, there are no rules-of-thumb for designing drug carriers akin to Lipinski's rule-of-five for orally active drugs. In this study, we produced liposomes and modified the surfaces with fourteen arginine-based molecules to determine the effects of surface modifications on drug release in Caco-2 cells using doxorubicin, an anti-cancer drug. The IC_{50} values for doxorubicin loaded in the modified and unmodified liposomes were compared to see how killing efficiency can be improved.

2. Materials and Methods

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All materials used in this study were purchased through Sigma and were used as received, unless stated otherwise. Fresh deionized (DI) water (Milli-Q, Thermo Scientific Nanopure, Waltham, MA) was used throughout this study.

2.1. Liposome Modification

Preparation and modification of these liposomes has been previously described.[11,39] Briefly, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, Inc., Alabaster, AL) (87.5 mg) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Inc.) (43.75 mg) were dissolved in chloroform (15.75 mL) and rotary evaporated at 40°C for 5 min in a 250 mL round bottom flask. The lipids were resuspended in 15 mL phosphate buffered saline (PBS, diluted from 10× solution to 10 mM, pH 7.4, Fisher, Pittsburgh, PA), dialyzed against DI water overnight, and freeze-dried in a lyophilizer (Labconco, 4.5 L, Kansas City, MO). Fourteen different molecules (**Figure 1**) were used to modify the liposomes: 2-amino-3-guanidinopropionic acid, 3-guanidinopropionic acid, nitroarginine, creatine (Fisher), carnitine, citrulline, 5-hydroxylysine, acetylglutamine, N-carbamyl-α-aminoisobutyric acid, acetylcarnitine, 2,4-diaminobutyric acid, acetylornithine, albizziin, and arginine (Amresco, Solon, OH). In a vial, 2 mL PBS, 10 mg of lyophilized liposomes resuspended in PBS at 5 w/v%, 2 mg surface modifier, and 20 mg N-(3-dimethylaminopropyl)-N′ -ethylcarbodiimide hydrochloride (EDC) were stirred overnight. This process was repeated for all 14 surface modifiers. The particles were dialyzed overnight against DI water and subsequently lyophilized.

2.2. Drug Loading

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Doxorubicin was used as a model drug. In 2 mL citric acid (150 nM, pH 4) 10 mg of the modified or unmodified (UM) liposomes described above were suspended and extruded 21 times using an Avanti Mini-Extruder. Liposomes were then neutralized to pH 7.4 with either NaOH or HCl. The doxorubicin solution and neutralized liposomes were incubated at 65°C. After 10 min, 200 µL doxorubicin (10 mg/mL in PBS) was added to the liposomes and incubated at 65°C for an additional 45 min. The loaded liposomes were centrifuged at 3000 rpm for 5 min. The supernatant was removed, and the pellet was suspended in 2 mL of clear media for a concentration of 250 µg/mL. Loading efficiency was determined by adding 50 µL of the supernatant to 50 µL PBS in a 96 well plate. Serial dilutions of 1 mg/mL doxorubicin were used to generate a standard curve. The amount of doxorubicin not entrapped in the liposome was determined by measuring the absorbance at 490 nm with a reference at 630 nm using a BioTek Synergy HT Multidetection Microplate Reader (BioTek, Winooski, VT).

2.3. Cell Culture

Caco-2 human colorectal adenocarcinoma cells (ATCC, Manassas, VA) were cultured at 37°C with 5% CO_2 in complete medium (CM), consisting of 10% fetal bovine serum, 1% penicillin, and 100 µg/L streptomycin in Roswell Park Memorial Institute (RPMI) medium. Cells were passaged every four to six days and subcultured between 6.7×10^3 and 2.7×10^4 cells/cm^2.

2.4. Cell viability

Caco-2 cells were seeded in 96-well plates at 1.6×10^5 cells/cm^2 in 100 µL CM in every well except the negative control. After 24 h, the supernatant was removed and replace with

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fresh CM and 10 µL 0.625% modified and unmodified liposomes. Cells without liposomes served as positive controls (PC) and liposomes in the absence of cells were used as negative controls. After a 48 h incubation, the medium was aspirated and wells were washed with PBS. To each well, 10 µL of 5 mg/mL (in DI water) methyl thiazol tetrazolium (MTT) and 100 µL CM were added to each well. The plate was incubated at 37°C for 4 h. Subsequently, 85 µL was removed from each well and 100 µL dimethyl sulfoxide (Fisher) was added to dissolve the insoluble formazan crystals. The plate was incubated at 37°C for 10 min. The optical density in each well was measured at 540 nm with a reference of 690 nm. Data was normalized to the positive controls.

2.5. IC_{50}

Caco-2 cells (1.6×10^5 cells/cm^2 in 100 µL CM in every well except the negative control) were seeded into a 96 well plate for 24 h. A serial dilution of liposomes loaded with doxorubicin was added to the plate. The positive control consisted of cells without liposomes. After 48 h incubation, an MTT assay was performed as described above.

2.6. Statistics and data analysis

Statistical analysis was performed using JMP statistical software (Cary, NC). Statistical significance of the mean comparisons was determined by a two-way ANOVA. Pair-wise comparisons were analyzed with Tukey's honest significance difference test. Differences were considered statistically significant for $p < 0.05$. Principal component analysis (PCA) was used to describe the covariance structure. In this work, PCA was used to explain relationships between the physicochemical properties of the modifier and the IC_{50} value of doxorubicin encapsulated

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in modified liposomes on Caco-2 cells. These relationships were visualized using linear combinations of the original variables and plotting them on axes that represent directions of maximum variance. Correlations exist for projections in similar (or opposite, for negative correlations) directions and of similar magnitude for the first and second principal components (PC1 and PC2) in two-dimensional space.

3. Results and discussion

3.1. Liposome modification and characterization

Liposomes where fabricated and modified with the arginine derivatives in **Figure 1** through carbodiimide chemistry. Alphabetical designations listed in **Figure 1** are used as labels for the modified liposomes for convenience. Following modification and extrusion, the zeta potential and size of the DOPE:DOPC liposomes were measured, as has been previously reported.[11] The zeta potential of the modified liposomes ranged from – 8.9 to – 33.9 mV with unmodified DOPE:DOPC liposomes having a zeta potential of – 16.8 $\pm$ 0.83, which is in line with previous reports.[40,41] The negative charge arises from the phosphate group present in the lipids. The liposomes were 83.5 to 108.8 nm in diameter, which results from the 100 nm filter used during the extrusion procedure. Cellular responses to particle internalization is size dependent, with particles <200 nm typically undergoing clatherin-mediated endocytosis and larger particles shifting towards a caveolae-mediated mechanism.[42] Size continues to be an influential parameter in biodistribution and clearance.[43] These liposomes were kept at a near constant diameter (~100 nm) to eliminate size of the liposomes as a possible variable influencing cell-liposome interactions.

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3.2. Modified liposomes are cytocompatible

DOPE:DOPC liposomes have been FDA approved for use as hepatitis A and influenza vaccines[44] and should have no impact on cell viability. However, chemically modifying previously cytocompatible materials can alter cellular responses, including cell viability. Cytotoxicity of the modified liposomes to Caco-2 cells was measured using an MTT assay. The viability of the cells was normalized to Caco-2 cells in the absence of particles (**Figure 2**). Unmodified liposomes (labeled as UM) were also included. All of the modified and unmodified liposomes resulted in cell viability ≥ 80%, suggesting minimal cytotoxicity.

3.3. Physicochemical properties of surface modified liposomes impact the IC_{50} of encapsulated doxorubicin

Modified and unmodified liposomes were loaded with doxorubicin using a transmembrane pH gradient to actively incorporate drug.[45] Using this method, doxorubicin precipitates in the aqueous core of the liposome through self-associations and interactions with salts.[46] The loading efficiency of doxorubicin in the modified and unmodified liposomes was > 93%, as has been previously reported.[11]

IC_{50} is a measure of the cytotoxic effects of the encapsulated doxorubicin. Caco-2 cells were incubated with doxorubicin loaded liposomes for 48 h and their responses were calculated as a percentage of cells not treated with the liposomes. IC_{50} values for each liposome modification were calculated using a sigmoidal dose response curve, shown in equation 1:

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} \quad (1)$$

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where A_1 is the upper limit of the dose curve, A_2 is the lower limit, x_0 is the IC_{50} and p is the steepness of the curve. The IC_{50} value for unencapsulated doxorubicin on Caco-2 cells was also measured (1.99 ± 0.019 μg/mL) and is in line with previously reported values.[47] Most liposomal formulations decreased the IC_{50} of doxorubicin compared to unencapsulated drug. Additionally, some modifications (A, C, F, K, L, M, and N) are more efficient than the unmodified liposome with lower IC_{50} values (**Figure 3**). Doxorubicin encapsulated in unmodified liposomes had an IC_{50} of 0.96 ± 0.16 μg/mL.

The goal in this study was to determine how chemical properties of surface modifiers could influence the IC_{50} value of liposomal doxorubicin. Relationships between lipophilicity, surface charge, surface tension, molecule flexibility, polar surface area, enthalpy, hydrogen bond acceptors and donors, acid dissociation values, and the isoelectric point of the modifier with the IC_{50} value of liposomal doxorubicin were examined. The surface modifier should have at least 4 hydrogen bond donors, a log *P* between -2 and -1, between 4 and 6 freely rotating bonds, and an isoelectric point above 5.5 to improve the toxicity of the encapsulated doxorubicin over that of the unmodified liposome. Relationships with the other mentioned physicochemical parameters were not observed. Many of these parameters are also found in Lipinski's rule of five and its variants.[48] The role of these parameters on improved drugability are discussed below.

Hydrogen bonding enables drug carriers to interact with the cell surface, potentially increasing the likelihood of internalization.[49] In addition to the number of hydrogen bonds available, the orientation of these bonds is also important.[50] Guanidinium functional groups are capable of forming bidentate hydrogen bonds with the negatively charged functional groups

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present on or associated with the cell membrane (namely carboxylates, phosphates, and sulfates).[51] Adding a methyl to the guanidinium group, as is the case with modification D, conserves charge but reduces the ability to form bidentate hydrogen bonds. Modification D had the highest IC_{50} value of all the liposomes tested, which suggests that hydrogen bond donation, particularly from bidentate groups, is influential in improving the efficiency of drug carriers. Modifications K and L are the only modifications that improve doxorubicin toxicity and do not have bidentate hydrogen bond donation capabilities. The functional groups on these modifications, however, are still capable of forming multiple hydrogen bonds. Hydrogen bonding capabilities facilitate cell entry through interacting with negatively charged phosphates, sulfates, and carboxylates on the cell surface[52,53] Previously, it has been shown that the surface chemistry of particles plays an integral role in the intracellular fate of particles,[54] likely resulting from the different internalization pathways used by the cell to endocytose the material.[55] The number of hydrogen bond acceptors was generally between four and six for all of the modifiers tested here, which did not provide a sufficient range to form a basis for exclusion of potential carrier candidates. Some versions of Lipinski's rule of five have suggested using a sum of both hydrogen bond donors and acceptors.[56] Again, since the number of acceptors was relatively constant, this parameter was not useful in excluding drug carriers. However, increasing the diversity and size of this library may allow for fine-tuning of these rules such that they are predictive.

Charge of drug delivery systems is an influential property on drug delivery, from interacting with the negatively charge cell surface[57] to escaping the endosome.[58] The isoelectric point of the modifiers that improve cytotoxicity in Caco-2 cells is above 5.5, which corresponds to the pH of the endosome.[59,60] This means that the modifiers will be positively charged both in

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the extracellular fluid and in the endosome, increasing their propensity to interact with the negatively charged cell membrane. The positive charge on these modifiers could facilitate proton influx, known as the proton sponge effect, leading to endosomal escape via endosomal bursting.[61] Another possibility is that the electrostatic interaction between the cationic liposomes and the negatively charged endosome allows the anionic lipids to diffuse into the liposome, thus forming a charge-neutralized liposome, known as the flip-flop mechanism.[62,63] Endosomal escape, through whichever method, is requisite for effective drug delivery. This may explain why the zeta potential of the liposomes is negatively correlated with the IC_{50} and why there is an empirical relationship between the isoelectric point and the IC_{50} stated above.

Lipophilicity influences absorption, distribution, metabolism, and excretion (ADME properties) of drug compounds.[64] It is widely assumed that drug partitioning in the lipophilic cell membrane is a rate-determining step in passive transport. Here, we are using liposomes which are capable of entering cells through non-clathrin, non-caveolae dependent endocytosis, along with clathrin or caveolae mediated endocytosis and macropinocytosis.[65] Permeability across Caco-2 monolayers has been linked to lipophilicity of the solute.[66] Predictive equations for Caco-2 cell permeability have relied on the lipophilicity of the drug molecule.[67] Unsurprisingly, the positively charged modifiers carnitine and acetyl-carnitine had IC_{50}'s > 1 μg/mL. The one modifier that does not follow this trend is nitroarginine, which has one of the lowest IC_{50} values (0.24 ± 0.03 μg/mL) and the lowest log *P* value (the octanol-water partition coefficient). The reason for the nitroarginine modification not following this trend is not clear at this time and illustrates the need for comprehensive studies on how surface modifiers alter interactions between cells and drug delivery vehicles.

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The flexibility of the modifier can alter ligand-receptor and ligand-protein binding, which can alter how the cell interacts with the modified drug carrier. 3-guanidinopropionic acid differs from 2-amino-3-guanidinopropionic acid by an α-amino group, which confers flexibility. The addition of this amino group also adds hydrogen-bond donating capabilities and increases the isoelectric point, both of which might also contribute to the diminished IC_{50} of 3-guanidinopropionic acid. Creatine, as mentioned above, loses its ability to form bidentate hydrogen bonds with negatively charged cell membrane species arising from the methylation of the guanidine group. The flexibility of the molecule also decreases. 5-hydroxylysine has the most freely rotating bonds and an $IC_{50} > 1$ μg/mL. Increased flexibility decreases ligand affinity on average by 0.5 kcal for every two rotatable bonds.[68] Previous studies have shown that increasing the number of rotational bonds corresponds to a decrease in membrane permeability.[56] With changing the flexibility (or any of the other physicochemical properties) of these modifiers, other changes occur, which further demonstrates the need to study libraries of compounds. Studying libraries allows determination of how physicochemical properties influence cell responses to drug delivery systems since multiple factors can be analyzed simultaneously and it is impossible to alter one physicochemical property without altering another.

Previously, we have examined how these same surface modifiers alter the IC_{50} for macrophages polarized with lipopolysaccharide to mimic the M1 pro-inflammatory phenotype (M(LPS)), with interleukin-4 to induce an M2-like pro-wound healing phenotype (M(IL-4)), and naïve M(0) cells that were not treated with biological activators.[11] Here, we were able to hypothesize relationships between physicochemical properties of the surface modifier and

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improved toxicity of doxorubicin, along with improved targeting to specific macrophage subpopulations through PCA. PCA is a statistical technique that combines high-dimensional data onto a lower dimensional space (in this case two dimensions) such that relationships between input variables (in this case the measured IC_{50} values and the properties of the modifiers) can be readily visualized. The PCs are chosen to maximize the variance of the projected points, which allows us to capture the maximum amount of original data on the newly calculated plot. M(LPS) and M(0) cells were both influenced by the number of hydrogen bond donors and the number of freely rotating bonds, while M(IL-4) cells were influenced by the zeta potential of the liposomes and the log *P* of the modifiers.

The relationships between the physicochemical properties of the modifiers and the IC_{50} values of encapsulated doxorubicin on Caco-2 cells is illustrated using informatics analysis (**Figure 4**). By projecting the original multidimensional data on a two-dimensional plot, the relationships between the variables, i.e. the modified liposomes, can be assessed. The score plot (**Figure 4A**) shows a wide distribution of values, which indicates that there is a variety of IC_{50} values obtained for the materials tested here. Groupings based on chemical structure can be observed. Modifications E and J contain quaternary ammonium groups and are far removed from the other groups. Another interesting detail is that modifications A and N are similar to modifications M and F with the exception that the urea group is replaced with a guanidine group. The spatial distances between these modifications, in terms of changing the functional group from a guanidine (modifications A and N) to a urea (modifications M and F) are similar. There is also a clustering of the terminal primary amines: modifications G, K, and L are centrally located. The acetylated modifiers (modifications H and L) are also spatially close. This shows

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that the IC_{50} values are chemistry dependent and chemical modification of drug carriers can be used to alter the efficiency of delivered drugs.

The relationships between the modifiers and IC_{50} values are illustrated in **Figure 4B.** The physicochemical properties of the modifiers were estimated using MarvinSuite (ChemAxon, Cambridge, MA) and a database.[69] These values included the isoelectric point, log *P*, the number of hydrogen bond donors and acceptors, the polar surface area, surface tension, log *D* at pH 5.5 and 7.4, the number of freely rotating bonds, and the hydrophilic-lipophilic balance (HLB). Log *D* is the octanol-water distribution coefficient in which both the ionized and non-ionized forms of the compound is measured, hence this value can change with pH. The previously measured zeta potentials of the modified liposomes[11] were also included. In this work the IC_{50} was negatively related to surface tension, which was not a parameter that was correlated to the IC_{50} values for any of the macrophage phenotypes. We can also see that log *P*, the isoelectric point, and the number of hydrogen bond donors are roughly orthogonal to the measured IC_{50} values. Since the described observations above indicate that surface modifiers should have at least 4 hydrogen bond donors, a log *P* between -2 and -1, between 4 and 6 freely rotating bonds, and an isoelectric point above 5.5 for more efficacious toxicity, are not linear relationships, but rather exclusionary empirical rules, it is not surprising that these values do not have linear relationships with the IC_{50} values. This is particularly true for log *P*, which is presented as a range of acceptable values. These relationships and how they differ from the correlated properties for different macrophage phenotypes illustrate how surface modifications can be used to target different cell types. This is not a particularly surprising finding and has been noted in several other studies. For example, using poly(β-amino ester)s for gene delivery

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to monkey kidney COS-7 fibroblasts was optimized using hydrophobic diacrylate monomers, in particular with a high number of hits using bisphenol A ethoxylate diacrylate as one of the monomer building blocks, resulting in hydrophobic polymers.[35] In contrast, that same monomer was not a hit for transfection in human umbilical vascular endothelial cells (HUVECs). Instead, 1,4-butanediol diacrylate was the most efficacious diacrylate used in the Michael-type addition.[70] Clearly, different rules must be generated to target different cell types using small molecules. This also demonstrates that small molecules can be effective at targeting specific cell types.

4. Conclusions

We have chemically modified DOPE: DOPC liposomes with 14 arginine derivatives to examine how materials properties influence the IC_{50} of encapsulated doxorubicin on Caco-2 cells. We were able to determine relationships between the surface modifier physicochemical properties and the toxicity of the encapsulated drug. A negative relationship between the IC_{50} values were found with the surface tension of the modifier. There may also be negative correlations with the number of hydrogen bond acceptors and the number of freely rotating bonds. We also suggested an empirical rule similar to Lipinski's rule of five for the drug carriers studied here on Caco-2 cells, which included at least 4 hydrogen bond donors, between 4 and 6 freely rotating bonds, an isoelectric point above 5.5, and a log *P* between -2 and -1. These results suggest that cell types can be targeted by altering the chemistry of the drug carrier and that rational design may be necessary for different cell types.

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Figure Legend

Figure 1. Chemical structures of molecules used for the modification of liposomal particles. The arginine derivatives shown here are lettered for easier identification in experiments and discussion throughout the paper.

Figure 2. The modified liposomes are cytocompatible. Cell viability of Caco-2 cells incubated with modified and unmodified liposomes. Data represents the mean value ± standard deviation (SD). n = 4. UM = unmodified liposome.

Figure 3. IC_{50} concentration for doxorubicin loaded modified liposomes on Caco-2 cells. Data represents the mean value ± SD. n = 8. Statistical analysis through two-way ANOVA and Tukey's HSD post-hoc test. (*) indicates $p < 0.05$ compared with unmodified liposomes. dox = doxorubicin.

Figure 4. Observation and loading plots of physiochemical materials properties and their influence on IC_{50} values of doxorubicin loaded modified liposomes on Caco-2 cells. PC1 explains 37.9% data variance and PC2 explains 23.5% data variance, which represents >60% of the original data information. (A) Observation plots of modified liposomes and (B) loading plots of the physicochemical properties of the modifiers in PC space. HBD = hydrogen bond donors, HBA = hydrogen bond acceptors, PSA = polar surface area, and HLB = hydrophilic lipophilic balance.

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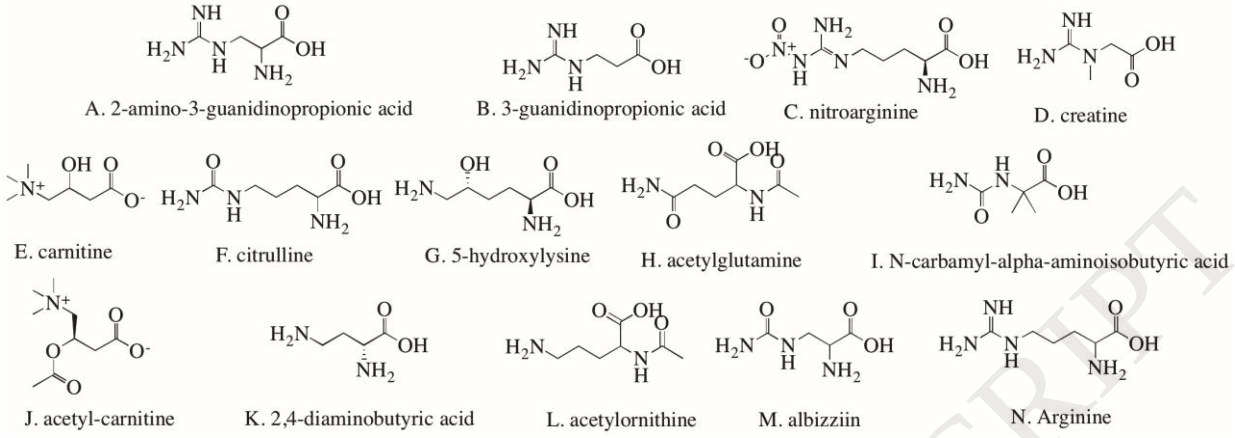
Figr-1



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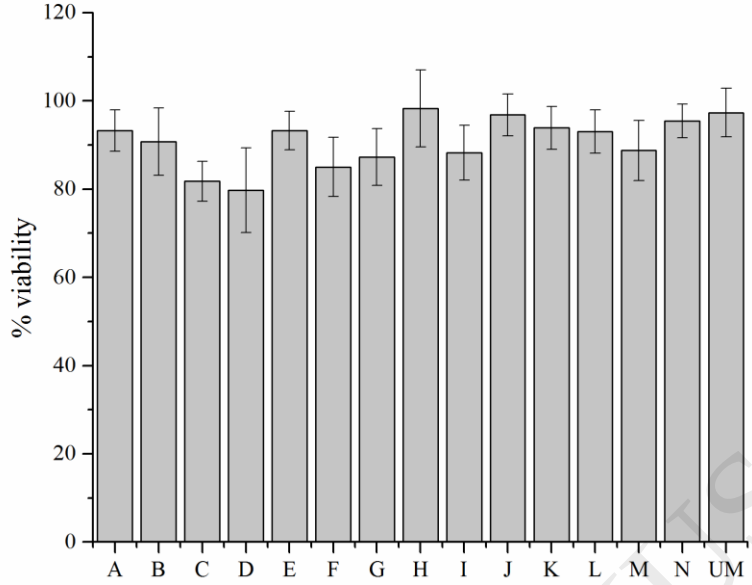

Figr-2

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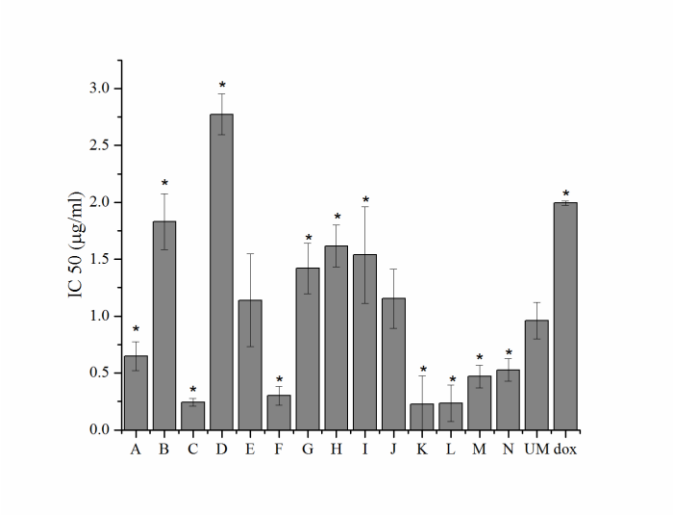

Figr-3

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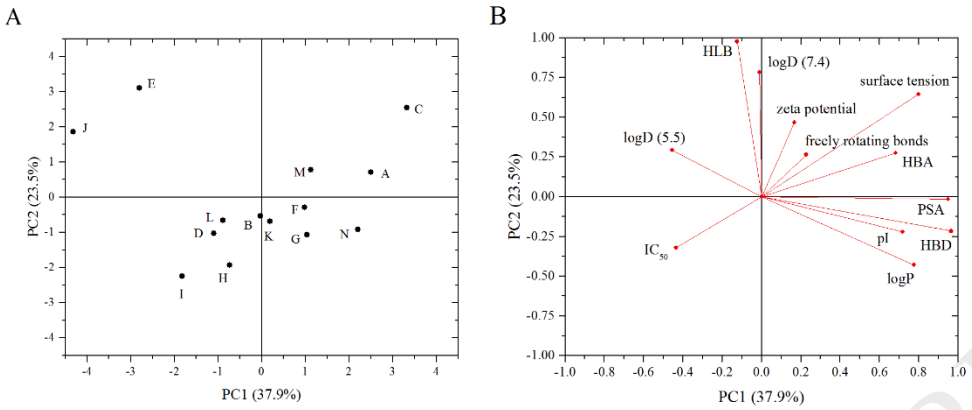

Figr-4

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