Pharmaceutical Research, Vol. 24, No. 12, December 2007 (© 2007)
DOI: 10.1007/s11095-007-9459-1

Expert Review

Multifunctional Nanoparticulate Polyelectrolyte Complexes

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Received June 28, 2007; accepted September 10, 2007; published online October 12, 2007

Abstract. Water-soluble, biodegradable, polymeric, polyelectrolyte complex dispersions (PECs) have evolved because of the limitations, in terms of toxicity, of the currently available systems. These aqueous nanoparticulate architectures offer a significant advantage for products that may be used as drug delivery systems in humans. PECs are created by mixing oppositely charged polyions. Their hydrodynamic diameter, surface charge, and polydispersity are highly dependent on concentration, ionic strength, pH, and molecular parameters of the polymers that are used. In particular, the complexation between polyelectrolytes with significantly different molecular weights leads to the formation of water-insoluble aggregates. Several PEC characteristics are favorable for cellular uptake and colloidal stability, including hydrodynamic diameter less than 200 nm, surface charge of >30 mV or <−30 mV, spherical morphology, and polydispersity index (PDI) indicative of a homogeneous distribution. Maintenance of these properties is critical for a successful delivery vehicle. This review focuses on the development and potential applications of PECs as multifunctional, site-specific nanoparticulate drug/gene delivery and imaging devices.

KEY WORDS: biomaterials; controlled release/delivery; endothelial targeting; *in vitro* models; polyelectrolyte complexes.

NANOPARTICULATE TECHNOLOGY

Nanotechnology is an area of science devoted to the manipulation of atoms and molecules leading to the assembly of structures in the nanometer (1 to 1,000 nm) scale size range. Research in the "nanorealm" began in physics and chemistry in the early 1970s but soon spread into medicine and biology. Specifically, a wide array of nanotechnologies is beginning to change the foundations of disease diagnosis, treatment, and prevention. These advanced innovations, referred to as nanomedicine by the National Institutes of Health (1), have the potential for widespread patient benefits. Because molecules and structures inside cells operate at the nano- and micro-scale, the evolution of nanomedicine as an offshoot of nanotechnology has become a key component for the future of research in medical intervention. A few of the current nanomedical approaches include carbon nanotubes that act as biological mimetics (2), polymeric nanoconstructs for tissue engineering (3,4), and nanoscale microfabrication-based devices (5). Furthermore, the use of nanoparticulate technologies as targeted forms of diagnostics, drug, and gene delivery is at the forefront of nanomedicine, and it has led to collaborative efforts between disciplines that were typically segregated: engineering and molecular biology, chemistry and virology, physics and surgery.

Nanoparticles (NPs), in this context, are defined as solid colloidal particles consisting of macromolecular compounds. They were initially devised as carriers for vaccines and anticancer drugs to limit the off-target tissue toxicity present in conventional methods. NPs can be fabricated from a multitude of materials, including synthetic polymers and biopolymers (proteins and polysaccharides). Drug integration of peptide segments, proteins, and/or small molecules with both targeting and therapeutic abilities into delivery systems in the form of nanoparticulate polymer matrices offers many benefits. These benefits include controlled drug release and protection, prolonged blood circulation times, and many other adjustable characteristics (6,7).

There are numerous engineered constructs, assemblies, architectures, and particulate systems being studied as drug delivery platforms. These include polymeric micelles, dendrimers, virus-derived capsid nanoparticles, polyplexes, and liposomes (8–12). Incorporation of therapeutic and diagnostic agents can be achieved by encapsulation, covalent attachment, or surface adsorption. Many carriers can be engineered for activation by pH, chemical stimuli, radiation, magnetic fields, or heat. Systems are being designed for multifuctionality that combine targeted tissue delivery, organelle trafficking, and imaging (13,14). These nanoparticles do not behave similarly; their behavior within the biological microenvironment, stability, and cellular distribution varies with their chemical makeup, morphology and size.

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The advantages of using biodegradable nanoparticles for drug delivery result from their two basic properties. First, NPs, due to their small size, penetrate within even small capillaries and are taken up within cells, which allows for efficient drug accumulation at the target sites in the body (15,16). Second, the use of biodegradable materials for NP preparation allow for the sustained drug release within the target site over a period of days or even weeks after injection (17), establishing many of the concepts described above. Table I introduces some nanoparticulate drug delivery approaches.

POLYMER-BASED NANOPARTICLES AND POLYELECTROLYTE COMPLEX DISPERSIONS

Polymer-based NP systems can be created through a variety of techniques; some involve the use of potentially toxic solvents and components. Emulsion, dispersion, and inverse microemulsion polymerization of biodegradable and non-biodegradable monomers comprise the most common fabrication platforms. Reaction environments frequently involve the application of mineral oils and strong organic solvents to maintain polymer stability and improve yields although unreacted monomers, initiators, and surfactants may compromise the biocompatibility of the final formulation (Table I, 11,17–41). Specifically, alkylcyanoacrylate nanostructures suffer from toxic breakdown products (42). These safety issues limit their potential application in biomedical related fields.

Water-soluble, biodegradable, polymeric, polyelectrolyte NPs have been developed to circumvent these processing limitations. Polymeric polyelectrolytes degrade at a slow rate, do not alter normal cell function, and use water as a solvent, a major advantage for products that may be applied as drug delivery systems in humans, (43,44). These nanoparticulate architectures, termed polyelectrolyte complex dispersions (PECs), result from strong electrostatic interactions between charged microdomains of at least two oppositely charged polyelectrolytes (45). The mixing of solutions of polyanions and polycations leads to the spontaneous formation of insoluble PECs under certain conditions. The formation of PECs are governed by the strength and location of ionic sites, polymer chain rigidity, precursor chemistries, pH, temperature, ionic strength, mixing intensity, and other controllable factors. PECs have been previously applied in gene delivery (46–48) and microencapsulation of various cell and tissue types (49,50). We have developed a biodegradable, polymer-based, nanosized PEC system, capable of functionalization (i.e. therapeutic, targeting, or imaging agent). This system has many potential applications including *in vitro* cellular uptake, gene delivery, targeting of neovasculature, and controlled *in vivo* biodistribution.

PEC ASSEMBLY MECHANISMS

The most predominant molecular forces for PEC assembly are strong electrostatic interactions. However, hydrogen bonding, hydrophobic interactions and van der Waals forces complement PEC formation, and they are related to the physical characteristics listed previously (51). Two major steps dictate PEC complexation: (1) the kinetic diffusion process of mutual entanglement between polymers, which occurs at relatively short times and depends on molar size differences, and (2) thermodynamic rearrangement of the already formed simplex aggregate due to conformational changes and disentanglement. The latter process occurs at rather long times leading to a source of instability in the PEC, and it is a consequence of phase separation in aqueous medium. Stop flow measurements have shown that the PEC formation takes place in less than 5 ms, nearly corresponding to the diffusion-controlled collision of polyion coils (52).

Three different types of aqueous PECs have been prepared (53):

- Soluble PEC, i.e. macroscopically homogeneous systems containing small PEC aggregates
- Turbid colloidal, PEC systems in the transition range to phase separation, exhibiting an observable light scattering or Tyndall effect
- Two-phase systems of supernatant liquid and precipitated PEC, which are readily separated as a solid after washing and drying (not desirable).

Two structural models for PECs are discussed in literature, dictated by the characteristics of the polyion groups, stoichiometry, and molecular weights: (1) the ladder-like structure, where complex formation takes place on a molecular level via conformational adaptation, and (2) the scrambled-egg model, where a large number of chains are incorporated into particle architecture (52). The ladder-like structure consists of hydrophilic single-stranded and hydrophobic double-stranded segments. These phenomena result from the mixing of polyelectrolytes having weak ionic groups and large differences in molecular dimensions. These properties can lead to populations of water-soluble and insoluble PECs, an unwanted consequence. The oppositely charged ions complex according to a "zip" mechanism where there is often insufficient ion pairing. In some cases, a high molecular

Table I. A Brief Summary of Current Drug Delivery Platforms

NP Platform	Size (nm)	Therapeutic Application
Polymeric	10–1,000	Brain tumors (7,18,19), bone healing (20), vaccine adjuvant (21), restenosis (17,22), diabetes (23)
Ceramic	<100	Photodynamic (24), insulin delivery (25)
Metallic	<50	Cancer (26,27), imaging (28)
Polymer Micelle	<100	Solid tumors (29–31), anti-fungal (32)
Liposome	50–100	HIV (33), tumors (34,35), vaccine delivery (36)
Dendrimer	<10	Bacterial infections (37), cancer (38,39), HIV treatment (40)

weight polyion with a weak charge density is titrated into a shorter, smaller molecular weight counterion (oligomer) non-stoichiometrically to form initially soluble PECs. Through continued addition of the high molecular weight polyion, insoluble PECs can form (54).

The scrambled-egg model refers to complexes that are the product of the combination of polyions with strong ionic groups and comparable molar masses yielding insoluble and highly aggregated complexes under a strict 1:1 stoichiometry. Fig. 1 shows these representations. As the stoichiometry is adjusted under dilute conditions (10^{-4} g/ml), colloidal PECs, exhibiting the Tyndall effect and consisting of a neutral and stoichiometric core surrounded by excess binding polyelectrolytes, are stabilized against aggregation, and they provide a practical nano- and micro-scale product (45). The excess polyelectrolyte provides stability in different solvent conditions (55), i.e. surplus cation bound to a neutralized anionic core leads to stability at low pH. Both the ladder and scrambled egg assemblies share the same steps of polyelectrolyte interaction, but they only result in the desired structures (insoluble stoichiometric complexes) under certain conditions. An important stride towards functional and practical use of PEC complexes for drug delivery is the design of assemblies that form under easily understood and controllable conditions such as simple stream mixing in a continuous or batch design.

We (56) showed that PEC formulations comprising precursors with similar, low molecular weights (LMW) yielded dispersions with suitable physicochemical characteristics as verified by photon correlation spectroscopy (PCS) and transmission electron microscopy (TEM), presumably due to efficient ion pairing and the presence of a steric stabilizer (Pluronic F-68). Anions employed were low molecular weight sodium alginate and chondroitin sulfate, while cations were poly(methylene co-guanidine) hydrochloride (PMCG), $CaCl_2$, and spermine tetrahydrochloride. Low molecular weight PECs fabricated with frequency-driven dispergation exhibited pH-independent stability, as validated by charge and size measurements. Representative morphology is shown in Fig. 2 via negative staining TEM. Table II describes the polyelectrolytes applied in our laboratory. Component concentrations used for preparation of PECs were 0.5 mg/ml while Pluronic F-68 was applied at 1% *m*/*v*.

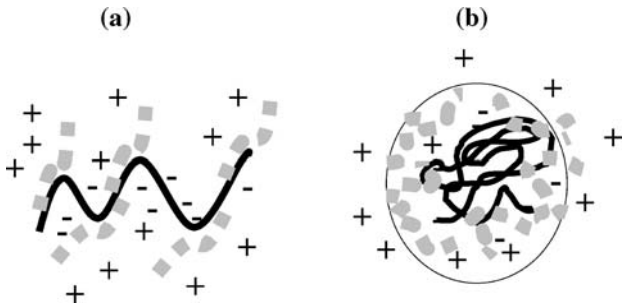

Fig. 1. Schematic representation of ladder and scrambled egg structures. Black represents the large polyion (*negative*) while *gray* represents a polyion of opposite charge (*positive*). **a** Shows the ladder representation where insufficient ion pairing occurs under certain stoichiometric conditions leading to macromolecular aggregates, insoluble, and soluble PECs. **b** Shows the scrambled egg model where polymers of comparable size complex yielding insoluble PECs under certain conditions.

As single entities, chondroitin sulfate and alginate are largely non-toxic, although they have been found to induce mitogenicity in rat insulinoma cells at high concentrations, 1 and 10 mg/ml, respectively (57). Spermine tetrahydrochloride has an observed EC_{50}>2 mM in CHO cells (58). Pluronic F-68 has been found to cause impairment of granulocyte function, while inducing cytotoxicity in HeLa at doses of 20 mg/ml (59). No current data exists on the toxicity of PMCG on mammalian cells, in spite of being used as a component of cell microencapsulation strategies (50,60). While their precursor polymers have demonstrated some toxicity, a PEC system based on this LMW formulation has been found to be non-toxic (61) and non-inflammatory after either intramuscular or subcutaneous injection as shown in Fig. 3.

PEC PHYSICOCHEMISTRIES AS MEDIATORS OF BIOLOGICAL INTERACTIONS

The size of nanoparticulate/PEC species is critical for cell binding and internalization (62–64). They generally have a higher intracellular uptake compared to microparticles (22). For example, Desai *et al.* demonstrated that 100 nm NPs exhibited a 2.5-fold greater uptake relative to 1 μm particles, and they had sixfold greater uptake relative to 10 μm particles in Caco-2 (human colon) cells (65). Increased intracellular uptake of NPs has been observed in Hepa 1–6, HepG2, and KLN 205 cell lines as well as perfused rat tissues (20,66).

Zeta potential, or mean surface charge, is a surrogate marker for the colloidal stability of PECs/NPs (67,68). The charge develops as a function of the excess polymer and is controlled by an ordered PEC assembly process. Zeta potential is rarely addressed mechanistically in the literature, since it is easily altered by environmental conditions, but it has important connotations for surface modification, size retention/aggregation, and targeting. The classical colloidal theory has long held that one of the major interactive forces that controls particle stability in aqueous liquid suspension is electrostatic forces at the particle surface represented by zeta potential (68,69). Simply put, the presence of significantly positive or negative surface charge causes charge repulsion and prevents further aggregation by virtue of fewer collisions and ionic attractions. This range of stable zeta potential in aqueous suspension has been empirically defined as greater than |±30| mV (69), and it is of great interest for biological and pharmaceutical systems to have sufficient zeta potential for preservation of colloidal stability and nano-scale size.

Variation of the particle surface charge could potentially control binding to tissue and direct NPs to cellular compartments both *in vivo* and *in vitro*. Cellular surfaces are dominated by negatively charged sulfated proteoglycans, molecules that play pivotal roles in cellular proliferation, migration, and motility (70). Cell surface proteoglycans consist of a core protein anchored to the membrane and linked to one or more glycosaminoglycan side chains (heparan, dermatan, keratan or chondroitin sulfates) to produce a structure that extends away from the cell surface. Glycosaminoglycans are highly anionic, and the interactions between proteoglycans and NP shells, if positively charged, tend to be largely ionic (71). Once inside the cell, degradation of polymers may occur, but targeting specific intracellular organelles is possible depending on the surface charge and attached ligands (22).

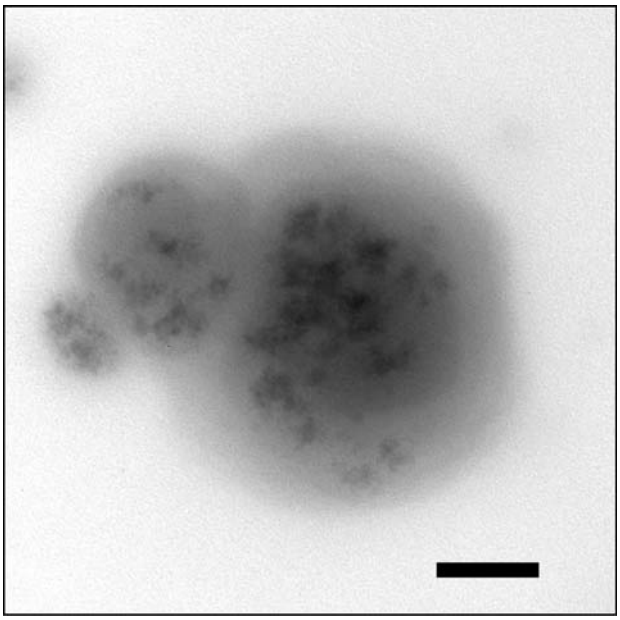

Fig. 2. Representative TEM micrograph of PEC prepared by non-stoichiometric, ultrasonic titration of low molecular weight sodium alginate (Mr=12 kDa) and chondroitin sulfate (Mr=15 kDa) into a cationic bath of spermine tetrahydrochloride (Mr=0.348 kDa), poly (methylene-co-guanidine) hydrochloride (PMCG; Mr=5 kDa), calcium chloride, and Pluronic F-68. Image was taken with a Phillips CM-12 120 keV electron microscope equipped with a CCD camera. Scale bar is 100 nm.

MODES OF PEC AND NANOPARTICLE INTERNALIZATION

NP endocytosis has been shown to be concentration, energy, time, size dependent, and saturable. Specific mechanisms may include phagocytosis, fluid phase pinocytosis, transport via clathrin-coated pits, caveolae-mediated transport, or non-endocytic pathways (72), processes that can be distinguished with various inhibitor strategies (73). Transcytosis and exocytosis are pathways that permit communication with the external environment (74). Uptake mechanisms are also cell-type dependent. Endocytosis results in internalization of the cell's plasma membrane to form vesicles that capture macromolecules and particles present in the extracellular fluid and/or bound to membrane-associated receptors. These vesicles then undergo a complex series of fusion events directing the internalized cargo to an appropriate intracellular compartment (uptake of fluids, macromolecules, particles and other ligands that sort to cellular processing pathways).

Intracellular uptake studies have largely focused on liposome (75–77) and polymer delivery systems (20,78,79). Kinetically, three specific modes of endocytosis have been implicated in the context of delivery systems: fluid-phase, adsorptive, and receptor-mediated endocytosis (80). Fluid-phase endocytosis refers to the bulk uptake of solutes in the exact proportion to their concentration in the extracellular fluid. This is a low-efficiency and nonspecific process. In contrast, during adsorptive and receptor-mediated endocytosis macromolecules are bound to the cell surface and concentrated before internalization. In adsorptive endocytosis, molecules preferentially interact with generic complementary binding sites e.g. heparan sulfute proteoglycans (HSPGs), lectin, or charged, surface-bound macromolecules. Fluid-phase endocytosis has a lower internalization capability compared to adsorptive endocytosis. It is also a saturable process; that is, the cellular uptake depends on the dose of nanoparticles. One more distinction is of practical aspect: both phagocytosis and macropinocytosis exhibit a non-saturable linear profile with the nanoparticle dose, as opposed to the more typical, saturable uptake mechanisms of endocytosis (e.g., in case of receptor-mediated endocytosis of a finite number of binding sites present on the cell surface; 81). This is an often neglected feature but of a great significance. Behrens *et al.* (82) observed a dose-dependent uptake for ~200 nm chitosan-coated polystyrene nanoparticles in a human intestinal cell line (Caco-2). Likewise, Panyam and Labhasetwar (72) described a nearly linear uptake of poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles in vascular smooth muscle cells, though uptake was partially obscured by rapid exocytosis. Non-specific, receptor independent uptake of LDL particles by macrophages also shows non-saturable kinetics, causing high levels of constitutive macrophage cholesterol accumulation (83) which is sensitive to cytochalasin D. Similar, non-saturable LDL uptake was observed for cultured human fibroblasts (84) as well as irreversibly glycated, albumin modified, LDL lipoproteins (85). The above observations are in accordance with the dose-independent elimination of PEG–PLGA nanoparticles in mice by the reticuloendothelial system (RES), while non-PEGylated nanoparticles followed non-linear and dose-dependent pharmacokinetics (86).

Flow cytometry can be utilized to extensively characterize and define the underlying mechanisms of PEC binding and uptake in biological models such as endothelial cells. Our laboratory has found that PEC internalization is through macropinocytosis (61) as defined by a series of inhibitor strategies including extracellular heparin (extracellular glycosaminoglycan competition; 71,87), reduced temperature (thermodynamics; 72), 2-deoxyglucose/azide (metabolism;

Table II. Polymers and Polyelectrolytes Applied in the Current Study

Polymer	Mr (kDa)	Source	Current Application
Sodium Alginate	12,000	Algal cell walls	Controlled release and bioadhesive systems (25)
Chondroitin Sulfate	15,000	Animal cartilage, ligaments, tendons	Osteoarthritis management (27)
Spermine tetrahydrochloride	348	Mammalian sperm	Cancer diagnosis and treatment (28)
Calcium chloride	111	Ubiquitous salt in all organisms	Cell, tissue polyelectrolyte maintenance (29)
PMCG	5,000	Synthetic	Microencapsulation (30–33)

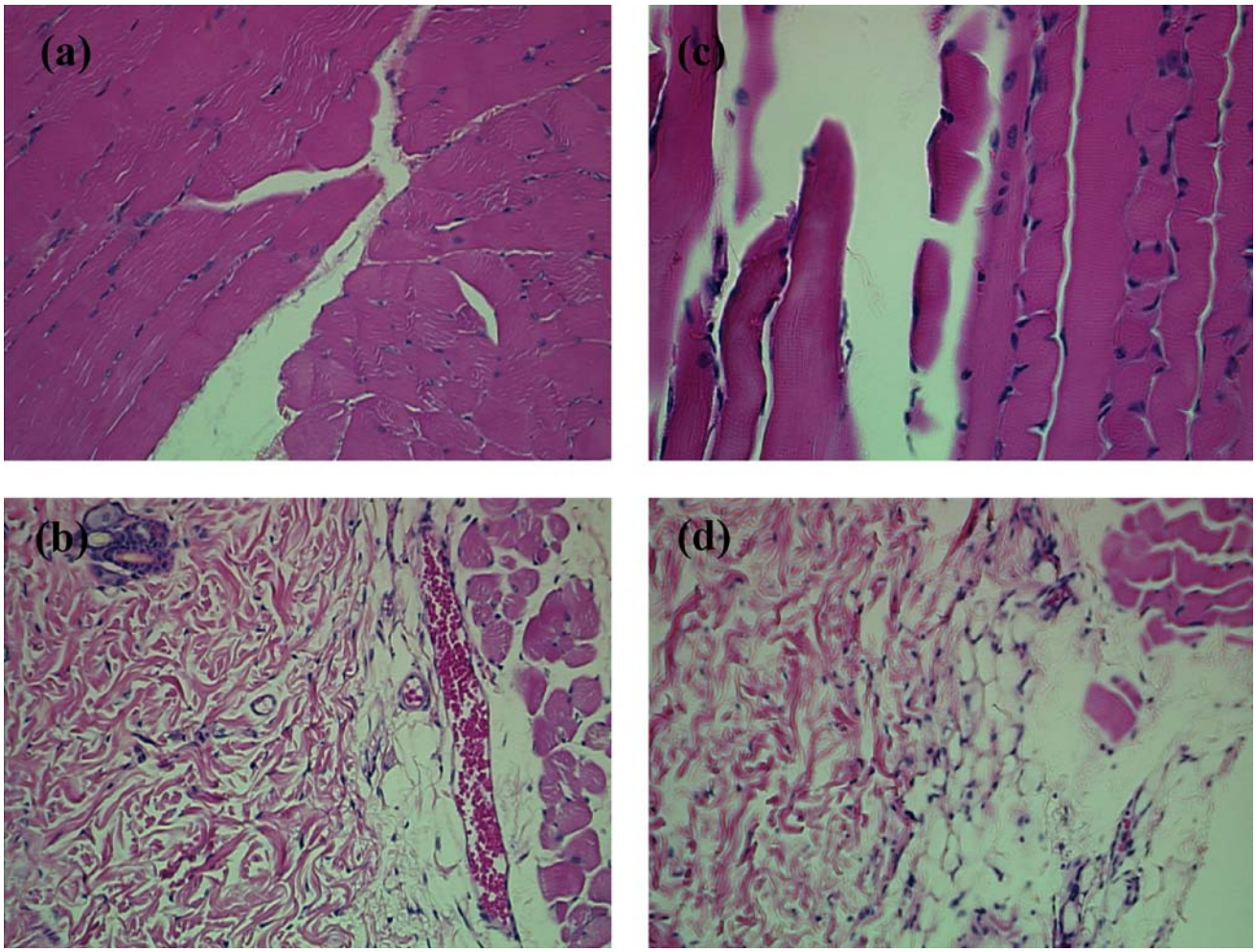

Fig. 3. Histological examination of intramuscularly (**a**, **c**) and subcutaneously (**b**, **d**) injected PECs and PBS 3 days after administration.

73), cytochalasin D (actin filaments; 88,89), HSPG biosynthetic effects (4-nitrophenyl xylopyranoside; 90,91), and surface receptor proteolysis (trypsin). Compartmentalization, uptake, and surface binding could be delineated through extracellular fluorescein isothiocyantate (FITC) quenching. In the absence of a targeting ligand, cationic PECs bind cells through strictly electrostatic interactions where cells provide an anionic sink to mediate the attachment. Additionally these studies incorporated, a novel, flow cytometric, Scatchard analysis protocol, in which the extent of receptor-controlled interactions was dictated by adherence to underlying assumptions of mass action and equilibrium binding (61). This flow cytometric strategy quantifies targeting and receptor-ligand interactions, features that are not available in direct, kinetic studies.

NANOPARTICULATE THERAPEUTIC DELIVERY

In recent years, biotechnology derived drugs including peptides, proteins, viruses, and monoclonal antibodies/fragments have become a central focus of pharmaceutical research and developmental efforts (92). The fate of these drugs after administration *in vivo* is determined by a combination of several processes: distribution, metabolism, and elimination when given intravenously (systemically) and absorption, distribution, metabolism, and elimination when given extravenously (locally; 93). Bioavailability, the ratio of drug accumulation at its site of action to the amount delivered to the body, is a significant limitation in the use of protein or peptide biologics. Typically, these molecules have a short half-life in blood plasma or other biological fluids as well as a high probability of off-target effects (94). Thus, the use of naked proteins/peptides *in vivo* has limited utility, necessitating advanced delivery systems that can act locally.

The carrier platform should be non-toxic, compatible with the drug applied, preserve its activity, and deliver the payload with reproducible pharmacodynamics. NP delivery systems have a number of advantages, including high stability *in vivo*, long-term payload release, and the capability of permeating through small capillaries and into cellular tissues (95). Drug incorporation into delivery systems offers many advantages, particularly the enhancement of the therapeutic index of many drugs, alteration of pharmacokinetics and biodistribution, and sustained release reservoirs. The use of a NP delivery vehicle can also stabilize and reduce the systemic toxicity of a therapeutic agent (96,97). The key requirements for an ideal delivery nanoparticle are (1) small size (50–200 nm), (2) high loading and entrapment efficiency, (3) slow complex

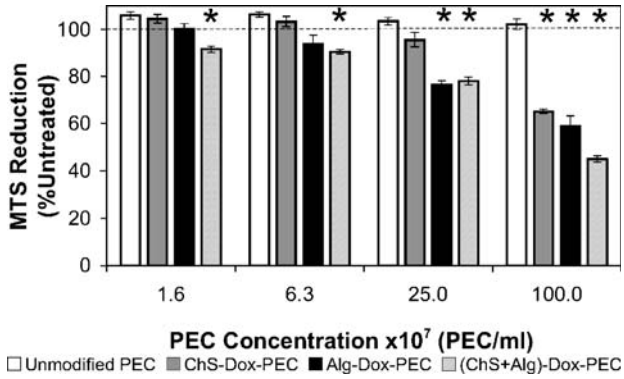

Fig. 4. Relative viability of HMVEC-1 cells incubated 72 h with various concentrations of PEC with and without core-loaded doxorubicin (*Dox*). Control cells are untreated. All results are given as the average ± standard error (n=4). *Asterisks* indicate statistical differences in MTS reduction (p>0.05) were seen by one-way ANOVA (deviations amongst experimental means) and Dunnet's test (experimental reductions less than the untreated control).

dissociation *in vivo*, and (4) optimized targeting to the desired tissue with limited non-specific uptake by other tissues (98). The development of formulations that can combine these benefits with a low cost, simple design is critical for highly efficient delivery systems. Our PEC vehicle is of particular interest because of the ease of incorporating a therapeutic agent within the complex. The therapeutic can simply be included in either the core or corona solutions prior to PEC fabrication.

There have been numerous examples of the use of a polyelectrolyte vehicle to deliver therapeutic payloads. Kabanov and coworkers demonstrated the enhanced transfection of mammalian cells with plasmid DNA via polyelectrolyte complexes formed between the DNA and poly(*N*-ethyl-4-vinyl)pyridinium with a simultaneous injection of Pluronic P85 (99). The entrapment and controlled release of several proteins was subsequently demonstrated in 1997 with chitosan/ethylene oxide-propylene oxide block copolymer complexes (100). Such studies using PECs as therapeutic delivery vehicles have provided a foundation for the current the PEC systems we have developed.

Entrapment within a nanoparticle protects the drug from inactivation and allows for a sustained release. For example, we have investigated the effect of loading doxorubicin (Dox) into PECs. Dox is first covalently conjugated to alginate (Alg) or chondroitin sulfate (ChS) that have been oxidized to form aldehyde groups. Cytotoxicity assays (MTS and Cytotox One) have been employed to verify the toxicity of the Dox conjugates is equivalent to free Dox. Loading of a single Dox-conjugate component within the PEC yields limited cellular toxicity. We are investigating additional methods to increase the therapeutic effect of the Dox-loaded PEC (Fig. 4).

We have also investigated the loading of viral vectors into our nanoparticle. Viruses, which directly transfer genetic material into the cell nucleus, have the additional limitations of off-target accumulation in liver sinusoids, Kupffer cells, and hepatocytes as well as a significant immune response (101,102). The ability of PECs to efficiently shuttle adenoviruses (AdVs) into cells to mediate the transfer of genes is an attractive feature that imparts a multifunctionality to these systems. This has been examined using PECs loaded with an AdV gene construct encoding a green fluorescent protein (GFP) and luciferase reporter genes. The PEC formulation showed a remarkable capacity to enhance adenoviral uptake and expression (Fig. 5a). GFP activity was significantly higher when comparing PECs encapsulation to free AdV, indicating the PEC vehicle is an efficient delivery method. The increased GFP activity was persistent as shown in Fig. 5b, suggesting the capacity of PECs to provide a sustained release of the vector which is advantageous for an effective therapeutic (46). The retention of virions is likely due to entanglement of relatively large virion particles within the polymeric chains of the complex whereby viral release occurs after cellular uptake. Possible mechanisms for enhancing viral potency include protection of entrapped virions from extracellular or intracellular degradation, use of an alternative or complementary cellular uptake pathway, and sustained delivery of virions.

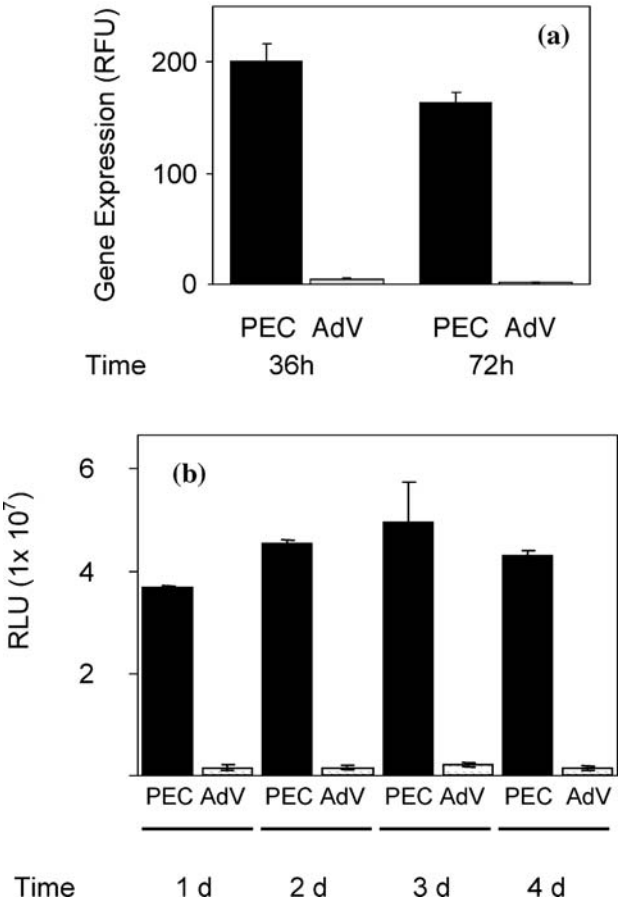

Fig. 5. PEC augment viral transduction *in vitro*. **a** Flow cytometric analysis of green fluorescent protein (*GFP*) expression in human microvascular endothelial cells (*HMVEC-1*) following either PEC–AdV gene transfer or free AdV infection. HMVEC-1 were incubated with PEC loaded with AdV or infected with free AdV for 6 h at 37°C, washed and then cultured in complete media. Expression of GFP was evaluated cytofluorometrically after 36 and 72 h continuous culture. Sustained, PEC-mediated viral luciferase expression in HMVEC-1 cells was incubated with equivalent titers of either PEC–AdV or free AdV for 6 h at 37°C. Afterwashing, cultures were collected every 24 h for 4 days, and lysates were assayed for luciferase activity (RLU). All results are given as the average ± standard error (n=3).

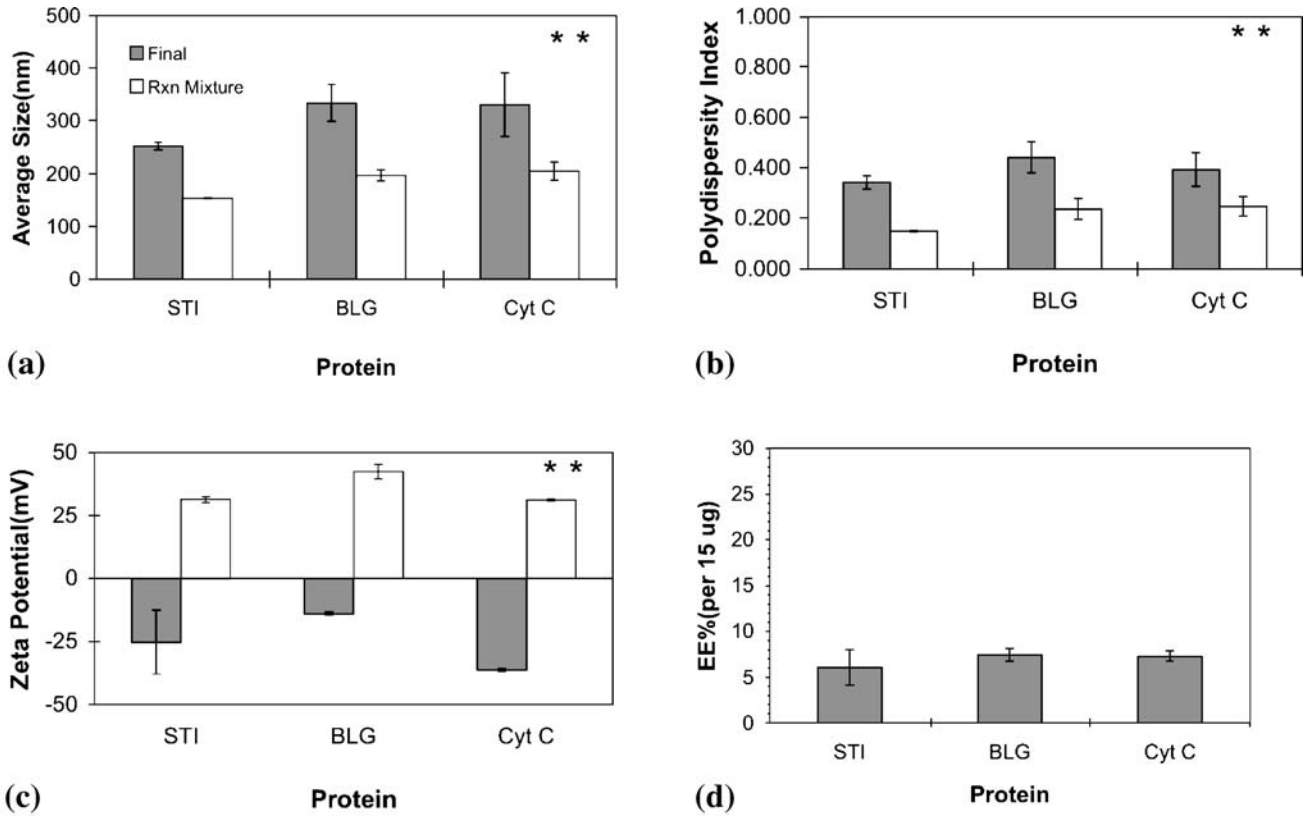

Fig. 6. Physicochemical properties of the reaction (*Rxn*) mixture and final preparations, in 100% FCS, for multi-component PECs prepared with various proteins loaded into the anionic solution. **a**, **b**, and **c** Correspond to hydrodynamic diameter, zeta potential, and polydispersity index, respectively, measured by PCS. The *double asterisks* indicate means (Rxn mixture *versus* final) that differ statistically by two-sample t-test at the 95% confidence interval (n=3). **d** Effect of protein on the protein entrapment efficiency (EE%) for PECs. Radioactive (I^{125}) protein associated with PECs was measured after particle preparation, isolation by centrifugation and resuspension in 100% FCS, for n=3. No statistical difference was indicated at $p<0.05$.

PEC PROTEIN ENTRAPMENT AND RELEASE

Proteins are often marginally stable and consequently easily damaged during their formulation as drugs. Encapsulation of proteins within biodegradable polymers has been shown to enhance the half-life *in vitro* (103,104) and *in vivo* (104–106). It is critical to control the liberation of drugs entrapped in polymeric supports under defined pharmacological and physicological conditions. PEC payloads can be delivered two ways: internalization of the PEC followed by release inside the cell, or particle docking at the cell surface and liberation through a bystander effect (107) non-internalizing ligands redisplayed (108).

Previous studies have investigated the drug release profiles from polymeric matrices as a function of pH (109), electric field (110), temperature (111), ultrasound (112), or light (113). Several PEC systems (77,100,108,114–116) have incorporated proteins and analyzed their release kinetics and entrapment. PECs allow the loading of proteins by polyion coacervation between charged groups. We have investigated the loading of 3 different iodinated proteins within PECs to trace their fate and address the effect of protein charge on entrapment and discharge in a simulated physiological environment under external sink conditions: 100% fetal calf serum (FCS) was added after replacement of fluid at each time point. Radioiodinated soybean trypsin inhibitor (STI), β-lactoglobulin (BLG), and cytochrome C (Cyt C) were selected because they possess high aqueous solubilities and a fairly wide range of isoelectric points (p*I*).

Sizes, polydispersity indices, and zeta potentials of PECs with Cyt C (p*I*=10.8), STI (p*I*=4.5), and BLG (p*I*=5.1) loaded into the anion core are shown in Fig. 6a–c for reaction (Rxn) mixtures and final formulations in FCS. There was no visible aggregation in FCS, but these properties were likely influenced by and altered due to the presence of heterogeneous serum particulates.

The effect of proteins with different pI on PEC loading is shown in Fig. 6d. Loading efficiencies ranged from 6.0% (STI) to 7.4% for Cyt C and BLG. The loading of proteins through intermolecular interactions and electrostatic forces between the proteins and PEC polyions has been found to be largely dependent on several molecular and environmental parameters: amount, molecular volume, polarity, charge, and degree of ionization (117). In this case, only the zeta potential and degree of ionization were studied; neither of which affected the incorporation.

Figure 7a shows the 7 day cumulative release, indicating a 'burst' release over the first 24 h and then a plateau, revealing an irreversibly bound phase. BLG-containing PECs exhibited a 43% release of protein after 1 day, while STI and Cyt C showed 34 and 21% released over the same period. Burst release is common in the PEC system, and it is affected by environmental (temperature, pH, concentration, diffusional gradients), and molecular (polymer molecular weight, internal cross-linking;

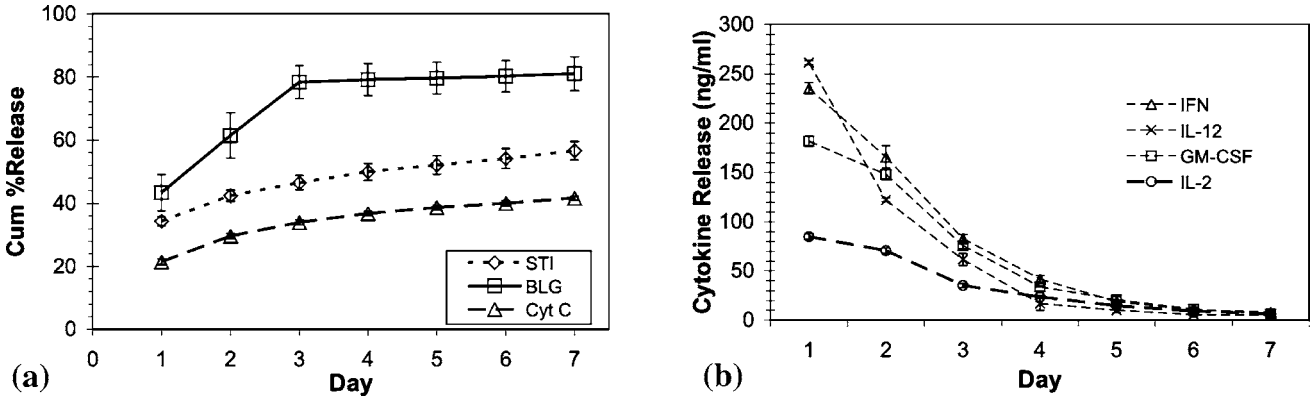

Fig. 7. *In vitro*, iodinated release profiles for **a** STI, BLG, Cyt C and **b** various cytokines into FCS at room temperature (n=3).

118) variables. STI, BLG, and Cyt C each demonstrated first-order release kinetics that approached zero-order relationships at longer times (t=4–7 days). The relatively low incorporation was likely due to the weak, amphipathic nature of the proteins. A significant fraction of STI, BLG, and Cyt C were unlikely to compete with stronger electotrolytes (PMCG, chondroitin sulfate, alginate, spermine) for sites in the complex, leading to rapid elution. According to the literature, alginate and chondroitin sulfate, constituents of the PEC core, are highly hydrophilic, owing to the presence of –OH, –COOH, and SO_4^- groups on the polysaccharide chains (108), while spermine and PMCG comprise a hydrophilic outer shell. Therefore, the entrapped proteins likely became sequestered outside of the inner core, but within the corona. The incorporation characteristics were consistent with the properties of chitosan/PPO PECs developed by Calvo *et al.* (100). The introduction of a more highly charged drug or protein would likely lead to higher entrapment levels (117).

Kamiya and Klibanov (114) have recently proposed that larger complex diameter leads to slower release. The current PEC formulations, comprised of polyions described in Table II, did not appear to adhere to that finding, as the release profiles were independent of the hydrodynamic diameter. Further PEC modification, before or after complexation, may be needed to reduce the burst effect. In spite of the low entrapment efficiency and burst release, PECs are still a promising candidate for drug delivery in instances where the vehicles are rapidly bound and taken up by cells. The drug could then be released intracellularly by diffusion or by dissociation of the PEC.

More clinically relevant cytokine payloads exhibit improved release kinetics when loaded in the PEC core. Granulocyte macrophage colony stimulating factor (GM–CSF), interleukin-2 (IL-2), interferon gamma (IFN) and interleukin-12 (IL-12) release into FCS at 20°C was measured by ELISA (Fig. 7b). In addition, released GM–CSF PECs stimulated antigen presentation and allogeneic T cell proliferation *in vitro* and an anti-tumor effect *in vivo* (data not shown).

STRATEGIES FOR PEC TARGETED DELIVERY OF THERAPEUTICS

The use of molecular targeting allows for the selective delivery of compounds to the tissue of interest, therefore increasing the therapeutic index and decreasing collateral effects (102,107). The concept of targeting always exploits phenotypic differences between the disease target and normal tissues that are then translated into a dose differential between the target and off-target sites. Current methods involve the use of antibodies and other ligands to deliver agents (peptides, nucleotides, hydrophobic drugs) to selected extracellular and intracellular targets (119). Antibodies have the advantage of high specificity, but they are expensive, time-consuming to produce, and have problems with stability and storage. Conversely, non-antibody ligands suffer from lower selectivity and or affinity, but they are inexpensive to manufacture and easy to handle (107).

The site-specific delivery to cells or organs is an attractive mode of treatment for increasing the therapeutic efficiency of drugs and reducing their toxicity (120). Several strategies have been developed to employ specific ligands that interact with the endothelial cell (EC) surface to mediate delivery of drugs selectively to the tumor microenvironment without affecting normal tissue (121–123). Tumor vasculature targeting is a promising strategy, as both primary tumor maturation and metastasis depend on the survival and growth of new blood vessels, termed angiogenesis. Additionally, intimate contact with the blood makes the tumor endothelial cell a uniquely accessible target within the tumor (124). Extensive research has led to the identification and isolation of several regulators of angiogenesis, some of which represent therapeutic targets (125,126).

Strategies for localization have included coupling of surface ligands to liposomal systems (35,127,128; active targeting) and the enhanced permeability and retention effect (EPR; 129,130; passive targeting), a unique pathophysiologic feature of tumor vasculature. Folate has been conjugated to dendrimers (39,131), polymeric (132,133), and liposomal (128) nanoparticles for specificity to cancer cells that overexpress the folate receptor. Other polymeric nanostructures (134,135), PECs (136), and liposomes (35) have been used to target cellular adhesion molecules, integrins, expressed on vascular endothelial cells in solid tumors. Liposomes (15), PECs (47), and polymer conjugates (134) have also utilized vascular endothelial growth factor (VEGF) for targeting the VEGF receptor on the vasculature. The use of an RGD based peptide, which has been shown to have a specific high affinity to integrins of neovasculature, would allow the development of a method to target malignant as well as wound tissue (137–140). We have begun investigating the ability to target PECs to integrins using an RGD

Table III. Targeted and Non-Targeted PEC physicochemistry in EC Growth Media

Platform	Size (nm)	PDI	Zeta Potential (mV)
Non-Targeted	192.3±16.6	0.393±0.186	−18.4±4.3
Targeted PEGp521	194.2±23.6	0.167±0.024	−25.2±5.6

containing peptide by FACS analysis. As predicted, the RGD-containing, FITC labeled PECs bound to HMVEC better than non-targeted PECs. We have detected an increased affinity to integrins by means of bivalent E-c(RGDfK)$_2$ peptide (data not shown).

Our group has more intensively investigated the ability to target our PEC system to the endothelium through the use of a thrombospondin-1 peptide sequence (TSP521). The matricellular protein, thrombospondin-1 (TSP-1) has been proposed to play a role in numerous biological processes including embryonic development, angiogenesis, and hemostasis (141,142). The ability of TSP-1 to regulate these processes has been attributed to its capacity to bind to matrix proteins, proteinases, growth factors, and cell surface receptors through multiple domains. The receptor-mediated binding and endocytosis of TSP-1 is controlled by HSPGs (143), cell surface receptors which are recognized by metastatic tumor cells upon their binding (144), and overexpressed in tumor milieu (70,145–147). Further clinical use of TSP-1 is limited by the problems with synthesizing large quantities of this large protein and the potential for off-target effects due to its complex structure (148). However, peptide sequences derived from certain TSP-1 domains show antiangiogenic activity and mimic the function of the intact macromolecule. The TSP521 peptide specifically binds HSPGs, and it has been shown to target the vasculature of an experimental glioma model (149). It also has been shown to impede the translocation of fibroblast growth factor-2 (FGF-2) to its tyrosine kinase receptor, resulting in inhibition of cell proliferation (150). These properties led to the selection of TSP521 as a potential neovascular targeting platform.

We incorporated TSP521 by passive, electrostatic entrapment after conjugation to PEG. The elongation achieves both a geometric and flexible presentation (119), decreased susceptibility to circulatory proteolytic enzymes, and improved phamacokinetic properties (151). PEGylation is widely used to prolong the biological half-life of polypeptides and proteins (151); however, in this application conjugation was designed to modulate the presentation of the peptide by increasing its molecular mass and flexibility. The self-assembly led to the spontaneous incorporation of PEGylated TSP521 (PEGp521), which was loaded into the anionic solution utilized for PEC fabrication. The loading efficiency for PEGylated peptide entrapment was 2%, verified isotopically and with fluorescence. Suspension of the formulation in a neutral buffer (HEPES, pH=7.4) led to stable suspensions and showed no statistical difference ($p<0.05$) in size and zeta potential compared to non-targeted PECs (Table III). The improved properties (reduced PDI) exhibited by PECs containing PEGylated TSP521 was due to the presence of PEG, which increased the resistance to salt-induced aggregation and provided steric stabilization (152). *In vitro*, the systems did not exhibit cytotoxicity as detected by MTS assay (data not shown).

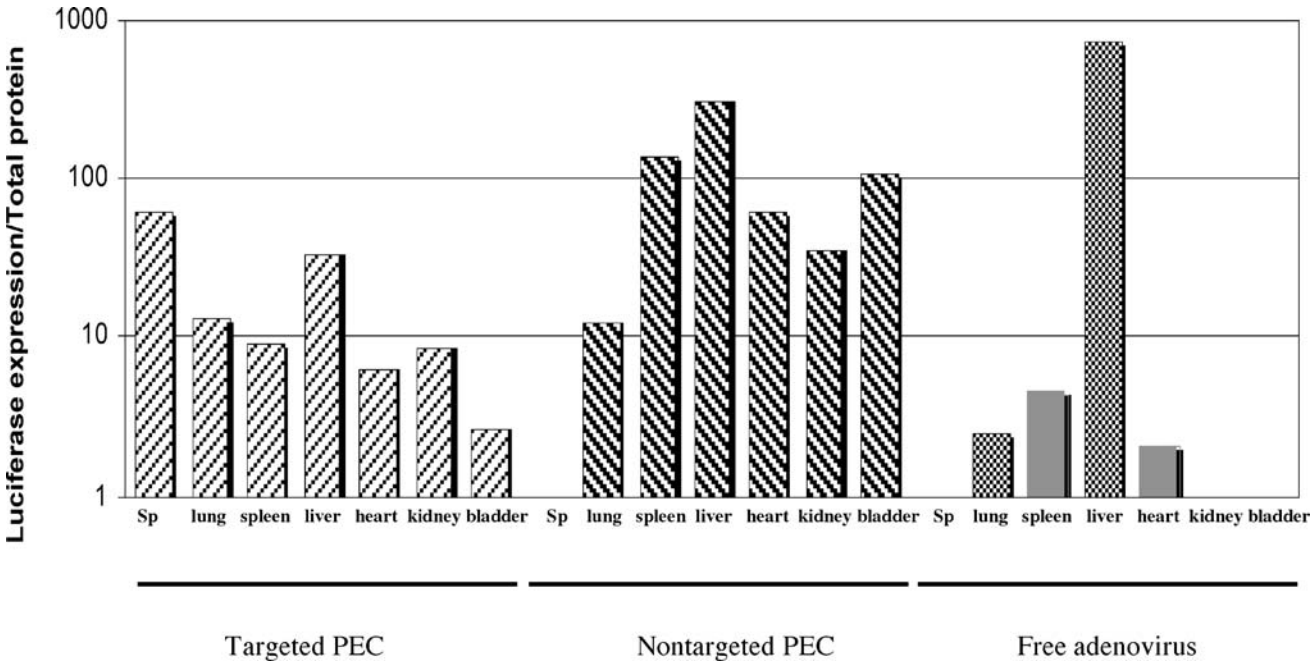

Fig. 8. Targeted delivery of PECs loaded with adenovirus. PEGylated TSP521 was concurrently loaded with an adenoviral construct carrying the firefly luciferase reporter gene. Accumulation in PVA sponges (*Sp*) indicated directed traffic to sites of neovascularization.

The combination of PEC targeting with AdV gene transfer represents another application for our polymeric nanoparticle. To assess the feasibility of this, the biological distribution of TSP521 targeted NPs and their ability to accumulate in areas of active neovascularization was investigated with PECs loaded with AdV gene construct encoded with the reporter gene for firefly luciferase. Luciferase activity in the polyvinyl alcohol (PVA) sponge, implanted on the ventral flanks of mice, was only detected in with the TSP521–PECs loaded with AdV vector (Fig. 8). The overall biodistribution was strongly altered by the presence of TSP521 peptide. Injection of free AdV showed that the virus was predominantly sequestered in the liver with no detectable expression in sponge granulation tissue. This demonstrates that the delivery of AdV vectors with our PEC vehicle results in tissue uptake and subsequent expression of the virus and establishes the viability of coupling a peptide (i.e. RGD) to our nanoparticulate system for *in vivo* tissue targeting agent.

The multifunctional targeting and delivery capabilities of NPs, combined with a polymeric, protective coating that decreases the toxicity of the payload to normal tissues, may overcome limitations of conventional cytotoxic treatment methods. Kim *et al.* employed folate targeting of poly (L-lysine)–poly(ethylene glycol) complexes to demonstrate the increased uptake of FITC labeled bovine serum albumin proteins by KB cells relative to folate receptor deficient A549 cells (133). Folate was also used to target polyamidoamine (PAMAM) dendrimers to KB tumors *in vivo* in order to deliver methotrexate, an anticancer drug (38). Suh *et al.* demonstrated the RGD targeting of polyethyleneimine/poly(ethylene glycol) (PEI–PEG) complexes in order to deliver DNA to HDMEC cells. RGD targeting resulted in a fivefold increase in transfection efficiency over non-targeted complexes (135).

The potential advantage of targeted delivery may result from an altered intracellular distribution. When a non-internalizing ligand is employed, the nanoparticle binds to target cells followed by a gradual release of the drug. The free drug is then taken up by the cell using standard uptake mechanisms. When an internalizing ligand is employed, the nanoparticle–drug is taken into the cell by receptor-mediated endocytosis and, assuming it is stable in the environment of the endosome, the drug is gradually released within the cell. The number of drug molecules that are delivered intracellularly are higher when an internalizing ligand is used as the diffusion and redistribution of the released drug seem to be higher for non-internalizing ligands, which leads to lower concentrations of drug being delivered to the target cells. It is probably for this reason that internalizing ligands have resulted in better therapeutic outcomes in animal models (38,153,154). For internalizing ligands, because not all of the nanoparticle drug will immediately be internalized into target cells, the opportunity for a bystander effect exists. A drug

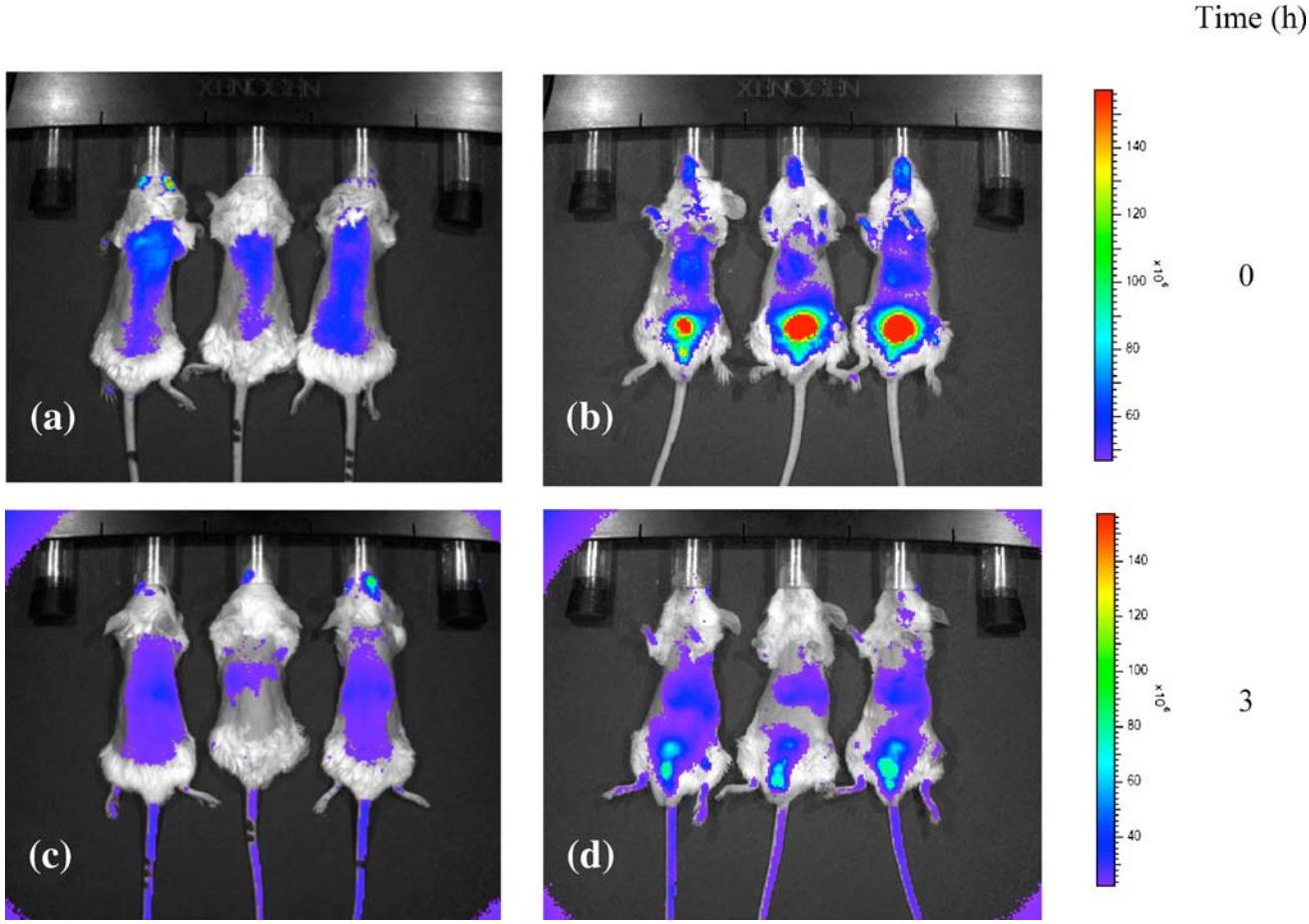

Fig. 9. *In vivo* fluorescence imaging of retro-orbitally injected AF750 PECs (**a**–**d**) immediately after injection and 3 h later in male BALBc mice. Dorsal imaging was performed in (**a**), (**c**), while ventral in (**b**),(**d**). The intensity of the signals, light flux, is denoted by the respective color bars in terms of pixels/second. 10 s exposures were applied followed by the overlay of photographic and fluorescence images.

that is released extracellularly diffuses inside the tumor mass followed by uptake into receptor-negative cells (107).

REAL TIME FLUORESCENT PEC IMAGING

The ability to localize PECs *in vivo* and *in vitro* to monitor tissue distribution is critical to understanding their biocompatibility and behavior (155). The use of fluorescently or radioactively labeled NP is the most common experimental approach found in the literature (73,89,120,156). Optical imaging modalities do not require ionizing radiation and are inexpensive (157). Additionally, bioluminescence and fluorescence approaches are highly sensitive and allow for high throughput screening because obtaining an image can be as short as seconds (158). Because of the strong tissue penetration ability of light in the near-infrared region (NIR), near-infrared fluorescence (NIRF) imaging has emerged as a powerful tool for small animal imaging. These fluorescence tracers emit or absorb light in the 650–900 nm wavelengths. NIRF imaging has a great potential for clinical use in providing both real-time surgical, functional, and molecular information on disease states. Water and biological tissues have minimal absorbance and autofluorescence in the NIR window, thus allowing efficient photon penetration into, tissue with low intra-tissue scattering. NIRF probes are therefore under active investigation and have been demonstrated as a viable method to noninvasively monitor disease states at the molecular level, localize cancer, and even assess the antitumor efficacy of new therapeutics (159–161).

The success of fluorescently labeled tumor-targeting peptides has initiated a quest for nanoparticulate formulations. Park's group demonstrated targeted cancer cellular uptake with FITC labeled folate PGLA nanoparticles (133). Kelly *et al.* (162) and Montet *et al.* (163) developed nano-imaging agents for visualization of vascular targets *in vivo*. Kelly used 30 nm magnetofluorescent nanoparticles endowed with VINP28, phage-display selected peptide, for targeting to VCAM-1 endothelial vascular adhesion molecule and obtained 54-fold T/B ratios in atherosclerotic lesions in mice. Montet employed the same vehicle (but of 10–100 nm size) decorated with a linear RGD–Cy5.5 peptide for targeting to integrins in BT-20 tumors but observed a 6.5 increase in the T/B ratio. In general, these studies found that the fluorophore labeled nanoparticles cleared from background tissue and organs over time while the signal was retained in the targeted tissue. These results have been concurrently translated into the therapeutic area with dendimers and liposomes (38,153).

The application of PECs as a drug delivery system must be proven both *in vitro* and *in vivo.* The fluorescent probes have reactive groups for the functionalization of amines, carboxyls, and other active chemical moieties on polymers and proteins. Therefore, AlexaFluor 750 (AF750), an amine reactive NIRF probe, has been incorporated into PECs for further examination of biodistribution. AF750 was successfully linked to the primary amine of PMCG, a component of the cationic solution. The resulting particles were stable after suspension in a slightly basic, hypotonic buffer with a size (185.78±63.34 nm), zeta potential (−30.9±1.00), and PDI (0.247±0.081), statistically consistent with unmodified PEC.

The *in vivo* application of AF750 PMCG-containing PECs was accomplished by retro-orbital injection of 100 μl of the above suspension (~3×10^9 PECs) and imaging at various time points up to 48 h in mice, using the Xenogen IVIS200 Imaging System. There was no acute reaction after intravenous administration, indicating bulk PEC biocompatibility. Intrajugular, tail vein, intraperitoneal, and direct heart injections were also assessed with no ill effects. Fig. 9 shows the PECs in circulation, and a large fraction of the fluorescent signal was rapidly cleared to the bladder. The images showed that this process occurred rapidly, between the time of injection and first imaging exposure (~10 min). Significant bladder fluorescence persisted at 3 h, but some signal was localized to the upper chest cavities (liver, lungs, spleen, kidneys). The bladder signal completely disappeared at 24 h. This urinary excretion was indicative of a partial destabilization of PECs by polyelectrolyte exchange reactions, leading to rapid clearance of (AF750) PMCG from the bloodstream. The low molecular weight of PMCG (5,000 Da) complex is well below the kidney filtration limits (164); however, the fluorescent signal could also be indicative of the dissociation and excretion of other PEC components. Following intravenous administration, naked PECs are exposed to a variety of factors that may have compromised their integrity and caused partial decomplexation, including interactions with proteoglycan-containing extracellular matrices and high concentrations of anionic serum proteins or other molecules present in blood plasma (165,166). Suspensions of AF750 PECs in 100% fetal calf serum supported the potential for dissociation/exchange, since 14% of the fluorophore conjugated PMCG was released in the first 30 min (data not shown).

After 24 h, the remaining, intact complexes became rapidly sequestered into highly perfused and vascularized organs that accumulate mononuclear phagocytes as a result of the immune response: liver, lungs, and spleen (Fig. 10). The fluorescence of each organ was imaged immediately after euthanasia and dissection, using region of interest (ROI)/light flux analysis. These organ-specific signals degraded over a 48 h period. Consistent with these findings, intravenous injection of colloidal carriers, such as liposomes and polymeric nanospheres, are recognized by the reticuloendothelial system as they circulate in the blood and bind opsoninizing macromolecules (167). There are many approaches to stabilize AF750 PECs against opsonization. One strategy would be to enshroud the PECs within a protective layer of hydrophilic polymers, such as PEG (77), in addition to the Pluronic F-68 already present. The surface modifications can be accomplished by activated linkages to sufficiently 'coat' the PECs, avoid phagocyte-mediated clearance, increase the circulatory time, and enhance complex stability. The imaging of AF750 PECs further established the feasibility of PECs as a template for nanoparticle-mediated targeted drug delivery.

CONCLUSIONS AND LIMITATIONS

The future of polymeric drug and gene delivery lies in developing non-toxic multi-component entities for combined targeting, imaging, and payload trafficking. PECs provide an inexpensive, biocompatible, versatile alternative system to current polymeric delivery strategies that apply organic solvents as reaction environments (Fig. 11). Common nanotechnological techniques, such as TEM and PCS, can easily provide information on PEC physical properties. PECs have

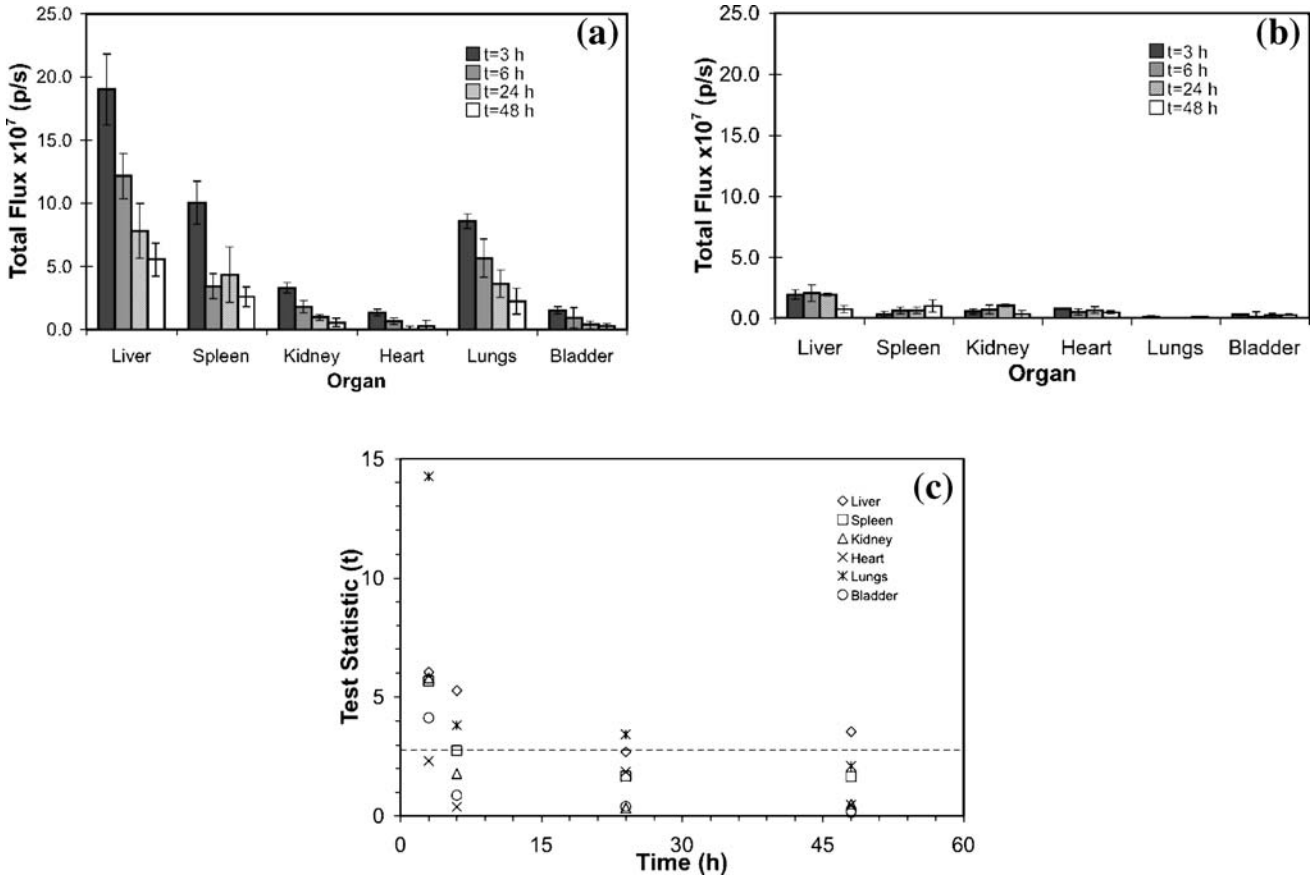

Fig. 10. Quantification of *ex vivo* organ distribution for **a** AF750 PECs and **b** PBS injected animals. The fluorescence was recorded as photons/second after creation of a region of interest for each organ. The background was subtracted by creating a region of interest away from the organ signals. The statistical significance of each fluorescence measurement (n=3) was tested against its PBS counterpart. The test statistic for each two-way comparison was plotted (**c**) as a function of time (n=3, p<0.05).

favorable and attractive physicochemical characteristics that are maintained at physiological pH and low concentration, serum-containing media: uniform, attractive size distributions, mean diameters statistically less than or equivalent to 200 nm, and surface charges indicative of stable colloidal suspensions. Additionally, these nanoparticulate architectures can entrap, release, and retain proteins, mimicking therapeutics, over the course of several days, leading to the possibility of a drug depot for intravenous or systemic administration. PECs exhibit little or no toxicity in a biological environment. Because of the PEC modular and chemical nature, surface amines and inner core hydroxyl groups permit the efficient incorporation of targeting moieties, chemical linkage of classically insoluble drugs (doxorubicin). The fluorophore incorporation strategy enables both visible and near-infrared fluorescence characterization of PEC fates in cell culture and small animals. Flow cytometry can be utilized to extensively characterize and define the underlying mechanisms of PEC binding and uptake, in endothelial cells and other targets (endothelial cells), through macropinocytosis. Compartmentalization, uptake and surface binding can be delineated through extracellular FITC quenching; another advantage of flow cytometry. In the absence of targeting ligands, cationic PECs bind cells through strictly electrostatic interactions where cells provide an anionic sink to mediate the attachment. Work in this laboratory has led to a novel, flow cytometric, Scatchard analysis protocol, where the extent of receptor–controlled interactions is dictated by adherence to underlying assumptions of mass action and equilibrium binding. This strategy for characterization for nanoparticulate architectures to define true targeting and receptor–ligand interactions; a critical feature due to misleading information from direct

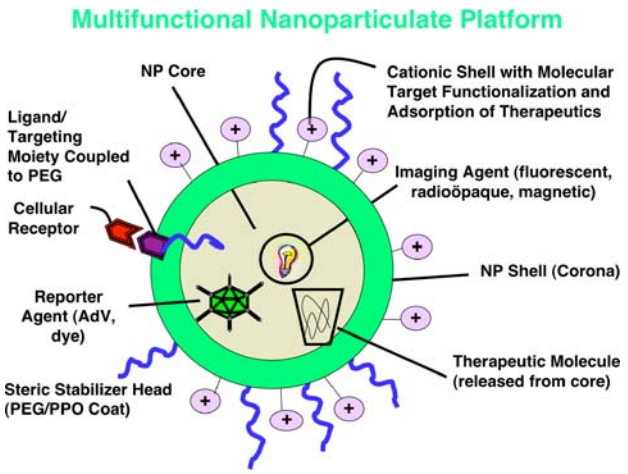

Fig. 11. Multifunctional NP platform with a negatively charged core and positively charged corona that can be loaded with a therapeutic agent to provide sustained release, imaging agent, or targeting agent.

kinetic studies. The Scatchard plots can screen targeted delivery systems, *in vitro*, before expensive animal models are used. This approach is ideal when radioactive nanoparticle components are not currently available or the incorporation of an isotope too cumbersome.

It is difficult to produce PECs that possess all ideal *in vivo* and *in vitro* performance related properties. Stability issues and off-target localization in liver, lungs, and spleen must be considered before clinical applications can be considered. Proper targeting ligand choice and presentation in combination with a corona adaptation can be improved through the use of PEG. PEG not only provides a coating to prevent opsonization and subsequent recognition by macrophages of the reticuloendothelial system, but can link a targeting moiety in a distal conformation to facilitate the appropriate receptor activation. First introduced by Gref (75), PEG introduction into poly(lactic-co-glycolic acid) nanospheres resulted in dramatic increases in blood circulation times and reduced liver accumulation in mice. Since that discovery in 1994, PEG incorporation into nano- and microparticulate biomaterials has been used extensively in countless studies to improve polymer stability and prevent protein fouling. In conclusion, PECs hold promise for a variety of combinatorial medical uses and could be utilized as targeted drug delivery formulation and also as a non-invasive, real-time imaging construct in humans.

ACKNOWLEDGMENT

We acknowledge support of National Institutes of Health Grants 1R01EB002825-01 (J.M.D. and A.P.), support from the Department of Veterans Affairs (J.M.D.), the University of Texas MD Anderson Odyssey Fellowship, and the TN Law Foundation (S.M.H.). In addition we would like to thank the Vanderbilt Institute for Nanoscale Science and Engineering (VINSE) for use of the Malvern ZetaSizer Nano ZS.

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