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Scalable Method to Produce Biodegradable Nanoparticles that Rapidly Penetrate Human Mucus

Qingguo Xu^{1,2}, Nicholas J. Boylan^{1,3}, Shutian Cai³, Bolong Miao^{3,4}, Himatkumar Patel¹, and Justin Hanes^{1,2,*}

¹The Center for Nanomedicine, Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21231, USA

²Department of Ophthalmology, The Wilmer Eye Institute, The Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21231, USA

³Department of Chemical & Biomolecular Engineering, The Johns Hopkins University 3400 N. Charles St, Baltimore, MD 21218, USA

⁴Institute of Biomedical Engineering, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300192, China

Abstract

Mucus typically traps and rapidly removes foreign particles from the airways, gastrointestinal tract, nasopharynx, female reproductive tract and the surface of the eye. Nanoparticles capable of rapid penetration through mucus can potentially avoid rapid clearance, and open significant opportunities for controlled drug delivery at mucosal surfaces. Here, we report an industrially scalable emulsification method to produce biodegradable mucus-penetrating particles (MPP). The emulsification of diblock copolymers of poly(lactic-co-glycolic acid) and polyethylene glycol (PLGA-PEG) using low molecular weight (MW) emulsifiers forms dense brush PEG coatings on nanoparticles that allow rapid nanoparticle penetration through fresh undiluted human mucus. In comparison, conventional high MW emulsifiers, such as polyvinyl alcohol (PVA), interrupts the PEG coating on nanoparticles, resulting in their immobilization in mucus owing to adhesive interactions with mucus mesh elements. PLGA-PEG nanoparticles with a wide range of PEG MW (1, 2, 5, and 10 kDa), prepared by the emulsification method using low MW emulsifiers, all rapidly penetrated mucus. A range of drugs, from hydrophobic small molecules to hydrophilic large biologics, can be efficiently loaded into biodegradable MPP using the method described. This readily scalable method should facilitate the production of MPP products for mucosal drug delivery, as well as potentially longer-circulating particles following intravenous administration.

Keywords

Drug delivery; Mucus-penetrating particles; Nanomedicine; Surface PEG density; Emulsification

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*Corresponding Author: The Center for Nanomedicine at Johns Hopkins University, 400 N Broadway, Robert H. and Clarice Smith Bldg., 6th Floor, Baltimore, MD 21231, USA, Tel: 410-614-6512, hanes@jhmi.edu (J. Hanes).

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1. Introduction

Localized delivery of therapeutics via biodegradable nanoparticles often provides advantages over systemic drug administration, including reduced side effects and prolonged drug levels at target sites [1–2]. However, controlled drug delivery at mucosal surfaces has been limited by the presence of the protective mucus layer [3–4]. Mucus is a viscoelastic gel that coats nearly all exposed epithelial surfaces not covered by skin, such as the respiratory, gastrointestinal, nasopharyngeal, and female reproductive tracts, and the surface of the eye. Mucus efficiently traps conventional particulate drug delivery systems via steric and/or adhesive interactions [5]. As a result of mucus turnover, most nanoparticle-based therapeutics delivered locally to mucosal surfaces suffer from poor retention and distribution, which limits their efficacy [3]. Biodegradable nanoparticles that penetrate deep into the mucus barrier provide improved drug distribution, retention and efficacy at mucosal surfaces [6].

Dense coatings of polyethylene glycol (PEG) allow nanoparticles to rapidly penetrate through highly viscoelastic human mucus secretions by minimizing adhesive interactions between nanoparticles and mucus constituents [7–10]. Biodegradable mucus-penetrating particles (MPP) have been prepared by physical adsorption of certain Pluronics, such as F127, onto pre-fabricated mucoadhesive nanoparticles prepared by nanoprecipitation [11]. In addition, MPP have been prepared by nanoprecipitation using diblock copolymers of poly(sebacic acid) and PEG (PSA-PEG) [12] or diblock copolymers of poly(lactic-co-glycolic acid) and PEG (PLGA-PEG) [13]. However, the scope of biodegradable MPP prepared by nanoprecipitation is limited because it requires dissolution of the drug and polymer in water-miscible solvents. Numerous hydrophobic drugs may benefit from local delivery by MPP, however, their poor solubility in water-miscible organic solvents limits efficient encapsulation into MPP by nanoprecipitation [14]. Other classes of emerging drugs, including nucleic acids, peptides and proteins, possess immense potential to treat diseases at mucosal sites [15]. However, these hydrophilic drugs cannot be easily formulated into MPP by nanoprecipitation. On the other hand, emulsification or emulsion solvent evaporation method, is widely used to efficiently encapsulate both hydrophilic drugs (water-in-oil-in-water double emulsion) and hydrophobic drugs (oil-in-water single emulsion) into biodegradable nanoparticles [15–18]. Emulsification method is one of most widely used scalable approaches to manufacture biodegradable nano/microparticle drug delivery systems in the pharmaceutical industry [19]. This method involves the emulsification of an oil phase in an aqueous phase containing emulsifiers (normally polyvinyl alcohol or PVA), and the evaporation of organic solvent from emulsion droplets resulting in the formation of solid nanoparticles [16, 19]. However, particles resulting from the emulsification method are mucoadhesive when the conventional emulsifier PVA is used [6, 20]. Here we report an emulsification method using off-the-shelf materials to encapsulate a wide range of drugs into biodegradable nanoparticles with mucus penetrating properties.

2. Materials & methods

2.1. Materials

Cholic acid sodium salt (CHA), Tween20, Tween80, hexadecyltrimethylammonium bromide (CTAB), dioctyl sulfosuccinate sodium (DSS), Saponin, Polyoxyl 35 hydrogenated castor oil (Cremophor EL) and D- α -tocopherol polyethylene glycol 1000 (Vitamin E-TPGS), were purchased from Sigma (St. Louis, MO). Poly(vinyl alcohol) (PVA; Mw=25 kDa with 88% hydrolysis and 6 kDa with 80% hydrolysis), and poly(ethylene-*alt*-maleic anhydride, 1:1 molar ratio) (PEMA) with Mw~400 kDa were bought from PolySciences (Warrington, PA). Sugar ester D1216 (SE) was a gift from Mitsubishi-Kagaku Foods Co. (Tokyo, Japan). Pluronic F127 and F68 were provided from BASF (Ludwigshafen,

Germany). Alexa Fluor 555 cadaverine was purchased from Invitrogen (Grand Island, NY). Poly(lactic-co-glycolic acid) (PLGA; LA:GA 50:50) with inherent viscosity of 0.15–0.25 dL/g (MW~15kDa) was purchased from Lakeshore Biomaterials (Birmingham, AL). PLGA-*b*-PEG diblock copolymers with PEG MW of 10, 5, 2 and 1 kDa, poly(D,L-lactic-acid)-*b*-PEG5k (PLA-PEG5k) and poly(ϵ -caprolactone)-*b*-PEG5k (PCL-PEG5k) with PEG MW 5 kDa were custom-synthesized by the Jinan Daigang Biomaterial Co., Ltd, (Jinan, China) and characterized by ^1H NMR and gel permeation chromatography (GPC), as shown in Supporting information (Table S1 and Fig. S1).

2.1. Preparation of nanoparticles

Alexa Fluor 555 cadaverine (AF555) was chemically conjugated to polymers as reported previously [21]. Nanoparticles were prepared by using the emulsification method. Typically, a mixture (total 50 mg) of PLGA-PEG5k and AF555-labeled PLGA-PEG5k was dissolved in 1 mL dichloromethane (DCM). The oil phase was poured into 5 mL aqueous solution containing 1% emulsifier under sonication (VibraCell, Sonics & Materials Inc., Newtown, CT) at 30% amplitude for 2 min in an ice-water bath to form the oil-in-water (o/w) emulsion. This emulsion was poured into another 40 mL aqueous phase of emulsifier solution under magnetic stirring at 700 rpm for at least 3 hours to allow solvent to evaporate. The solvent was further evaporated by placing the solution in a vacuum chamber for 30 min. The final nanoparticle suspensions were filtered through 1 μm syringe filter, centrifuged at 20,000 g for 25 min and thoroughly washed with DI water. Emulsifiers including CHA, DSS, CTAB, PVA, PEMA, Saponin, SE, Tween20, Tween80, F127, F68, Cremophor EL and Vitamin-E TPGS were tested at a concentration of 1% w/v. CHA solutions at 0.01%–0.5% w/v were also used to make nanoparticles by the emulsification method.

In order to evaluate the effect of PEG MW (1, 2, 5 and 10 kDa) on mucus-penetrating property of nanoparticles prepared by the emulsification method, CHA was selected as the representative low MW strong emulsifier. PLGA-PEG nanoparticles with different PEG MW at approximately 6wt% PEG content were prepared in 0.5% CHA solution. In order to achieve overall 6wt% PEG content for PLGA-PEG10k nanoparticles, blends of PLGA-PEG10k (21.6 wt% PEG) and PLGA15k were utilized.

For the preparation of drug encapsulated nanoparticles, curcumin and bovine serum albumin (BSA) were used as model hydrophobic drug and hydrophilic biologics, respectively. PLGA-PEG5k and curcumin were dissolved in DCM, and the preparation of curcumin-loaded nanoparticles by single o/w emulsification method was similar to that for preparation of unloaded nanoparticles. The prepared curcumin-nanoparticles can be visualized in mucus because of curcumin's intrinsic fluorescence. BSA was used as a model hydrophilic drug because it is representative of large molecule biologics. BSA-FITC and BSA (10% ratio of BSA-FITC) were dissolved in 0.2 mL aqueous solution. This solution was added to 1 mL of 100 mg/mL PLGA-PEG5k in DCM solution during probe sonication (30% amplitude, 1 min with 1 s pulse) in an ice-water bath. The resultant water-in-oil primary emulsion was immediately added to a second water phase (5 mL 1% saponin solution) under sonication (20% amplitude for 2 min). The water-in-oil-in-water double emulsion was transferred to another 40 mL 1% saponin solution with magnetic stirring for 3 hours. Nanoparticles were filtered through 1 μm syringe filter, washed and collected by centrifugation. BSA-FITC allowed the possibility to track BSA-loaded nanoparticles in mucus. The target drug loading for curcumin nanoparticle and BSA nanoparticle was 9.1% and 16.7%, respectively.

2.2. Estimation of emulsification capability

The emulsification capability of each emulsifier was estimated by its ability to prevent the formation of aggregated particles. PLGA-PEG5k (MW ~83 kDa) was used as the model

diblock copolymer, and it was dissolved in DCM at 50 mg/mL. PLGA-PEG5k nanoparticles were prepared by the emulsification method using different emulsifiers at the same method described above. Aggregated particles were collected by centrifugation at 500 g for 20 min, and the remaining nanoparticles in the supernatant were collected by centrifugation at 30,000 g for 25 min. The weight ratio of unaggregated nanoparticles (W_{NP}) to aggregated particles (W_{AGG}) was calculated, and used as the index to estimate the emulsification capability of the emulsifier, as follows:

$$\text{Emulsification capability} = W_{NP} / (W_{NP} + W_{AGG}) \times 100\%$$

2.3. Characterization of nanoparticles

Diameter and ξ -potential (surface charge) of nanoparticles were measured using Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA). Nanoparticles were suspended in 10 mM NaCl solution. Particle morphology was characterized using a H7600 transmission electron microscope (Hitachi, Japan). TEM samples were prepared by dropping a dilute suspension of nanoparticle on a TEM grid and allowing to air dry.

2.4 Quantification of surface PEG density

The surface PEG density on nanoparticles was determined by ^1H NMR using Bruker 400 REM instrument at 400 MHz [22–24]. Relaxation time was set at 10 s, and ZG at 90° . Nanoparticles were directly prepared in 0.5% CHA D_2O solution and suspended in D_2O with 1wt% 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (Sigma, St Louis, MO) as internal standard for ^1H NMR analysis. The ^1H NMR spectra of nanoparticles in D_2O are shown in Fig. S2. A known weight of 5 kDa PEG (Sigma, St Louis, MO) homopolymer in D_2O with 1% 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt was serially diluted to different concentration to set up the calibration curve (Fig. S3) for the PEG signal in ^1H NMR, and this calibration curve was used to calculate the surface PEG content on nanoparticles. A 0.2 mL solution of nanoparticles in D_2O was lyophilized and weighed. By assuming all surface PEG chains were full length of 5 kDa PEG, the surface PEG density was calculated as the number of PEG molecules per 100 nm^2 surface area on nanoparticles. Control ^1H NMR experiments were also performed with PLGA nanoparticles prepared by the same method and there were no detectable CHA peaks for PLGA nanoparticles (Fig. S2). PEG density, $[\Gamma]$, is the number of PEG molecules on nanoparticle surface per 100 nm^2 . It can be calculated by dividing the total PEG content (M_{PEG} in mole) detected by ^1H NMR by the total surface area of all nanoparticles as follows:

$$[\Gamma] = \frac{M_{PEG} \times 6.02 \times 10^{23}}{W_{NP} / d_{NP} / \frac{4}{3}\pi\left(\frac{D}{2}\right)^3} \div 4\pi\left(\frac{D}{2}\right)^2 \times 100$$

Where W_{NP} is the total mass of nanoparticles, d_{NP} is the density of nanoparticle (here we assume the density of nanoparticles is equal to the density of polymer, 1.21 g/mL for PLGA), D is the particle diameter as measured by the dynamic light scattering.

According to reference [25], full surface mushroom coverage $[\Gamma^*]$ is the number of unconstrained PEG molecules occupy 100 nm^2 of particle surface area. In order to determine $[\Gamma^*]$, we estimated the surface area occupied by a single PEG chain. Using random-walk statistics, a single PEG chain occupies an area at the interface given by a sphere of diameter:

$$\xi=0.76m^{0.5}[\text{\AA}]$$

where m is the molecular weight of the PEG chain. The surface area occupied by one PEG molecule can be determined from $(D/2)^2$. Thus PEG 5kDa has an unconstrained molecule sphere with diameter of 5.4nm, and occupies a surface area of 22.7 nm². Then the number of PEG molecules to fully cover 100 nm² surface area, $[\Gamma^*]$, is 4.4. $[\Gamma/\Gamma^*]$ as an index to measure the PEG density on the nanoparticle surface, where the values <1 indicates low PEG density where PEG molecules are in a mushroom conformation; whereas values>1 indicate high PEG density where PEG molecules are in a brush-like conformation. Similarly, $[\Gamma^*]$ for PEG 10 kDa, 2 kDa and 1 kDa is 2.2, 11 and 22, respectively.

2.5 Human cervicovaginal mucus (CVM) collection

CVM was collected as previously described [8]. Briefly, undiluted cervicovaginal secretions from women with normal vaginal flora were obtained using a self-sampling menstrual collection device following a protocol approved by the Institutional Review Board of the Johns Hopkins University. The device was inserted into the vagina for 60 s, removed and placed into a 50 mL centrifuge tube and centrifuged at 1000 rpm for 2 min to collect the secretions.

2.6 Multiple particle tracking (MPT) of nanoparticles in CVM

The tracking of fluorescently labeled nanoparticles in fresh CVM was performed as published [26]. Briefly, 0.6 μ l of nanoparticles at suitable dilution was added to 20 μ l mucus within a custom-made chamber slide and incubated at room temperature for 1 hour prior to microscopy. The trajectories of nanoparticles in CVM were recorded by using multiple particle tracking (MPT). 20 s movies were captured at a temporal resolution of 66.7 ms using a silicon-intensified target camera (VE-1000, Dage-MTI) mounted on an inverted epifluorescence microscope equipped with 100x oil-immersion objective (N.A., 1.3). Tracking movies were analyzed using MetaMorph software (Universal Imaging, Glendale, WI). Time averaged mean square displacement (MSD) and effective diffusivity for each particle were calculated as a function of time scale. $\langle \Delta r^2(\tau) \rangle = [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2$, where x and y represent the nanoparticle coordinates as a function of time and τ is the time lag. Curcumin-loaded nanoparticles and FITC-BSA-loaded nanoparticles were tracked in human CVM in the same manner using the fluorescence from either encapsulated curcumin or BSA-FITC. Particle penetration into a physiologically 30 μ m thick mucus layer was modeled using Fick's second law and diffusion coefficients obtained from tracking experiments [11–12].

2.7 Drug encapsulation

The encapsulation efficiency of curcumin in nanoparticles was measured by dissolving the freeze-dried nanoparticles in DMSO and measuring the absorbance at 430 nm using Biotek Synergy MX plate reader (Winooski, VT). The drug content was determined by comparing to the curcumin calibration curve (concentration range 0–50 μ g/mL). Absorbance of blank nanoparticle in DMSO at the same polymer concentration was subtracted. The encapsulation efficiency of BSA-FITC was analyzed after alkaline digestion, as described previously [27–28]. A known amount of freeze-dried nanoparticles underwent complete hydrolysis in 1 M sodium hydroxide. The resultant solution was analyzed using Biotek Synergy MX plate reader at 490 nm excitation wavelength and 525 nm emission wavelength. Standard solutions containing the same amount of polymer and increasing amounts of BSA-FITC at the same processing condition were prepared. The amount of BSA in the nanoparticles was determined by comparing to the BSA-FITC calibration curve.

Drug loading (DL) and encapsulation efficiency (EE) were calculated as follows:

$$DL (\%) = \frac{\text{Weight of drug}}{\text{Weight of nanoparticles}} \times 100\%$$

$$EE (\%) = \frac{\text{Experimental drug loading}}{\text{Target drug loading}} \times 100\%$$

3. Results and discussion

3.1 Preparation and characterization of mucus penetrating particles

We discovered that PEG-containing diblock copolymers can self-assemble to form dense, muco-inert PEG coatings on the surface of emulsion droplets formed by the emulsification method, but only if low molecular weight (MW) emulsifiers are used in place of PVA. As a proof-of-principle, we labeled diblock copolymers of poly(lactic-co-glycolic acid) and PEG 5 kDa (PLGA-PEG5k) with fluorescent dye, and then formulated fluorescently-labeled PLGA-PEG5k nanoparticles using various emulsifiers, including Pluronic F127 and F68, Cremophor EL, Vitamin E-TPGS (TPGS), PVA, poly(ethylene-*alt*-maleic anhydride) (PEMA), cholic acid sodium salt (CHA), dioctyl sulfosuccinate sodium (DSS), hexadecyltrimethyl ammonium bromide (CTAB), saponin, Tween20, Tween80, and sugar ester D1216 (SE) (Table 1). We then observed nanoparticle transport dynamics in fresh, undiluted human cervicovaginal mucus (CVM). PLGA-PEG5k nanoparticles produced using high MW PVA and PEMA were immobilized in CVM, as evident by the highly constrained, non-Brownian time-lapse traces of the particles in mucus (Fig. 1A and Video S1). In contrast, nanoparticles produced using low MW emulsifiers, such as anionic CHA and DSS, cationic CTAB, and neutral saponin, Tween20 and SE, readily penetrated CVM, as evident by their diffusive, Brownian trajectories that span large distances within 3 s (Fig. 1B and Video S2). As a result, low MW emulsifiers produced a several-thousand-fold increase in the mean-square-displacement ($\langle \text{MSD} \rangle$) of nanoparticles in CVM, at a time scale of 1 s, relative to nanoparticles prepared with high MW emulsifiers (Fig. 1C). Furthermore, PLGA-PEG5k nanoparticles containing low MW emulsifiers penetrated CVM with average effective speeds less than 10-fold slower than the same particles in water (Table 1).

In the absence of PEG, PLGA/CHA nanoparticles had a highly anionic surface charge and were mucoadhesive, likely as a result of hydrophobic interactions between nanoparticles and mucus (Table 1). In contrast, the surface charge of mucus-penetrating PLGA-PEG5k/CHA nanoparticles was near neutral, suggesting the formation of a dense PEG coating that masked the negative charge of PLGA and CHA. The concentration (range: 0.01–1% w/v) of low MW emulsifiers had no significant effect on nanoparticle surface charge and mucus penetrating property (Table S2), likely because the PEG corona completely shielded these small molecules on the particle surface. In addition, the inherent charge associated with the emulsifier had no effect on the PLGA-PEG5k nanoparticle surface charge and mucus penetrating property (Table 1), further supporting the role of the PEG corona in shielding these low MW emulsifier molecules on the particle surface (Scheme 1). Furthermore, the choice of emulsifying agent influenced the extent of PEG brush formation during the emulsification process. While both PLGA-PEG5k/PVA nanoparticles and PLGA-PEG5k/CHA nanoparticles have near neutral surface charges, only the CHA formulations are mucus-penetrating (Fig. 1D). PVA and PEMA differ from the other emulsifiers as they contain a linear hydrophobic backbone decorated with hydrophilic side groups. When PVA or PEMA stabilizes the oil/water interface, the hydrophobic polymer backbone can polyvalently adhere at the oil/water interface where they are in intimate contact with the protruding PEG brush. Residual PVA was detected on PLGA nanoparticles after preparation by the emulsification method using PVA [29]. Thus high MW emulsifiers, including PVA

and PEMA, may disrupt the architecture of the PEG molecules on the particle surface, thereby rendering the particles mucoadhesive (Scheme 1). In the case of PLGA-PEG5k/PEMA nanoparticles, the negative surface charge (-42 mV) suggests disruption of the PEG coating, as the charge likely originates from PEMA molecules exposed on the surface (Table 1).

PLGA-PEG5k nanoparticles prepared by the emulsification method, using either low MW or high MW emulsifiers, were spherical in shape (Fig. 2). We also tested other PEG-containing diblock copolymers, poly(D,L-lactic-acid)-*b*-PEG5k (PLA-PEG5k) and poly(ϵ -caprolactone)-*b*-PEG5k (PCL-PEG5k). For both, we observed immobilization for nanoparticles made using PVA and rapid mucus penetration for nanoparticles made using a low MW emulsifier, CHA, with effective diffusivities similar to those measured for PLGA-PEG5k nanoparticles (Fig. 3). Based on measured diffusivities at short time scales, we estimate that a majority of PLGA-PEG5k/CHA, PLA-PEG5k/CHA and PCL-PEG5k/CHA nanoparticles (50–70%), regardless of the core materials, will penetrate physiologically thick mucus layers within 60 min, compared to less than 1% of nanoparticles prepared by the emulsification method using the conventional high MW emulsifier PVA (Fig. 1E, Fig. 3G and Fig. 3H). The presence of PEG coatings on nanoparticles shielded both the low MW emulsifiers and the nanoparticle core materials. Thus, similar mucus-penetrating property can be achieved for nanoparticles made of different PEG-containing block copolymers. The wide range of PEG-containing block copolymers suitable for preparing MPP by the emulsification method can help to tailor the encapsulation efficiency and release profiles for various cargo therapeutics [15, 30–31].

However, not all low MW emulsifiers are suitable for preparing MPP by emulsion processes. For example, Cremophore EL, Tween80, Vitamin-E TPGS, Pluronic F127 and F68 were unable to fully stabilize the emulsion droplets during particle preparation, resulting in the formation of varying degrees of large aggregates. In order to more thoroughly assess the effect of emulsifiers, we estimated their emulsification capability by measuring the percentage of non-aggregated PLGA-PEG nanoparticles prepared in 1% w/v aqueous solution of each emulsifier. The percentages of non-aggregated PLGA-PEG nanoparticles were dependent on the emulsifiers (Fig. 4). While the non-aggregated nanoparticle thus prepared were mucus-penetrating (data not shown), the non-aggregated nanoparticle fraction in the prepared particles was as low as 30%. For industrial application, particles with low aggregate fraction are required. Thus, the ability to produce MPP from PEG-containing block copolymers by the emulsification method is critically dependent on both the MW and emulsification capability of the emulsifier (Fig. 4). The emulsifiers must be strong enough to stabilize the emulsion droplets, yet be small enough to be completely shielded at the particle surface by the PEG corona.

3.2 Effect of PEG molecular weight on mucus-penetration

PLGA-PEG/CHA nanoparticles with a wide range of PEG MW (1, 2, 5, and 10 kDa), prepared by the emulsification method, all rapidly penetrated mucus (Table 2). The content of PEG in PLGA-PEG (1–10 kDa) nanoparticles were all controlled around 6wt% (Table S1). The nanoparticle surface charge was inversely proportional to the PEG MW and varied from -18 mV (1 kDa PEG) to -2.3 mV (10 kDa PEG). Surface charge has been used as an indicator for surface PEG coverage on nanoparticles [32–33], and previously we hypothesized a critical threshold for the particle surface charge between -10 and -7 mV measured under neutral pH to govern the mucoadhesive versus mucoinert properties of latex particles [9]. The rapid mucus penetration of PLGA-PEG1k/CHA nanoparticles with surface charge of -18 mV in the current work shows that the surface charge threshold may vary depending on the core materials used, as we predicted previously [9]. Therefore, we quantified the surface PEG coverage by directly measuring the surface PEG density, $[\Gamma]$

(number of PEG per 100 nm²) by ¹H NMR [22–24]. The $[\Gamma]$ values of PLGA-PEG nanoparticles decreased as PEG MW increased. However, the ratio $[\Gamma/\Gamma^*]$ [25, 34] of surface PEG density to the theoretical PEG density required for the formation of a brush-like PEG coating $[\Gamma^*]$ was greater than 2 (Table 2) regardless of PEG MW, indicating the presence of a dense brush-like coating of PEG on the surface of PLGA-PEG (1–10 kDa)/CHA nanoparticles. The formation of a dense PEG brush on the particle surface is generally required for mucus penetration [9, 35].

Our previous work on latex polystyrene (PS) beads when conjugated with PEG showed that only low MW PEG (1–5 kDa) produced MPP, while high MW 10 kDa PEG failed to render PS particles mucus-penetrating [9]. Interestingly, in the current work we found that the PLGA-PEG nanoparticles with 10 kDa PEG rapidly penetrated mucus with diffusivities close to that of nanoparticles with 2 kDa PEG and 5 kDa PEG (Table 2). We speculate the difference between the current and previous work may arise from the different formulation methods used to produce MPP. Part of PEG could be imbedded inside of PLGA-PEG particle core in the current work resulting in PEG coatings at the particle surface without full length 10 kDa. In comparison, PEG chains were only located on the surface of PS-PEG nanoparticles in our previous work [9]. Furthermore, in our previous work, the surface PEG coverage on PS particles could be over-estimated by the fluorimetric assay method used, which is a crude assay compared to the NMR-based method used here [9]. Therefore, the real PEG density on PS particles surface-modified with 10 kDa PEG may have not been as dense as achieved on PLGA-PEG10k/CHA nanoparticles by the emulsification method. In the emulsification method, 10 kDa PEG can form highly dense PEG coatings on PLGA-PEG nanoparticles with $[\Gamma/\Gamma^*]$ of 3.0 (Table 2), thereby limiting the contact of mucus components with nanoparticles. By optimizing the PEG conjugation method [36], we have prepared 100 nm and 200 nm PS particles with 10 kDa PEG coating (PS-PEG10k) that can rapidly penetrate fresh undiluted human mucus (unpublished data).

The rapid penetration of human mucus by PLGA-PEG/CHA nanoparticles with PEG MW range from 1 kDa to 10 kDa allows us to expect a much wider molecular weight range of PEG than we previously tested may allow nanoparticles to be mucoinert. The PEG MW range for producing MPP needs to be further studied. Factors such as the particle size, core material used, type of mucus used, and surface PEG density achieved all may affect the determination of PEG MW range for MPP.

3.3 Drug encapsulation into MPP

Next, we confirmed that both hydrophobic and hydrophilic drugs can be efficiently encapsulated into PLGA-PEG MPP. Two model compounds: curcumin, a hydrophobic small molecule drug (MW=368 Da), and BSA, a hydrophilic protein (MW=66 kDa), were encapsulated using an o/w single emulsion into PLGA-PEG5k/CHA nanoparticles and a w/o/w double emulsion into PLGA-PEG5k/saponin nanoparticles, respectively. The encapsulation efficiency of both curcumin and BSA into MPP using low MW emulsifiers was similar to that achieved with conventional particles (CP) made by the emulsification method using PVA (Table 3). Curcumin and BSA-loaded nanoparticles made using PLGA-PEG5k with CHA rapidly diffused in mucus at rates only 6 and 36-fold slower than in water at $\tau=1$ s, respectively (Fig. 5A and Table 3). On the other hand, nanoparticles prepared using PLGA-PEG5k with PVA were immobilized in CVM (Fig. 5B), with transport rates more than 2,000-fold slower than in water (Table 3). A substantial fraction, up to 40% and 30% of curcumin-MPP and BSA-MPP, respectively, are expected to penetrate physiologically thick mucus layers within 60 min, compared to < 1% of PVA-coated nanoparticles (Fig. 5C).

Alonso and coworkers have studied PLA-PEG nanoparticles prepared by the same emulsification method using CHA to load proteins and plasmid DNA for nasal

administration, and up to 35% and 90% encapsulation efficiency were achieved for model protein antigen, tetanus toxoid (TT), and plasmid DNA (pCMV- β Gal), respectively [37–38]. The findings in the current work that various low MW emulsifiers (negatively charged, positively charged and neutral) can produce MPP by the emulsification method, significantly expand the types of MPP products that can be made with capability to load a wide spectrum of therapeutic agents. Certain positively charged drugs, such as doxorubicin, moxifloxacin and vancomycin, that may interfere with negatively charged emulsifiers, can fail to be encapsulated into MPP by the emulsification method using CHA emulsifier. However, other low MW emulsifiers with strong emulsification capability and neutral charge, such as saponin and sugar ester, may help to encapsulate these drugs into MPP.

Our findings highlight numerous potential advantages of mucus penetrating nanoparticles prepared by the emulsification method for mucosal drug delivery applications. First, the most commonly used emulsifier, PVA, can be replaced with low MW emulsifiers to prepare biodegradable nanoparticles with similar drug encapsulation efficiency by the emulsification method. These nanoparticles rapidly penetrate human mucus barriers, whereas PVA-coated nanoparticles do not. Thus, the most widely used industrial production method for controlled release particles can be applied to manufacture well-PEGylated nanoparticles for drug delivery applications [16, 39]. Second, we expect MPP prepared by the emulsification method can rapidly penetrate other mucosal surfaces, such as those on the epithelial surface of the eyes, nose, lungs, gastrointestinal tract and more, since CVM shares similarity in chemical content and rheological properties with other mucus fluids [5]. Indeed, we have found that MPP prepared by the emulsification method can rapidly penetrate normal airway mucus collected during surgery and sputum expectorated by cystic fibrosis (CF) patients (unpublished observations). Third, challenging hydrophilic drugs, including proteins, peptides and nucleic acids, can be encapsulated into MPP by the emulsification method. Fourth, hydrophobic drugs, which are difficult to dissolve in water-miscible organic solvents, could be successfully formulated into biodegradable MPP by the emulsification method. Improved pharmacokinetics and therapeutic efficacy of hydrophobic drugs could be expected through their mucosal delivery within MPP [6].

4. Conclusion

In summary, we report a biodegradable drug delivery platform that can rapidly penetrate the mucus barrier, thereby potentially improving the distribution and pharmacokinetics of both hydrophobic and hydrophilic drugs at mucosal surfaces. This biodegradable mucus-penetrating drug delivery platform was constructed by an industrially scalable process, the emulsification method, using low MW emulsifiers with strong emulsification capabilities. Biodegradable mucus penetrating particles that can deliver hydrophilic biologicals and/or hydrophobic drugs will likely facilitate the treatment of numerous diseases and conditions that affect mucosal tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Transport of biodegradable nanoparticles prepared by the emulsification method with different emulsifiers in human CVM. Representative trajectories of (A) conventional particles (CP) and (B) mucus-penetrating particles (MPP). (C) ensemble-averaged geometric mean square displacements (<MSD>) as function of time scale for representative CP (PLGA-PEG5k/PVA) and MPP (PLGA-PEG5k/CHA). (D) Distributions of the logarithms of individual particle effective diffusivities (D_{eff}) at a time scale of 1 s for the representative CP (PLGA-PEG5k/PVA) and the representative MPP (PLGA-PEG5k/CHA). (E) Estimated fraction of particles capable of penetrating a physiological 30 μm thick mucus layer over time. Data represent three independent experiments with 120 nanoparticles tracked for each experiment. Error bars are presented as standard error of mean (s.e.m).

Fig. 2.
The TEM images of the representative (A) MPP, PLGA-PEG5k/CHA nanoparticles and (B) CP, PLGA-PEG5k/PVA nanoparticles. The scale bar is 200 nm.

Fig. 3.

Transport of PLA-PEG5k and PCL-PEG5k nanoparticles in human CVM (A, B) representative trajectories. (C, D) Ensemble-averaged geometric mean square displacements ($\langle \text{MSD} \rangle$) as function of time scale. (E, F) Distributions of the logarithms of individual particle effective diffusivities (D_{eff}) at a time scale of 1 s. (G, H) Estimated fraction of particles capable of penetrating a physiological 30 μm thick mucus layer over time. Data represent three independent experiments with 120 nanoparticles tracked for each experiment. Error bars are presented as s.e.m.

Fig. 4.

Correlation of the mucus-penetrating properties of PLGA-PEG5k nanoparticles prepared by the emulsification method to MW and emulsification capability of the emulsifiers. MPPs are indicated by black solid symbols and nanoparticles found to be mucoadhesive (CP) are indicated by open symbols. Red solid symbols represent nanoparticles that are mucus-penetrating but cannot be efficiently prepared without aggregation by emulsification method. The shaded region represents the range of emulsifier MW and emulsification capabilities that produces stable nanoparticles with low aggregation and ability to penetrate mucus.

Fig. 5. Transport of drug loaded MPP and CP in human CVM. A) Ensemble-averaged geometric mean square displacement $\langle \text{MSD} \rangle$ as a function of time scale. B) Distributions of the logarithms of individual particle effective diffusivities (D_{eff}) at a time scale of 1 s. C) Estimated fraction of particles predicted to be capable of penetrating a 30 μm thick mucus layer over time. Data represent three independent experiments with 120 nanoparticles tracked for each experiment. Error bars are presented as s.e.m.

Scheme 1.

The development of biodegradable nanoparticles by the emulsification method. Green chains represent the PEG, grey spheres represent the nanoparticle core, red lines represent high MW emulsifiers (such as PVA), and red dots represent low MW emulsifiers (such as CHA). The use of low MW emulsifiers resulted in dense PEG coatings on nanoparticles allowing their rapid penetration through mucus (mucus penetrating particles, MPP), however, conventional high MW emulsifiers in the emulsification method will disrupt PEG coatings rendered the adhesive interaction with mucus elements and immobilization in mucus (conventional particles, CP).

Table 1
Characteristics of biodegradable nanoparticles prepared by the emulsification method using different emulsifiers.

Polymer	Emulsifier	Emulsifier MW [Da]	Diameter [nm]	ζ -potential [mV]	D_w/D_m [a]
PLGA-PEG5k	DSS	444	136 $\pm$ 5	-5.5 $\pm$ 0.5	3.9
	CHA	430	115 $\pm$ 11	-3.7 $\pm$ 0.4	5.1
	CTAB	364	77 $\pm$ 3	-4.6 $\pm$ 0.7	5.6
	Saponin	1.8k	108 $\pm$ 1	-7.0 $\pm$ 0.7	10
	SE	540	97 $\pm$ 3	-4.2 $\pm$ 0.3	6.8
	Tween20	1.2k	156 $\pm$ 7	-3.8 $\pm$ 0.3	3.5
	Tween80	1.3k	152 $\pm$ 6	-4.2 $\pm$ 0.3	6.8
	F127	12.5k	169 $\pm$ 8	-2.4 $\pm$ 0.2	4.2
	F68	8.4k	162 $\pm$ 5	-3.3 $\pm$ 0.3	4.2
	TPGS	1.5k	204 $\pm$ 7	-4.8 $\pm$ 0.3	5.6
PLGA15k	Cremophor	2.1k	232 $\pm$ 4	-3.5 $\pm$ 0.1	3.6
	PVA	25k	156 $\pm$ 8	-2.9 $\pm$ 0.3	40,000
	PEMA	400k	185 $\pm$ 6	-42 $\pm$ 1.6	23,000
	PVA	25k	175 $\pm$ 5	-2.6 $\pm$ 1.0	19,000
	CHA	430	144 $\pm$ 6	-72 $\pm$ 2.2	41,000

^[a]Ratios of the ensemble average diffusion coefficients in water (D_w) compared to in mucus (D_m) at a time scale of 1 s.

Table 2

Characteristics of PLGA-PEG/CHA nanoparticles with different MW PEG prepared by the emulsification method using CHA emulsifier.

PEG MW [kDa]	Diameter [nm]	Z-Potential [mV]	PEG density [Γ] [a] (#PEG/100nm ²)	[Γ/Γ^*] [b]	D_w/D_n
10	124 $\pm$ 6	-2.3 $\pm$ 0.1	6.7	3.0	9.6
5	107 $\pm$ 3	-4.2 $\pm$ 0.3	13.9	3.3	4.4
2	128 $\pm$ 1	-12 $\pm$ 0.9	26.2	2.5	5.0
1	134 $\pm$ 5	-18 $\pm$ 1.2	45.0	2.3	7.7

a/PEG density [Γ] indicates the number of PEG molecules per 100 nm². The surface PEG content was quantified by ¹H NMR of nanoparticles in D₂O.

b/Ratio of PEG density to full surface coverage [Γ/Γ^*]. Full surface coverage [Γ^*] indicates the theoretical number of unconstrained PEG molecules required to fully coat a 100 nm² surface. ($[\Gamma/\Gamma^*]<1$ indicates mushroom regime with low surface PEG density, whereas >1 represents brush regime with high surface PEG density) [25].

Table 3

Physicochemical properties of MPP and CP encapsulated with model hydrophobic drug (curcumin) and model hydrophilic biologics (BSA).

Formulation	Emulsifier	Method	DL [%]	EE [%]	Diameter [nm]	PDI ^[a]	ζ-potential [mV]	D _w /D _m
Curcumin MPP	CHA	o/w	4.5	49	156±12	0.086	-5.1±0.5	6
Curcumin CP	PVA	o/w	4.3	47	151±11	0.029	-3.3±1.1	2400
BSA MPP	Saponin	w/o/w	11.4	68	164±1	0.238	-4.5±0.4	36
BSA CP	PVA	w/o/w	11.5	69	218±22	0.184	-2.2±0.8	5100

^[a]PDI is the polydispersity index measured by Zetasizer.