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Size Selectivity of Intestinal Mucus to Diffusing Particulates is Dependent on Surface Chemistry and Exposure to Lipids

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Abstract

Intestinal mucus provides a significant barrier to transport of orally delivered drug carriers, as well as other particulates (e.g., food, microbes). The relative significance of particle size, surface chemistry, and dosing medium to mucus barrier properties is not well characterized but important in design of delivery systems targeted to the intestinal mucosa. In this study, multiple particle tracking (MPT) was used to study diffusion of 20- to 500-nm diameter carboxylate- and polyethylene glycol- (PEG-) functionalized polystyrene model carriers through intestinal mucus. The impact of exposure to mucus in buffer vs. a partially digested triglyceride mixture was explored. Effective diffusivity of particles in intestinal mucus decreased with increasing particle size less than and more than theoretically (Stokes-Einstein) expected in a homogenous medium when dosed in buffer and model fed state intestinal contents, respectively. For example, effective diffusivity decreased 2.9- vs. 20-fold with increase in particle size from 100 to 500 nm when dosed to mucus in buffer vs. lipid-containing medium. Functionalization with PEG dramatically decreased sensitivity to lipids in dosing medium. The results indicate that reduction of particle size may increase particle transport through intestinal mucus barriers, but these effects are strongly dependent on intestinal contents and particle surface chemistry.

Graphical Abstract

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INTRODUCTION

Mucus lines wet epithelial surfaces in the body, and as such is an important barrier to penetration of molecular, particulate, and microbial species. Complex mucus gels containing mucin glycoprotein, sloughed cells, bacteria, lipids, salts, proteins, and other macromolecules^{1–3} provide a defensive barrier above the respiratory, reproductive and gastrointestinal (GI) tracts. However, the specific interactions and mechanisms underlying the barrier properties of mucus are generally not well characterized.

Oral delivery is the most utilized route for drug administration.⁴ Small molecule (< 500 Da) drugs are candidates for oral delivery due to potential to be absorbed in the GI tract.⁵ Oral delivery of proteins has been explored using polymeric nanoparticle carriers to provide protection against the harsh acidic environment in the stomach and hydrolytic degradation in the gastrointestinal tract.^{6,7} Particle size plays a central role in oral delivery of nanoparticles. For example, it was shown that 100 nm diameter nanoparticles were taken up in the GI tract to a greater extent than 1000 nm nanoparticles.⁸ In a hydrogel such as mucus, the mesh pore size (10–200 nm)³ sets the threshold beyond which particle diffusion is suppressed. Researchers have found, consistent with this concept, that particle mobility decreased with increasing particle size in sputum mucus from cystic fibrosis patients.⁹ However, the opposite effect was seen in cervical mucus, with larger polymeric nanoparticles having more rapid transport. In addition, the mobility of larger particles in cervical mucus was enhanced by coating the particles with polyethyleneglycol (PEG).^{10,11} These findings suggest that while relative mucus mesh and particle size is an important factor in controlling particle penetration at mucosal surfaces, there are other important phenomena to consider, including the impact of the heterogeneous nature of the pore network and intermolecular interactions, impacting the complex permeability properties of mucus gels. It was recently demonstrated that 100 nm bile salt coated particles diffuse more rapidly through porcine intestinal mucus than 500 nm bile salt coated particles.¹² The impact of particle size on transport of particles

through intestinal mucus has not been exhaustively studied, yet is significant to the design of oral particulate carrier systems.

It is interesting and important to consider how the intestinal mucus barrier may change with exposure to intestinal contents, such as lipids in food. Endogenous lipids are present in mucus at a mass percentage up to 2% and account for mucus hydrophobicity.^{13,14} Researchers have shown that lipids contribute to reduced diffusion of drugs in pig intestinal mucus by using a small scale tracer method.^{15,16} Additionally, endogenous lipids were found to contribute to the viscoelastic nature of mucus. Extraction of lipids from dog gastric mucus reduced the viscosity by 80%.¹⁷ The significance of endogenous lipids to mucus properties motivates exploration of the potential impact of exogenous lipid mixtures originating from food or drug delivery systems on intestinal mucus barriers. We recently demonstrated that food associated lipids representative of postprandial intestinal contents altered mucus barrier properties, as indicated by a 10 – 142-fold reduction in the transport rate of 200 nm microspheres through mucus, depending on their surface chemistry.¹⁸ The mechanism and role of potential interactions between exogenous lipids and mucus, however, remain unclear. For example, it is not certain whether the bile salt micelles present in fed state intestinal contents stay intact within mucus. Bile salt micelles originating from bile salts and phospholipid increase in concentration in the fed state GI environment and are known to be important to the solubilization¹⁹ and absorption of orally delivered compounds.²⁰ It was recently demonstrated that bile salts can coat particles and emulsion droplets, significantly impacting their ability to diffuse through intestinal mucus.^{21,22} It is not clear if bile salt micelle structures stay intact within mucus, and thus if bile micelles interact with the intestinal epithelial surface. Elucidation of bile micelle transport phenomena in mucus would facilitate mechanistic understanding and prediction of the impact of food-associated bile micelles on orally delivered compound absorption.

In this study, the impact of lipids associated with eating on transport through mucus of particles varying in size and surface chemistry has been investigated using real-time multiple particle tracking (MPT). The results shed light on significance of altered mucus structure and/or altered intermolecular interactions to fortified mucus barriers in the presence of lipids. This information is helpful in designing oral delivery systems for optimal penetration of intestinal mucus in fed and fasted states. MPT, a powerful technique that reveals particle-environment interactions, has been utilized in a number of drug and gene carrier transport studies,^{3,9,10,23,24} including recent studies of particle transport through intestinal mucus.^{25,26} Diffusion of food-derived model bile micelles in mucus was also studied to assess if bile salt micelles stay intact or dissociate within the mucus layer using fluorescence recovery after photobleach (FRAP). As bile salt micelles (~10 nm) are too small to be individually imaged using particle tracking techniques, FRAP enabled analysis of the diffusion of micelles in mucus.²⁷

MATERIALS AND METHODS

Collection and preparation of native mucus samples

Native porcine intestinal mucus was extracted from animals fasted overnight within 2 h of sacrifice. The jejunum was isolated and mucus was transferred into 2 ml sample vials stored at -80°C until experimentation as previously described.²⁸

Preparation and characterization of microspheres

Particle solutions were prepared using yellow-green fluorescent FluoSpheres (Invitrogen Molecular Probes, Carlsbad, CA). Twenty- to 500 nm carboxyl modified particles were covalently modified with diamine PEG as described previously.³ Particle sizes and surface potentials (ζ -potentials) were determined by dynamic light scattering using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY). Particle sizes were measured at room temperature by dynamic light scattering in various test media at a concentration of 0.0025% wt/vol diluted 10X in distilled water.

Test media preparation

Simulated intestinal contents were prepared using maleate buffer at physiologically relevant pH level (6.5) and Ca^{2+} concentration (10 mM), and model bile components at levels representative of the fed state²⁹, including bile salt (sodium taurodeoxycholate, NaTDC) and phospholipid (L- α -phosphatidylcholine, lecithin from egg yolk). To simulate food-associated lipid intake, soybean oil, sodium oleate and monoglycerol were added to the model bile/maleate buffer solution (Table 1). Preparations were stirred for 3 hours at 300 rpm at 37°C .

Bile salt micelle preparation

Model bile solutions for FRAP experiments were prepared both above and below the critical micelle concentration (CMC) using a fluorescent component. The preparations contained maleate buffer (Table 1) together with NaTDC and fluorescently labeled (2-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Dodecanoyl)-1-Hexadecanoyl-sn-Glycero-3-Phosphocholine) (β -BODIPY FL C12-HPC), (Molecular Probes, Inc. Eugene, OR) as a model of the human bile. The CMC of the NaTDC and phospholipid system in maleate buffer, at pH 6.5 and temperature of 37°C has been determined using a spectroscopic technique. Bile salt micelles above CMC were prepared by adding 12 mM NaTDC (BS) and 4 mM β -BODIPY FL C12-HPC (PL), and bile salt micelles below CMC were prepared by adding 3 mM and 1 mM BS/PL in maleate buffer.

Multiple particle tracking of microspheres in mucus

Particle transport rates were measured by tracking the trajectories of fluorescently labeled microspheres with a frame rate of 30 fps for 20 s using a 12.5 megapixel cooled Olympus DP70 digital color camera (Olympus, Center Valley, PA) mounted on an inverted Olympus IX51 microscope attached with X-Cite 120 fluorescence system (EXFO, Mississauga, Ontario, Canada). 200 μl of mucus was added onto non-fluorescent 8-well polystyrene medium chambers (Thermo Fisher Scientific, Rochester, NY). 10 μl of diluted particle

suspension (0.0025% wt/vol) was deposited with minimal perturbation onto native mucus. The mucosal specimens were covered and equilibrated for 2 h at 25°C in a humid chamber prior to microscopy. Trajectories of n~100 microspheres were analyzed for each experiment and three experiments were performed from three different mucus specimens for each experimental setup to account for mucus variability. Trajectories for each particle type were generated using the feature point detection and tracking algorithm of the ParticleTracker ImageJ plugin.³⁰ For each set of experiments, mucus from 3 independently collected vials of mucus, originating from at least two animals, were utilized.

Analysis of particle trajectories

The centroids of particle coordinates were transformed into time-averaged mean squared displacement (MSD) and effective diffusivities (D_{eff})

$$\text{MSD} = [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \quad (1)$$

$$D_{\text{eff}} = \text{MSD} / (4\tau) \quad (2)$$

where $x(t)$ and $y(t)$ represent the nanoparticle coordinates at a given time, and ζ is the time scale.^{9,10,24} The extent of particle interaction with the mucus gel network was determined by fitting particle MSD vs. time scale to

$$\text{MSD} = 4D_0\tau^\alpha \quad (3)$$

where α is the anomalous exponent indicating particle motion obstruction and D_0 is time independent diffusion coefficient. Effective diffusion coefficients were also used to estimate the fraction of microspheres expected to penetrate intestinal mucus layers of given thickness using a numerical integration of Fick's second law:

$$\delta C / \delta t = D_{\text{eff}} \delta^2 C / \delta x^2 \quad (4)$$

where C is the concentration of particles, t is time and x is position.³¹ The initial condition of particles present at the apical side ($C(x,0) = 1$ when $x = 0$, $C(x,0) = 0$ otherwise) and boundary conditions of constant particle concentration at the apical side ($C(0,t) = 1$) were applied. While this analysis can only be applied accurately to a homogenous medium that can be modeled as a continuum, and it is recognized that mucus is not homogenous, this analysis can provide an approximation for the amount of particles dosed to a mucus surface that can penetrate through the mucus within a given period of time.

Confocal microscopy settings for FRAP

FRAP experiments were conducted to explore whether bile salt micelles stay intact within mucus. Imaging was performed on a Zeiss LSM 700 laser scanning confocal microscope utilizing a 40X Plan-Apochromat, NA 1.4 objective. Fluorescent bile salt micelles were excited with 488-nm light from a 30-mW argon laser set at 50% output. 12-bit 512x512 images were obtained with 5% laser transmission to avoid photobleaching, at a digital zoom

of 1, and with a pixel dwell time of 3.15s. The circular region of interests (ROI) representing the bleaching region was 20 pixels in diameter. Irreversible photobleaching of the bleach region is performed with 40 iterations using 100% laser transmission. The first bleach was after 5 scans of the large ROI. Each experiment consists of a time series of 600 scans of the ROI, each taking 200 ms, with a 1.5-s delay between scans.

FRAP data analysis

Theoretical estimated diffusion coefficients of bile salt micelles and particles in water were calculated using the Stokes-Einstein (SE) equation:

$$D_0 = k_B T / 6\pi\eta a \quad (5)$$

where k_B is the Boltzmann constant, T is temperature, η is the fluid viscosity, and a is the particle radius (1). Hankel transform method³² was applied to calculate experimental micelle diffusion coefficients using Matlab.

$$Fv(k) = \int_0^\infty f(r) J_v(kr) r dr \quad (6)$$

where Fv is the Bessel function. Theoretical estimated diffusion coefficients of single phospholipid molecules (phospholipid molecules that do not form micelles) were calculated using the Wilke-Chang equation:

$$D_{AB} = 7.4 \times 10^{-8} \frac{\sqrt{\varphi_B MW_B}}{\eta_B \bar{V}_A^{0.6}} T \quad (7)$$

where D_{AB} is the diffusivity of compound A in solvent B (cm^2/s), is the constant which accounts for solvent/solvent interactions (2.6 for water), T is temperature (K), η_B is the viscosity of solvent B (g/mol), MW_B is molecular weight of solvent B (g/mol), and $\bar{V}_A$ is the molar volume of compound A.

RESULTS and DISCUSSION

Size dependence of particle transport across intestinal mucus

The size selectivity of gastrointestinal mucus was explored by investigating diffusion of 20-, 40-, 100-, 200-, and 500 nm diameter carboxylate-modified particles using multiple particle tracking. The measured trajectories of particles were converted to mean squared displacements (Figure 1A) as a function of time and used to calculate effective diffusion coefficients. Transport rate through mucus decreases with increasing particle size from 20 to 500 nm diameter. At a time scale of 10 s, effective diffusivities of 20-, 40-, and 100 nm particles were $0.13 \mu\text{m}^2/\text{s}$, $0.09 \mu\text{m}^2/\text{s}$ and $0.06 \mu\text{m}^2/\text{s}$ (Figure 1B) with α values of 0.85, 0.83, and 0.79 respectively. Particles larger than the reported mesh pore size of mucus (~ 200 nm) expectedly exhibit subdiffusive motion ($0.2 < \alpha < 0.9$)³³, with effective diffusion coefficients of 200-, and 500 nm particles of $0.026 \mu\text{m}^2/\text{s}$ and $0.021 \mu\text{m}^2/\text{s}$, and α values of 0.68 and 0.63, respectively (Figure 1B). Comparison of the theoretically expected reduction in diffusivity resulting from increase in particle size (according to the SE relation, which

applies to freely moving particles) to experimentally measured changes in diffusivity in mucus reveals important insight into particle-environment interactions. Considering 20 nm (the smallest size) particles as a reference, 40, 100, 200, and 500 nm particle effective diffusion coefficients were predicted to be reduced by 1.8-, 4.3-, 8.5- and 20-fold, respectively, according to the SE relation. However, experimental effective diffusion coefficients in mucus were 1.4-, 2.2-, 5-, and 6.2-fold lower. Thus, particle diffusion rates are less impacted by size in mucus than if diffusing freely, likely due to intermolecular interactions. In addition, physical barrier effects such as volume exclusion and path tortuosity may slow transport of “trapped” particles but also force larger particles to diffuse in larger pores within the heterogeneous mucus network.³⁴

Mucus gel barriers control pathogen and environmental particulate access to epithelia. They can also impact effective drug delivery as carriers can be trapped in mucus and not able to penetrate through to underlying cells. The thickness of the mucus lining the gastrointestinal tract varies from 10 to 250 μm .^{35,36} In order to gain insight into how measured differences in diffusivity may translate to changes in particle penetration through mucus, the percent penetration of a dosed particle population across a mucus barrier of a given thickness can be theoretically estimated using a mathematical model based on Fick’s second law and effective diffusivities of microspheres (Figure 1C). Particles 20–100 nm in size were predicted to be able to penetrate mucus layers up to $\sim 200 \mu\text{m}$ thick in about an hour, a time frame on the same order as the mucus turnover rate, reported to be 50–270 min.³⁷ However, particles larger than the reported mucus mesh size are not predicted to be able to penetrate over $\sim 100 \mu\text{m}$ thick mucus layer.

Influence of food-associated lipids on size selectivity of particle transport through mucus

As the intestinal lumen environment is dynamic, changing notably upon food ingestion, the potential effects of food-associated lipids on size selectivity of microsphere transport through GI mucus were studied. Particle transport through GI mucus was markedly hindered when dosed in fed state intestinal contents relative to dosing in buffer (Figure 2A). The ensemble-average particle transport rates decreased with time, as expected in a heterogeneous medium such as mucus (Figure 2B). Size selectivity of mucus was markedly increased by exposure to lipids. Diffusion rate of 500 nm particles was 2.5- and 20-fold lower than that of 100 nm particles when dosed to mucus in buffer and fed state medium, respectively (Table 2, Figure 2A).

The decreased effective diffusivity in mucus from 100 to 500 nm particles is thus less than the 4.6-fold difference expected due purely to particle size (as estimated by the Stokes-Einstein equation) when dosed to mucus in buffer, but greater than expected when dosed to mucus in fed state medium. Interestingly, particle diffusion rates for 200- to 500 nm particles dosed to mucus in fed state medium were similar, supporting a “cut off” size at which particles are excluded from certain pores within the mucus matrix. Based on calculated diffusivities, it is predicted that 100 nm particles dosed in fed state medium will not penetrate a 50 μm thick mucus layer (Figure 2C), while they would penetrate a 150 μm thick mucus layer if dosed in maleate buffer.

While it is recognized that intestinal contents will vary considerably from the simulated media used here, and other relevant factors, such as GI transit, are neglected, the results support the significance of intestinal mucus and dosing medium on ability of particle carriers to penetrate intestinal mucus barriers. It is noted that if orally dosed particles are intended for absorption or uptake at the epithelial surface, the mucus barrier is only the first barrier to uptake that needs to be considered, and particle size and surface properties are also certainly expected to impact cellular uptake. It is also noted that contents in simulated media may interact with the particle surface, altering effective particle size. There was a moderate change in measured particle size and surface charge in the simulated intestinal media relative to buffer (Supplementary Figure S1). However, a change in size of this magnitude would not account for the measured impact of lipids on particle transport through mucus, and the moderate decrease in zeta potential observed in simulated fed state medium would not be expected to hinder transport.¹⁸

Influence of surface chemistry on particle transport

Surface functionalization with polyethylene glycol (PEG) has been reported to enable particles to diffuse through mucus at a rate similar to diffusion through water. It has been hypothesized that this occurs by reduction of particle-mucus interactions.^{3,31} Particles modified with PEG overcome several mechanisms of transport barriers including hydrophobic and electrostatic interactions.³⁸ The effects of surface chemistry on size selectivity and sensitivity of particle diffusion through GI mucus to lipids were investigated by covalently attaching PEG to 200 and 500 nm particles. When dosed to mucus in buffer, 200 nm carboxylate-modified particles were 6-fold slower than PEG-modified particles. However, 200 nm carboxylate-modified particles were 575-fold slower than the same size PEGylated particles when dosed to mucus in lipid-containing media. In contrast to negatively charged carboxylate-modified particles, PEGylated particle transport was not markedly impacted by lipids. Diffusion rates of 200- and 500 nm size PEG-modified particles dosed to mucus in fed state were only 1.5- and 2.5-fold lower at a time scale of 10 s than the same size particles in maleate buffer. (Figure 3B) Furthermore, estimated particle penetration indicates similar passage percentage when dosed to mucus in buffer or in fed state, as shown in Figure 3C. Predicted penetrations across a 50 μm thick mucus layer of PEG-modified particles dosed in maleate buffer and fed state were 64% and 62%, respectively, for 200 nm particle and 17% and 7%, respectively, for 500 nm particles.

Microscopic transport of micelles in intestinal mucus

The mechanism by which lipids impact barrier properties of mucus is not entirely clear. Fed state intestinal contents include microemulsions and micelles. In an effort to understand the interactions between exogenous lipids and mucus, we explored whether model bile micelles stay intact within mucus. The microscopic diffusivity of $\sim 5\text{--}10\text{ nm}^2$ ²⁹ bile salt micelles was studied using FRAP. Fluorescence intensities were captured in mucus both before and after bombarding a ROI with laser light for a given time to bleach fluorescence, which then subsequently recovered due to diffusion into the ROI (Figure 4).

When performing FRAP, uniform bleaching efficiency allows to maintain consistent signal to noise ratios in the regions of very low intensity that occur immediately following the

bleach period. Bleaching efficiency was reasonably uniform in the samples analyzed, averaging approximately 70–80% (data not shown). The measured time scale of fluorescence recovery (Figure 5A) was used to estimate diffusion coefficients. Most of the bleaching was recovered after 60 seconds (Figure 5A).

Theoretically estimated diffusivities of micelles in water ($9.6 \times 10^{-7} \text{ cm}^2/\text{s}$) were over 3-fold higher than experimental diffusivities of micelles in mucus (above CMC) ($2.9 \times 10^{-7} \text{ cm}^2/\text{s}$); suggesting that micelle diffusion is hindered by diffusion through mucus, in spite of the large nominal mucus pore size (200 nm) relative to micelle size. Additionally, micelle diffusivities when dosed above the CMC were 13- and 20-fold lower than both the theoretically estimated and experimentally measured diffusivities of lipid molecules (dosed below CMC), supporting the likely presence of intact micelles in mucus (Figure 5B).

CONCLUSIONS

The heterogeneous mesh network of intestinal mucus results in decreased transport rates with increasing particle size from 20 to 500 nm, but the size selectivity depends on the particle surface chemistry and the presence of food-associated lipids in the dosing medium. The transport of carboxylate-functionalized particles decreases with increasing size to a lesser extent than theoretically expected (Stokes-Einstein) when dosed in buffer, but to a greater extent than theoretically expected when dosed in lipid-containing medium. PEGylation markedly decreases the impact of lipids on particle transport through mucus. Taken together, the observed impact of lipids on size selectivity of carboxylate-functionalized particle transport through mucus, the proposed role of PEGylation in reducing particle-mucus interactions, and the dramatic impact of PEGylation on particle transport through mucus in the presence of lipids, suggests that lipids impact both effective mesh pore size and molecular interactions within mucus gels. In general, it is predicted that particles in the size range studied should be able to cross an intestinal mucus layer of physiologically relevant thickness (~100 μm) in the timeframe of mucus turnover (~1 hour) to some extent, again dependent on size, surface chemistry, and presence of lipids in intestinal contents. The results thus emphasize the importance of considering size and surface chemistry in designing oral delivery carriers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Ensemble $\langle \text{MSD} \rangle$ and B) D_{eff} vs time scale plots for carboxylate-modified microspheres dosed to mucus in Maleate Buffer, C) Estimated particle penetration through mucus layer over time using Fick's second law.

Figure 2.

A) Ensemble $\langle \text{MSD} \rangle$ and B) D_{eff} vs time scale plots for carboxylate-modified microspheres dosed to mucus in maleate buffer and fed state, C) Estimated particle penetration through mucus layer over time using Fick's second law.

Figure 3.

A) Ensemble $\langle \text{MSD} \rangle$ and B) D_{eff} vs time scale plots for 200-, and 500 nm in diameter PEGylated particles dosed to mucus in maleate buffer and fed state, C) Estimated particle penetration through mucus layer over time using Fick's second law.

Figure 4.

Images of GI mucus before (Pre) and at 0, 60 seconds after photobleaching. Scale bar, 10 μm

Figure 5.

A) Normalized, individual experimental intensity recovery curve. B) Measured and calculated diffusion coefficients of bile salt micelles in mucus. Stokes-Einstein equation was used to calculate diffusion coefficients of micelles, and Wilke-Chang equation was used to calculate diffusion coefficients of lipid molecules. Data represent mean $\pm$ standard error of three experiments, * denotes statistically significant differences ($P < 0.05$).

Table 1

Bio-relevant media dosed to mucosal surfaces.

Maleate Buffer	Triz-Ma	100	mM
	NaCl	65	mM
	CaCl ₂	10	mM
	NaN ₃	3	mM
	NaOH	40	mM
Model Bile	NaTDC	12	mM
	Lecithin	4	mM
Lipid	Soybean Oil	35	mM
	Sodium Oleate	30	mM
	1-Oleoyl-rac-glycerol	15	mM

Table 2

Ratios of ensemble average diffusion coefficients of particles in mucus at $\zeta = 10$ s.

Dosing Medium	Surface Chemistry	D(500 nm)/D(200 nm)	D(500 nm)/D(100 nm)
Maleate Buffer	Carboxylate	0.931	0.408
Fed State	Carboxylate	0.832	0.051
Maleate Buffer	PEG	0.089	NA
Fed State	PEG	0.047	NA