Anti-PEG antibodies alter the mobility and biodistribution of densely PEGylated nanoparticles in mucus

Christine E. Henry, Ying-Ying Wang, Qi Yang, Thuy Hoang, Sumon Chattopadhyay, Timothy Hoen, Laura M. Ensign, Kenetta L. Nunn, Holly Schroeder, Justin McCallen, Thomas Moench, Richard Cone, Steve R. Roffler, Samuel K. Lai

A R T I C L E   I N   F O R E I G N

Article history:
Received 2 March 2016
Received in revised form 24 June 2016
Accepted 12 July 2016
Available online xxxx

Keywords:
PEGylation
IgG
IgM
Mucosal immunity
Nanomedicine
Diffusion

A B S T R A C T

Antibodies that specifically bind polyethylene glycol (PEG) can lead to rapid elimination of PEGylated therapeutics from the systemic circulation. We have recently shown that virus-binding IgG can immobilize viruses in mucus via multiple low-affinity crosslinks between IgG and mucins. However, it remains unclear whether anti-PEG antibodies in mucus may also alter the penetration and consequently biodistribution of PEGylated nanoparticles delivered to mucosal surfaces. We found that both anti-PEG IgG and IgM can readily bind nanoparticles that were densely coated with PEG polymer to minimize adhesive interactions with mucus constituents. Addition of anti-PEG IgG and IgM into mouse cervicovaginal mucus resulted in extensive trapping of mucus-penetrating PEGylated nanoparticles, with the fraction of mobile particles reduced from over 95% to only 34% and 7% with anti-PEG IgG and IgM, respectively. Surprisingly, we did not observe significant agglutination induced by either antibody, suggesting that particle immobilization is caused by adhesive crosslinks between mucin fibers and IgG or IgM bound to individual nanoparticles. Importantly, addition of corresponding control antibodies did not slow the PEGylated nanoparticles, confirming anti-PEG antibodies specifically bound to and trapped the PEGylated nanoparticles. Finally, we showed that trapped PEGylated nanoparticles remained largely in the luminal mucus layer of the mouse vagina even when delivered in hypotonic formulations that caused untrapped particles to be drawn by the flow of water (advection) through mucus all the way to the epithelial surface. These results underscore the potential importance of elucidating mucosal anti-PEG immune responses for PEGylated therapeutics and biomaterials applied to mucosal surfaces.

Statement of Significance

PEG, generally considered a ‘stealth’ polymer, is broadly used to improve the circulation times and therapeutic efficacy of nanomedicines. Nevertheless, there is increasing scientific evidence that demonstrates both animals and humans can generate PEG-specific antibodies. Here, we show that anti-PEG IgG and IgM can specifically immobilize otherwise freely diffusing PEG-coated nanoparticles in fresh vaginal mucus gel ex vivo by crosslinking nanoparticles to the mucin mesh, and consequently prevent PEG-coated nanoparticles from accessing the vaginal epithelium in vivo. Given the increasing use of PEG coatings...
1. Introduction

All exposed surfaces in the human body not covered by skin, including those of the respiratory, gastrointestinal and urogenital tracts, are covered with a layer of mucus secretions. In addition to mucus secreted by goblet cells and mucus-secreting glands, mucus also contains various proteins, lipids, cells and other constituents, all of which contribute to the proper functioning of mucus as a lubricant and diffusional barrier against foreign particles [1]. Mucus is continuously secreted and cleared, which enables it to function as a constantly renewed filter that can rapidly eliminate any foreign particulates trapped within the dense network of mucin fibers [2].

To overcome the mucus barrier, we and others have engineered nanoparticles with minimal affinity to mucus constituents [3–6], which can undergo rapid diffusion in the low viscosity interstitial fluids between mucin fibers [7] as well as transport through the mucus gel by advective bulk fluid flow [8]. The most popular approach to formulate such mucus-penetrating particles has been to coat particles with a dense layer of polyethylene glycol (PEG) [3,4,9]; the resulting ‘mucus-penetrating particles’ have been shown to markedly improve the delivery of chemotherapy particles against cervical cancer [10], microbicides against vaginal Herpes transmission [11], and DNA in the lung airways [12].

These successes have expanded the use of PEGylation, which is already routinely employed to prolong the circulation kinetics of proteins, liposomes and polymeric nanoparticles [13–16], to mucosal applications. The increasing use of PEG in drug delivery applications, including those that require repeat dosing, has led to investigations of potential immune responses upon repeated or prolonged exposure to PEGylated therapeutics. Since the late 1990s, several groups have reported that a second dose of PEG-modified liposomes is generally rapidly cleared from the blood and accumulates in the liver when injected into the same rat or mouse at several-day intervals [17,18]. This accelerated blood clearance phenomenon, which results in markedly reduced efficacy for some PEGylated therapeutics, was later found to be mediated by induced antibodies that specifically bind PEG [19–21]. Schellekens et al. [22] recently raised considerable concerns regarding the anti-PEG antibody phenomena by summarizing some of the variations and apparent contradictions across different studies, and astutely highlighted the lack of validated and standardized assays as a major barrier in correctly characterizing anti-PEG antibody responses in biological specimens. Nevertheless, given the sheer volume of observations for anti-PEG antibody response by numerous independent investigators across different animal models and different PEGylated systems, it is perhaps more likely that the variable observations reflect an incomplete understanding of the complexity of anti-PEG phenomena in vivo rather than solely inadequately supported conclusions based on artifacts from ELISA and other assays.

It is important to also note that all in vivo studies in the literature were performed in animals that had no prior exposure to PEG. In contrast, humans are increasingly exposed to PEG found in everyday products including detergents, food products and cosmetics. Due to this repeated exposure, a substantial proportion of the human population likely has pre-existing anti-PEG antibodies. In 2003, Armstrong et al. reported anti-PEG IgG and IgM in 22.5% and 8.6% of serum samples, respectively [23], and those proportions have likely increased over the past decade. Indeed, pilot studies in our lab suggest pre-existing anti-PEG immunity may be even more prevalent [24].

Possible flaws with detection assays notwithstanding, these findings clearly underscore the importance of evaluating the influence of pre-existing anti-PEG immunity in any human studies of PEGylated therapeutics. Furthermore, because PEG exposure may occur at mucus membranes (e.g., via oral exposure to PEG in dental care products, gastrointestinal exposure to PEG in food additives, or vaginal exposure to PEG in personal lubricants or vaginal creams), mucosal anti-PEG responses capable of altering the biodistribution and pharmacokinetics of topically applied PEGylated carriers are a distinct possibility.

An emerging mechanism of mucosal immune protection, pioneered by our group, is the immobilization of individual viruses due to antibody-mucin interactions, which in turn prevents viral translocation across mucus [25]. Previous work has shown that the interactions between antibodies and mucus are low-affinity and transient; for example, the diffusion of IgG and IgA molecules (diameter ~10 nm) in human mucus (pores ~340 ± 70 nm [7]) is slowed only ~5–20% compared to in buffer [26,27]. We found that this seemingly negligible affinity is sufficient to trap HSV-1 virions in human cervicovaginal mucus (CVM) with sub-neutralizing potency, presumably because the array of virion-bound IgG ensures a sufficient number of transient low-affinity bonds between the virus/IgG complex and mucus at any given time [25]. Furthermore, a non-neutralizing IgG facilitated effective protection against vaginal Herpes infection in mice, but that protection was abolished when vaginal mucus was removed. These results indicate that virus-binding antibodies in mucus can directly alter the mobility of virions in mucus, leading to a markedly reduced flux arriving at the epithelium [27]. We thus sought to evaluate here whether anti-PEG antibodies in mucus would similarly trap PEGylated nanoparticles in mucus and impede therapeutic delivery.
Previously characterized [30]. The binding avidity for anti-PEG IgG (3.3) has been reported to be 1.3 N/A (Table 1) [4].

- [amino(polyethylene glycol)-5000] (DSPE-PEG 5k, Nanocs, New York, NY) with 2 kDa methoxy poly(ethylene glycol) amine (PEG; Rapp Polymere, Tuebingen, Germany) via a carboxyl-amine reaction, as published previously [4,28]. Particle size and ζ-potential were determined by dynamic light scattering and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). Size measurements were performed at 25 °C at a scattering angle of 90°. Samples were diluted in 10 mM NaCl solution, and measurements were performed according to instrument instructions. High density PEGylation (>1 PEG/nm²) was verified using the fluorogenic compound 1-pyrenylidiazomethane (PDMA) to quantify residual unmodified carboxyl groups on the polystyrene beads [28]. PEG conjugation was confirmed by a near-neutral ζ-potential (Table 1) [4].

### 2.3. Anti-PEG IgG and IgM antibodies

Mouse anti-PEG IgG (3.3) and IgM (AGP-4) antibodies are commercially available from Academia Sinica [29] and have been previously characterized [30]. The binding avidity for anti-PEG IgG (3.3) has been reported to be 1.3 x 10⁻⁷ M at room temperature [31], while that for anti-PEG IgM (AGP-4) is at least 1.4 x 10⁻¹⁰ M [32]. To confirm the specificity of the anti-PEG antibodies used in our studies, we performed a competitive ELISA. Medium binding EIA/RIA 96-well plates (Corning, Corning, NY) were coated with 50 μg/mL of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-5000] (DSPE-PEGsil, Nanocs, New York, NY) in PBS overnight at 4 °C. Anti-PEG IgG or control IgG antibody was added at 6 μg/mL, while anti-PEG IgM or control IgM antibody was added at 2 μg/mL. The antibodies were incubated with the plate in the absence or presence of free diol PEGsil (Sigma-Aldrich, St. Louis, MO). Bound antibody was detected using goat anti-human IgG HRP (Santa Cruz, Biotechnology, Dallas, TX) or anti-human IgM HRP (Rockland Immunocchemicals, Pottstown, PA) and then 1-step Ultra TMB substrate (Thermo Scientific, Waltham, MA), and absorbance at 450 nm was read using a Spectramax M2 ( Molecular Devices, Sunnyvale, CA). No PEG-containing detergent was used in any of the ELISA assays.

- Anti-PEG IgG and IgM binding to PS-PEG nanoparticles were confirmed by dot blot assay. Two microliters of PS or PS-PEG beads were blotted onto nitrocellulose membranes. The membranes were incubated with 1:2000 dilutions of anti-PEG IgM, control IgM (sc-58070 Vancomycin (2F10); Santa Cruz Biotechnology, Dallas, TX), anti-PEG IgG, or control IgG (sc-101339 Biotin (33); Santa Cruz Biotechnology, Dallas, TX), followed by incubation with a 1:10,000 dilution of HRP-labeled goat anti-mouse IgM (Life Technologies, Carlsbad, CA) or a 1:7000 dilution of HRP-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX). Bound secondary antibody was detected using an ECL kit (BioRad, Hercules, CA), and imaged using a FluorChem E system (ProteinSimple, San Jose, CA). The same anti-PEG and control IgG and IgM were used in both multiple particle tracking and in vivo studies.

#### 2.4. Multiple particle tracking

For particle tracking studies, anti-PEG or control IgG or IgM, as listed above, was first added to 20 μL of mCVM in custom-made chambers, followed by addition of dilute particle solutions (10⁻⁶–10⁻¹⁰ particles/mL, 5% v/v). To confirm the specificity of anti-PEG antibodies to PEG, free PEG (8 kDa, 0.2 mg/mL; Sigma-Aldrich, St. Louis, MO) was also added to mCVM prior to the addition of particles. Samples were incubated 1 h at 37 °C before microscopy. The trajectories of the fluorescent particles in mCVM were recorded using an EMCCD camera (Evolve 512; Photometrics, Tucson, AZ) mounted on an inverted epifluorescence microscope (AxioObserver D1; Zeiss, Thornwood, NY), equipped with an Alpha Plan-Apo 100×/1.46 NA objective, environmental (temperature and CO₂) control chamber and an LED light source (Lumencor Light Engine DAPI/GFP/543/623/690). Videos (512 × 512, 16-bit image depth) were captured with MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) at a temporal resolution of 66.7 ms and spatial resolution of 10 nm (nominal pixel resolution 0.156 μm/pixel). The tracking resolution was determined by tracking the displacements of particles immobilized with a strong adhesive, following a previously described method [33]. Particle trajectories were analyzed using MATLAB software as described previously [34]. Sub-pixel tracking resolution was achieved by determining the precise location of the particle centroid by light-intensity-weighted averaging of neighboring pixels. Trajectories of n > 40 particles per frame on average (corresponding to n > 100 total traces) were analyzed for each experiment, and four independent experiments were performed in mCVM collected from different mice. The coordinates of particle centroids were transformed into time-averaged mean squared displacements (MSD), calculated as <Δr²(t)> = [x(t + t) − x(t)]² + [y(t + t) − y(t)]² (where t = time scale or time lag), from which distributions of MSDs and effective diffusivities (Deff) were calculated, as previously demonstrated [35,36]. MSD may also be expressed as MSD = 4Dt², where a, the slope of the curve on a log-log scale, is a measure of the extent of impedance to particle diffusion (a = 1 for pure unobstructed Brownian diffusion; a < 1 indicates increasing impedance to particle movement as α decreases). Mobile particles were defined as those with Deff > 1.5 x 10⁻¹⁵ m²/s at t = 0.2667 s (this t value corresponds to a minimum trajectory length of 5 frames), based on multiple datasets of mobile and immobile nanoparticles (e.g., PS and PS-PEG nanoparticles) in human mucus [7,37]. For particles ~100 nm in size, a Deff > 1.5 x 10⁻¹⁵ m²/s effectively means that the particles move a distance greater than their diameters within 0.2667 s.

#### 2.5. Distribution of nanoparticles in the mouse vagina

To evaluate PEGylated nanoparticle trapping in vivo, we used female 6–8 week old CF-1 mice (Harlan, Frederick, MD) pretreated with 100 mg of 17β-estradiol benzoate (Sigma-Aldrich, St. Louis, MO) injected subcutaneously two days before the experiments [11]. Anti-PEG or control (anti-vancomycin) IgM was first administered to the mouse vagina in two doses: 10 μL of 150 μg/mL antibody in 0.5× normal saline, and after a 10 min interval, another 10 μL dose in normal saline. The mildly hypotonic medium in the first dose results in advective transport of antibody close to the epithelium [8], which, combined with the second dose in isotonic medium, ensures antibody was well distributed throughout the luminal layer. After another 10 min interval, 20 μL of either PS-PEG or control PS (0.025% w/v) was administered in a slightly hypotonic medium (0.75× normal saline). Assuming the native volume of mCVM is approximately 20 μL, and that ~50% of applied antibody is lost due to drip out or absorption across the vaginal...
epithelium due to advective flow, the final antibody concentration after two 10 μL doses of 150 μg/mL would be ~50 μg/mL.

Mice were sacrificed after 10 min, and the entire vagina was then gently removed and frozen in Tissue-Tek™ O.C.T.™ Compound (Sakura Finetek U.S.A., Inc., Torrance, CA). Transverse sections were obtained at various points along the length of the tissue (between the introitus and the cervix) with a Microm HM 500 M Cryostat (Microm International, Walldorf, Germany). The thickness of the sections was set to 6 μm to achieve single-cell layer thickness. The sections were then stained with ProLong Gold antifade reagent (Invitrogen, Grand Island, NY) with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei and retain particle fluorescence. Fluorescence images of the sections were obtained with an inverted fluorescence microscope at 10× magnification. We quantified the distribution of particles in tissue sections using Image J software (National Institutes of Health) to extract pixel intensities in the red (PS-PEG) or green (PS) channel, and compared total fluorescence between the vaginal lumen and within 20 μm of the epithelial surface. The same procedures were performed for n = 3–4 mice per condition, and particle distribution was averaged over multiple sections per mouse. Mice were anesthetized for the duration of experiments. All experimental protocols were approved by the Johns Hopkins Institutional Animal Care and Use Committee.

2.6. Statistical analysis

Data averages are presented as means with standard error of the mean (SEM) indicated. Statistical significance was determined by a one-tailed, Student’s t-test (α = 0.05).

3. Results

Due to both passive transudation and active transport by the MHC class I-related neonatal Fc receptor [38], IgG, rather than IgA, is the predominant immunoglobulin in human CVM secretions (>10-fold more IgG than IgA) [39]. However, the routes of PEG exposure that may lead to gradual induction of anti-PEG IgG in humans are not yet known and would be difficult to produce in mice. Therefore, we first tested whether exogenous murine anti-human PEG antibodies are not yet known and would be difficult to produce in mice. We added free PEG in excess that allows quantitative measurements of hundreds of individual particles. PS-PEG particles in control mCVM were only slowed ~5-fold compared to their theoretical speeds in water (Table 1), but after two 10 μL doses of 150 μg/mL prior to the addition of nanoparticles, a large fraction of PS-PEG became immobilized (Movie S2), similar to uncoated (mucoadhesive) PS particles (Movie S3). Since control IgG did not trap PEGylated nanoparticles, the observed trapping is unlikely to be caused by non-specific binding of IgG to PEG or alterations of the mCVM microstructure due to addition of IgG antibodies. Instead, these results imply that anti-PEG antibodies can specifically bind to and trap PEGylated nanoparticles in mucus. To further validate the specificity of anti-PEG IgG binding to PEG coatings, we tested whether saturation with free PEG would prevent immobilization of the beads in mCVM. Indeed, when we added free PEG in excess of the anti-PEG antibody concentration, PS-PEG became freely mobile (Fig. S1, Movie S4).

We quantified the speeds of PS-PEG in mucus treated with antibody binding to PEG. (A,B) Binding of (A) anti-PEG or control IgG and (B) anti-PEG or control IgM antibodies to PEG-coated plates at different concentrations of free PEG (C-F) Dot blot assay demonstrating binding of anti-PEG antibodies to PS-PEG vs. control PS beads. PS and PS-PEG beads were blotted onto nitrocellulose membrane and incubated with (C) anti-PEG IgG, (D) control IgG, (E) anti-PEG IgM, or (F) control IgM.

Please cite this article in press as: C.E. Henry et al., Anti-PEG antibodies alter the mobility and biodistribution of densely PEGylated nanoparticles in mucus, Acta Biomater. (2016), http://dx.doi.org/10.1016/j.actbio.2016.07.019
Brownian diffusion caused by anti-PEG IgG was also reflected by logarithms of individual particle effective diffusivities (Deff) at a time scale of IgG (Fig. 3B; at a time scale of 0.2667 s). The impediment to free squared displacement (<MSD>) by in good agreement with previous reports [11,40]. The addition of anti-PEG IgG indicates a statistically significant difference (p < 0.05). Distributions of the logarithms of individual particle effective diffusivities (Deff) at a time scale of 0.2667 s. Log Deff values to the left of the dashed line correspond to particles with displacements of less than ~100 nm (i.e., less than the particle diameter) within 0.2667 s. These small motions are consistent with particles permanently stuck to the mucus gel, and most likely reflect thermal motions of the gel itself. Data represent the ensemble average of three independent experiments, with n > 40 particles per frame (n > 100 particle traces per experiment) on average for each experiment.

We next sought to test whether an agglutinating antibody may trap PEGylated particles even more extensively. Secretory IgA (sIgA) is another common immunoglobulin found in mucus. The polymeric nature of sIgA, as well as IgM, has long been suggested to facilitate “immune exclusion”, the agglutination of microorganisms by polymeric immunoglobulins into clusters too large to diffuse through mucus [41]. The relevance of sIgA- and IgM-mediated immune exclusion in the female reproductive tract is vividly illustrated by agglutination of otherwise vigorously motile sperm, which make little to no forward progress after agglutination and cannot ‘swim’ through mucus [42]. Unfortunately, anti-PEG sIgA is not commercially available and cannot be readily generated in the lab. Therefore, we evaluated the effects of anti-PEG IgM on the diffusion behavior of PEGylated nanoparticles in mucus.

Once we validated the specificity of nanoparticle trapping by anti-PEG antibodies (Movie S2, Movie S7), we determined the extent to which anti-PEG IgM immobilized the nanoparticles by quantifying the particle density distributions. Using the S6, while saturation of mCVM with free PEG prevented immobilization of PS-PEG by anti-PEG IgM (Fig. S2, Movie S7), again validating the specificity of nanoparticle trapping by anti-PEG antibodies binding to PS-PEG. The <MSD> of PS-PEG in mCVM with anti-PEG IgM was 150-fold lower than for the same particles in mCVM treated with control IgM (Fig. 4B), and reflects a drop in...
the mobile fraction from 97% to 7% (Fig. 4C). In comparison, mucus-adhesive PS particles exhibit a mobile fraction of 3.5% on average in mCVM (Fig. 2). Anti-PEG IgM also reduced the average α value of PS-PEG from 0.79 to 0.45 (p < 0.05 vs. control), underscoring the extent of antibody-mediated immobilization. Interestingly, we did not observe any nanoparticle agglutination in mCVM with anti-PEG IgM; agglutination was only observed when nanoparticles were pre-mixed with anti-PEG IgM prior to addition to mucus (Fig. S3).

Lastly, we wanted to evaluate whether the observed changes to the diffusion of PEGylated nanoparticles in physiological mucus specimens ex vivo would also alter nanoparticle distribution at mucosal surfaces in vivo. We therefore topically administered anti-PEG or control IgM to the mouse vagina, followed by addition of PS-PEG in hypotonic medium. We observed that a substantial fraction of PS-PEG was drawn advectively through luminal mucus of PS-PEG in hypotonic medium. We observed that a substantial fraction of PS-PEG was drawn advectively through luminal mucus in mice dosed with control IgM, with a significant fraction penetrating deep into the rugae (Fig. 5A). In contrast, PS-PEG nanoparticles were largely trapped in luminal mucus in mice that received anti-PEG IgM; far fewer particles reached the vaginal epithelium or penetrated into the rugae (Fig. 5B), similar to mucus-adhesive carboxylated latex beads (Fig. 5C). We further quantified particle distribution in the vaginal lumen vs. coating the epithelium (within 20 μm of the epithelial surface). The ratio of particles in the lumen vs. epithelium was approximately 1 for PS-PEG with control IgM, indicating on average 50% of particles were within 20 μm of the epithelium (Fig. 5D). In contrast, for both PS-PEG with anti-PEG IgM and uncoated latex beads, nearly 15-fold more particles were in the lumen than in proximity to the epithelium. These results are consistent with previous observations that nanoparticles that interact with and bind to the mucin mesh fibers overlaying the epithelium are unable to penetrate the mucus layer and reach the epithelium [11].

4. Discussion

The emergence of anti-PEG antibodies represents a potential Achilles’ heel to the increasingly common use of PEG in nanomedicine. Rodent as well as large animal studies have clearly illustrated that anti-PEG immunity directly abrogates the extended circulation times that PEGylation generally affords to therapeutics [17,43–46]. Anti-PEG immunity may also result in serious complications beyond poor pharmacokinetics. Indeed, anti-PEG response as a result of repeated weekly injections of PEGylated liposomes containing synthetic oligodeoxynucleotides led to significant morbidity and mortality in mice [20]. In human clinical studies of PEG-uricase for the treatment of gout, the presence of anti-PEG antibodies was associated with early elimination and poor outcomes [47,48]. While these studies all highlight the systemic effects associated with anti-PEG response, potential mucosal anti-PEG response has not been evaluated to date. Our finding that anti-PEG IgG or IgM can directly and specifically impede the mobility, and consequently alter the biodistribution, of PEGylated nanoparticles at mucosal surfaces adds yet another potential pitfall of anti-PEG immunity in vivo (Fig. 6).

More antibodies are secreted into mucus than into blood or lymph [42,49,50]; thus, a major part of the physiological immune response is likely intended to occur in the mucus secretions
coating exposed surfaces of the respiratory, gastrointestinal and urogenital tracts. However, the notion that secreted antibodies can work together with mucus to impede the mobility of pathogens and foreign particulates and reduce the flux arriving at the epithelium remains largely unrecognized. This is in part a consequence of the exceedingly weak affinity between antibodies and

Fig. 5. Representative transverse 50 μm thick frozen tissue sections showing the distribution of (A,B) PEG-coated nanoparticles in the mouse vagina treated with (A) control or (B) anti-PEG antibody, or of (C) uncoated PS nanoparticles in the mouse vagina. Red corresponds to PEG-coated nanoparticles, green corresponds to uncoated PS nanoparticles, and blue corresponds to DAPI-stained cell nuclei. Scale bars indicate a length of 200 μm. (D) Image-based quantification of particle distribution in the vaginal lumen vs. in close proximity to the epithelium (within 20 μm of the epithelial surface). * indicates a statistically significant difference (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: C.E. Henry et al., Anti-PEG antibodies alter the mobility and biodistribution of densely PEGylated nanoparticles in mucus, Acta Biomater. (2016), http://dx.doi.org/10.1016/j.actbio.2016.07.019
mucus, which would suggest that individual antibodies are incapable of crosslinking a pathogen or particle to mucus. It was not until very recently that virus-binding IgG antibodies were shown by both experiments [25] and by computational modeling [27] to be capable of immobilizing viruses in mucus via multiple bonds between mucins and N-glycans on IgG-Fc, which in turn reduces the flux of viruses arriving at the epithelium in vivo. Our observed correlation between limited PEG-coated nanoparticle diffusion due to anti-PEG antibodies in mucus and their exclusion from reaching the vaginal epithelium is consistent with the poor mucus penetration and distribution observed previously with conventional latex nanoparticles in the mouse vagina [11]. The immobilization of PEGylated nanoparticles by anti-PEG antibodies in mucus offers further evidence that trapping by antibody-mucin crosslinking may be a universal mechanism of mucosal immunity across different animal species.

We found that anti-PEG IgM was substantially more potent in trapping individual PEGylated nanoparticles in mucus than anti-PEG IgG. This may be due to a number of reasons. First, anti-PEG IgM has a higher binding avidity to PEG than anti-PEG IgG (see Section 2), which likely translates to greater antibody accumulation on the surface of PEGylated nanoparticles. Second, IgM likely possesses greater avidity to mucins than IgG. This can be inferred from the diffusivity of individual IgM molecules in mucus, which is slowed nearly 50% compared to in buffer, whereas individual IgG molecules are slowed only 5–20% in mucus compared to in buffer [26]. Because both IgG and IgM are far too small relative to the mucin mesh spacing [7] to be slowed substantially by steric hindrance, IgM must be slowed to a greater extent than IgG due to more and/or stronger adhesive interactions with mucus constituents. The greater mucin-binding avidity of IgM implies fewer particle-bound IgM would be needed to immobilize a PEGylated nanoparticle compared to IgG; indeed, greater trapping of PS-PEG was achieved with IgM than IgG even at a lower concentration.

A classical mechanism of immune defense is “immune exclusion”, which describes the agglutination of microorganisms in the gut by secreted polyvalent IgA or IgM into clusters too large to diffuse through mucus [51]. Although mucus is highly viscoelastic at the macroscale [1,52], PEGylated nanoparticles can readily diffuse through mucus at rates comparable to their rates in water [49], and thus are not precluded from colliding with each other and becoming agglutinated. Interestingly, we observed negligible levels of agglutination in all of our microscopy studies. This is likely because even a single particle-bound IgM can substantially impede the Brownian motion of the particle (assuming the binding of IgM to PEG does not alter IgM affinity to mucins, a single IgM bound to the nanoparticle may slow it down by ~50%), and lower particle mobility directly reduces the frequency of colliding with other particles, a necessary first-step to agglutination. Thus, IgM-induced agglutination is unlikely to be prevalent when the kinetics of IgM accumulating onto the nanoparticle surface is substantially faster than the rate of collision between nanoparticles. This directly challenges the general dogma that antibody-mediated agglutination—often supported by micrographs showing the formation of large immune complexes upon incubation of antibodies and pathogens in buffer in the absence of mucus—is a dominant mechanism of mucosal immunity by polymeric immunoglobulins.

In addition to a lack of standardized quantitative assays to characterize anti-PEG antibodies, a major contention to reports of anti-PEG phenomenon in the literature is that the observations may not require antibodies that specifically bind PEG polymer [22]. Indeed, Schellekens et al. suggested that the accelerated blood clearance observed in many studies could perhaps be attributed to complement activation and/or non-specific accumulation of IgM and IgG onto the surface of PEGylated drugs or drug carriers. Although it is impossible to draw direct comparisons between studies performed in blood vs. mucus, we showed here that the mobility of PEG-coated nanoparticles was specifically retarded by anti-PEG IgG and IgM, because (i) addition of control IgG and IgM antibodies did not comparably alter bead motions, (ii) addition of free PEG competitively inhibits trapping of beads by anti-PEG antibodies, and (iii) the total dose of anti-PEG IgG or IgM in mucus represents only a very small fraction of the total IgG and IgM present [25]. Although it is likely that not all reports of accelerated blood clearance of PEGylated agents are caused solely by anti-PEG antibodies, our studies underscore the ability of anti-PEG IgG and IgM to specifically bind PEGylated particles in physiologically relevant environments (i.e. mucus), and in turn alter the biological fate of the PEGylated particles.
The concentration of approximately 50 µg/mL anti-PEG antibody used in our in vivo studies (see Section 2) is likely in the range of concentrations that can be achieved under physiological conditions. Kwaida and coworkers recently showed that ~50 µg/mL of serum anti-PEG IgM can be induced following injection of PEG-liposomes at a dose of 5 nmol of phospholipids per mouse [53]. Much of the antibody in secretions coating the epithelial surface is derived from serum transudation, and thus is likely similar in composition to the systemic antibody repertoire. Indeed, despite the common belief that IgA is the main immunoglobulin found in mucus, IgG is in fact the dominant antibody isotype in vaginal secretions [38,39], similar to in serum. In addition, although PEG-specific antibody levels in human vaginal secretions have never been measured, we have previously found Herpes-specific antibodies at up to 20–30 µg/mL levels in native vaginal secretions from human donors [54].

The vast majority of the literature on anti-PEG immunity focuses on the acute induction of anti-PEG immune response by specific PEGylated liposomes or nanoparticle therapeutics. The possibility of pre-existing anti-PEG response is rarely addressed. Because most of the population has yet to receive PEGylated therapeutics, the presence of PEG-specific antibodies in bodily secretions such as mucus most likely results from induction by alternative routes of exposure. Since PEG and other PEG-containing polymers are often found in soaps and other detergents, a hypothetical route of exposure could be via open cuts and wounds cleaned with PEG-containing soaps, or bleeding gums at the oral mucosa exposed to PEG-containing toothpaste. The combination of PEG and surfactants that are toxic to cells may provide a danger signal that inadvertently induces immune cells to generate antibodies against PEG. Alternatively, PEG is also frequently found in numerous foods as well as oral and topical products. Repeated exposure to PEG at the oral, gastrointestinal and vaginal mucosa, including exposure accompanied by surfactants, could potentially induce systemic and/or mucosal anti-PEG immunity over time, with significant implications for the delivery of PEGylated therapeutics via relevant routes of administration.

5. Conclusion

PEG is widely used in nanomedicine as a stealth coating polymer to improve circulation time and therapeutic efficacy. However, a growing body of evidence generated over the past two decades shows that systemic exposure to PEG can induce anti-PEG antibodies that not only significantly alter the systemic circulation kinetics of PEG-modified nanotherapeutics, but may also directly result in adverse outcomes. Our finding that anti-PEG antibodies can also trap PEG-coated nanoparticles in mucus secretions in vivo and alter particle biodistribution at mucosal surfaces in vivo further suggests that mucosal anti-PEG immunity may be an under-recognized challenge. Mucosal exposure to PEG and the prevalence of anti-PEG antibodies in human mucus is not yet well understood. In light of the large arsenal of PEGylated therapeutics that are FDA-approved or in clinical development for both systemic and mucosal applications, we believe further investigations into the origin and characteristics of anti-PEG immunity are of critical importance to support ongoing efforts in translational nanomedicine development.

Disclosures

Mucomune LLC seeks to harness antibody-mucin interactions to improve protection against or treatment of infections at mucosal surfaces, and has licensed intellectual property from the University of North Carolina - Chapel Hill (UNC-CH). SKL is a founder of Mucomune. SKL and YYW own company stock. SKL's relationship with Mucomune is subject to certain restrictions under University policy. The terms of this arrangement are being managed by UNC-CH in accordance with its conflict of interest policies.

Acknowledgement

Financial support was provided by the PhRMA Foundation Pre-doctoral Fellowship (Q.Y.), National Science Foundation CAREER Award (DMR-1151477, S.K.L.), The David and Lucile Packard Foundation (2013-39274, S.K.L.), National Institutes of Health grants R21EB017938 (S.K.L.), U19AI096398 (S.K.L. and R.C.), 1F32AI102535 (YYW), and Diversity Supplement AI096398 S1 (KLN), and startup funds from the Eshelman School of Pharmacy and Lineberger Comprehensive Cancer Center (S.K.L.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Durai B. Subramani for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.07.019.

References


