The Effect of Intestinal Bacteria Adherence on Drug Diffusion Through Solid Films Under Stationary Conditions

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Purpose. To study the *in vitro* and *in vivo* the role of surface bacterial adhesion on the diffusion of model drugs at stationary conditions.
Methods. Salicylic acid (SA) diffusion through ethyl cellulose (EC) films was measured *in vitro* in side-by-side diffusion cells with and without *E. coli* of intestinal origin. Insulin (I) release from paper strips coated or uncoated with pectin films, with or without antibiotic treatment, was measured *in vivo* in conscious rats after cecal implantation by comparing blood glucose levels at T_{max} of the pharmacodynamic effect.
Results. During five hours of diffusion studies which were performed immediately following incubation of EC films with bacteria, the diffusion rate of SA throughout the films was 2.72-fold lower in the presence of bacteria compared with the diffusion rate in the control studies conducted without bacteria. The mean blood glucose levels dropped in the rat to 40.6 ± 21.6% of glucose basal levels within 2.4 ± 1.4 h when uncoated I solid carriers were used. Glucose levels did not change for pectin-coated dosage forms. After antibiotic treatment which prevented the formation of bacterial biofilm on the surface of the I solid dosage forms, blood glucose levels dropped to 22.0 ± 4.7% and 50.9 ± 20.5% of glucose basal levels within 7.4 ± 2.6 h and 1.8 ± 0.9 h for pectin uncoated or coated dosage forms, respectively. Maximum bacterial adherence occurred at stationary conditions (RPM = 0), while at maximum agitation (200 RPM), almost no adherence occurred.
Conclusions. (*a*) Bacterial adherence slows down the diffusion rate of SA through EC films; (*b*) Under stationary conditions bacterial adherence may also interfere with drug release from biodegradable (pectin) films; (*c*) Successful functioning of biodegradable colon-specific delivery systems depends on agitation and surface friction in the lumen of the colon.

KEY WORDS: bacterial adhesion; colonic delivery; ethyl cellulose; insulin; pectin.

INTRODUCTION

Solid dosage forms for oral delivery of drugs to the distal parts of the small intestine and the colon [mainly for the local treatment of ulcerative colitis and Crohn's disease with 5-aminosalicylic acid (5-ASA)] are usually coated with pH-sensitive polymers, such as Eudragit® S or L or ethyl cellulose (1–3). Recently, the use of biodegradable polymers which could be used either in the form of a coating material (4–7) or in the form of matrix hydrogels (8–10) has been suggested. The assumed advantage of biodegradable carriers for colon-specific drug delivery is their independence on GI pH gradients or transit time variabilities. As shown by Ashford and co-workers, the *in vivo* disintegration of tablets coated with Eudragit®S as analyzed by γ-scintigraphy in volunteers was extremely variable in both time and position (11). In contrast, polymers susceptible to enzymatic degradation in the colon depend solely on enzyme activity which, in turn, depends on the microbial count in the colonic ecosystem. A potential restriction of this targeting approach would be acute diseased states (*e.g.*, diarrhea in colitis), in which the microbial population in the large intestine is reduced. However, Lendrum and co-workers indicated that sulfasalazine, a common 5-ASA prodrug, is active even when colonic bacterial count is reduced as measured by stool culturing in IBD patients (12).

Another obstacle for the proper functioning of colonic drug delivery systems may be bacterial adherence to the surface of solid dosage forms in the large bowel. In a previous study (13) we showed that the dissolution of pectin tablets in the presence of the pectinolytic bacteria *K. oxytoca* was retarded, probably because of the formation of bacterial biofilm on solid or gelling surfaces. While the role of surface bacterial adherence in colonic delivery has never been studied, the interference of this phenomenon in related areas is well documented. For example, bacterial adherence to nine contraceptive devices and biliary stents has been shown to intervene with their regular performance (14,15).

The overall objective of this study was to investigate how drug diffusion from typical and model solid drug platforms is affected by the presence of bacteria *in vitro* and *in vivo*. More specifically, the study goals were: (*a*) to study *in vitro* the role of surface bacterial adhesion on the diffusion of a model drug (salicylic acid) through ethyl cellulose films; (*b*) to investigate *in vivo* how pectin-coated solid dosage forms of insulin function, at stationary conditions, in the cecum of conscious rats, and (*c*) to study *in vitro* the effect of agitation on bacterial adherence to the surface of pectin films. Ethyl cellulose was selected as being a typical synthetic coating material for sustained release dosage forms, which, by definition, are required to function in the large bowel. Pectin was selected because of recent suggestions that it may be used as a natural, biodegradable solid carrier for colon-specific drug delivery purposes (16,8).

MATERIALS AND METHODS

All materials and reagents were purchased from Sigma, St. Louis, MO, unless otherwise mentioned in the text. All solvents were analytical or HPLC grade.

In Vitro Studies with Ethyl Cellulose and Pectin Films

Preparation of Ethyl Cellulose (EC) Films

Using vigorous stirring 6 g of EC (Ethocel N-100, Hercules, Delaware) and 4 g of polyethylene glycol 400 (Carbowax® Sentry®, Union Carbide, Danbury, CT) were dissolved in 100 ml of chloroform. The latter was used as a plasticizer. Films were prepared by casting on glass plates and drying (17).

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ABBREVIATIONS: AP, aprotinine; 5-ASA, 5-aminosalicylic acid; CFU, colony forming units; EC, ethyl cellulose; I, insulin; OD, optical density; SA, salicylic acid; SDC, sodium deoxycholate.

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The dried EC films were cut into circular pieces (areas of 12.55 cm^2) with a mean thickness of 36 ± 2.5 μ.

Permeability studies in bacterial rich media were performed in side-by-side diffusion cells in which the above EC films were mounted. The volume of each compartment was 25 ml. Sterile saline (25 ml) was placed in the donor compartment of the diffusion cell. The acceptor compartment of the diffusion cell was filled with 23 ml of nutrient broth and was inoculated with 2 ml of bacterial suspension (*Escherichia coli* isolated from fresh beagle dog feces; the species was identified by the Department of Clinical Microbiology, Hadassah Medical Center) in the same nutrient broth. The diffusion cells were incubated overnight at 37°C to allow bacterial adherence to the film. The optical density (OD) in the acceptor compartment was measured prior to the initiation of each diffusion study. The studies started only after an OD value of at least 0.26 was reached, which, in validation studies, was found to be equal to an average value of 1.3×10^8 CFU of *E. coli* as measured by viable count. The nutrient broth in the acceptor compartment was then replaced by 25 ml of sterile saline and the saline in the donor compartment replaced with 25 ml of sterile saline containing 150 mg% of salicylic acid (SA). The diffusion cells were shaken for the next 5 hours on a rotating bath (50 rpm) at 37°C, and samples (100 μl) were withdrawn at predetermined time intervals from a sampling port in the acceptor compartment for SA analysis. Aliquots of sterile saline were added to replenish each volume withdrawn.

Scanning electron micrographs (SEM) of surfaces from the EC films were made from the acceptor compartment aspects after each study. Specimens were sputtered with gold- palladium, and examined with a Philips SEM 505 at accelerating voltage of 20 R.V.

SA Analysis

To each withdrawn sample 0.9 ml of saline was added, vortexed and centrifuged. SA concentrations in the supernatant solution were determined spectrophotometrically (Uvikon 930, Kontron Instruments, Switzerland) at 298 nm.

Preparation of Pectin Films

Pectin (apple, degree of esterification: 57.9 ± 1.1%, BDH, Poole, U.K.) aqueous solution (4%w/v) was cast onto Petri dishes and allowed to dry at ambient temperature in an aseptic laminar flow hood (3 days). The films thus obtained, 37.2 ± 1.94 μ thick, were kept dry until adherence studies.

Bacterial Adherence on Pectin Films and the Effect of Agitation Rate

The above isolated fecal *E. coli* was grown on MP-7 medium (18) (no yeast extract) to allow for pectinolytic activity. The induced bacteria were transferred to sterile vials each containing 10 ml of nutrient broth. Adherence studies were performed on 10 × 10 mm pieces of pectin films which were secured to the vials' lids using a thin nylon thread. In separate studies the vials were agitated for 10 minutes at 50, 100, 150, and 200 rpm at 37°C. Each film was then taken out, rinsed gently with sterile saline and dissolved in 10 ml of sterile saline at 37°C to form a bacterial suspension. 100 μl of each bacterial suspension thus obtained was plated on appropriate solid media for viable counts.

In Vivo Studies with Solid Insulin Carriers Coated by Pectin Films

Insulin (I) solution (Actrapid® Novo, Denmark, 30 I.U.) was mixed with an aqueous solution of sodium deoxycholate (SDC), Sigma, 10 mg/ml) and 1000 I.U. of aprotinine (AP, Trasylol®, Bayer, AG Germany) (19). The mixture was soaked onto 5 × 25 mm filter paper strips (no. 4, Whatman, U.K.) and dried to form I-solid carriers. The I-solid carriers were then coated on both sides with the pre-cast pectin films and their edges "glued" carefully with 10 ml of sterile distilled water. These pectin-coated I-solid carriers were kept desiccated until further treatment ("Formulation 1"). In addition, two other formulations were prepared: filter paper strips soaked with I, SDC and AP *without pectin coat* ("Formulation 2") and placebo formulation made of filter paper strips soaked with SDC and AP, coated with pectin but *not containing* I ("Formulation 3"). The three formulations are summarized in Table I.

Rats Studies

Male Sabra rats (200–220 g) were fasted overnight and anesthetized (100–200 μl of sodium pentobarbital 6%w/v). In separate studies laparotomy was performed, the cecum was exposed and Formulations 1, 2 or 3 (see Table I for details) were implanted in the rat ceaca through a 1 cm incision. The cecum was then sutured (3/0 silk suture) gently to prevent bleeding, re-located and the abdominal cavity was sealed with 9 mm autoclips (Clay Adams, N.J.).

Two groups of studies were performed using the three formulations: with (control) or without antibiotic treatment. The antibiotic treatment included three days of oral administration of a mixture of neomycin sulphate (1 mg/kg rat body weight) and 0.8 mg of metronidazole, administered via an intragastric intubation. The purpose of the antibiotic treatment was to check how absence of bacteria affects drug release from the implanted formulations. The various studies are summarized in Table II.

Blood samples (30 μl) were withdrawn from the rats' tail veins every whole hour by capillary tube over a period of 9 hours for determination of blood glucose levels. In addition, the basal glucose level of each rat was determined prior to each experiment.

The research adhered to the *Principles of Laboratory Animal Care* (NIH publication #85-23, revised 1985).

Table I. The Three Formulations of I-Solid Carriers Used in the *In Vivo* Studies with Pectin Films

Formulation	I[a](I.U.)	SDC[b](mg/ml)	AP[c](I.U.)	Pectin coat
1	30	10	1000	yes
2	30	10	1000	no
3	no	10	1000	yes

Note: All carriers were 5 × 25 mm filter paper strips.
[a] I = insulin.
[b] SDC = sodium deoxycholate.
[c] AP = aprotinine.

Table II. A summary of the Study Design in Which Formulations 1–3 Were Implanted in the Rat Cecum

Study number	Formulation number	Antibiotic treatment
I	1	no
II	2	no
III	3	no
IV	1	yes
V	2	yes

Insulin Analysis

30 μl of blood were mixed with 270 μl of trichloroacetic acid and centrifuged for 15 minutes at 5000 rpm. 100 μl from the supernatant liquid were mixed with 2.5 ml of glucose oxidase reagent (Boehringer Mannheim, Germany). The intensity of the developed color was determined spectrophotometrically at 600 nm after 30 minutes.

A typical blood glucose level profile in the rat after cecal implantation of Formulation 2 after antibiotic treatment (Study V) is shown in Figure 1.

The results of blood glucose determination in the rats after cecal implantation of the various formulations with or without antibiotic treatment are expressed as (***a***) peak glucose levels (calculated in % of glucose basal levels), and (***b***) the time (in hours) to reach the peak glucose levels.

Statistical Analysis

A Kruskal-Wallis test was performed to check whether the various groups of rats and microbiological experiments were from different populations. A difference was considered to be statistically significant when the p value was less than 0.05. When the difference between the groups was validated, a Mann-Whitney U test was used to analyze the significance of the differences between the obtained data ($p < 0.05$).

RESULTS

After overnight incubation of EC films with *E. coli* a profound adherence of the bacteria ($10^8 - 4 \times 10^8$ CFU, as measured by viable count) to the surface of the films could be observed visually by SEM analysis (Figure 2). In control studies (no bacteria) the surface of the EC films was found to be free from bacteria. As can be seen from Figure 2 (top), most of the EC films surface was covered with bacterial biofilm, leaving some exposed film areas. Closer inspection of the films which was focused on the a free film area (Figure 2, bottom) reveals the pores in the EC films that, as a result of the *E. coli* adherence, were blocked by the bacterial biofilm which covered most of the surface area of the examined film.

The effect of the surface adherence of the *E. coli* on SA diffusion through the EC films is demonstrated in Figure 3. During five hours of diffusion studies which were performed immediately following incubation of the films with bacteria, the diffusion rate (as calculated from the slope of the straight diffusion profile of the control study and the slope of the approximation to straight line of the diffusion profile in the presence of bacteria) of SA throughout the films was 2.72-fold lower in the presence of bacteria compared with the diffusion rate in the control studies which were conducted without bacteria.

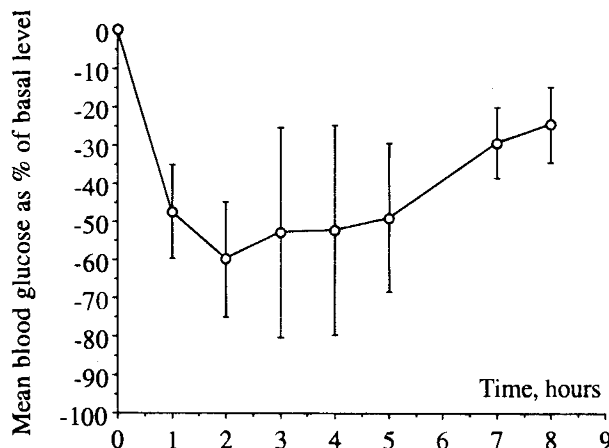
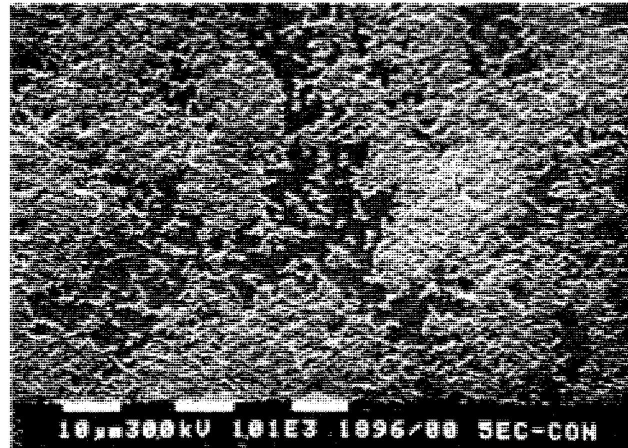


Fig. 1. A typical reduction in glucose blood levels in the rat after cecal implantation of filter paper strips soaked with I, SDC and AP after antibiotic treatment (Study V). Shown are mean values of eight studies ± S.D.



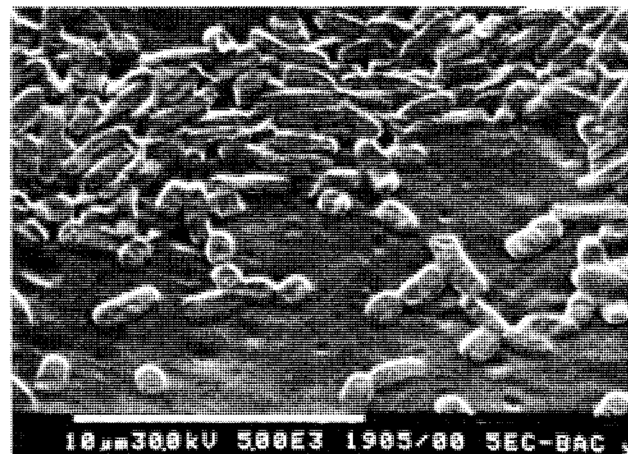


Fig. 2. A typical SEM micrograph (acceptor compartment aspect) of EC film taken at the end of an SA diffusion study when the acceptor compartment contained 10^7 CFU of fecal *E. coli* (A: magnification ×1000, B: magnification ×5000).

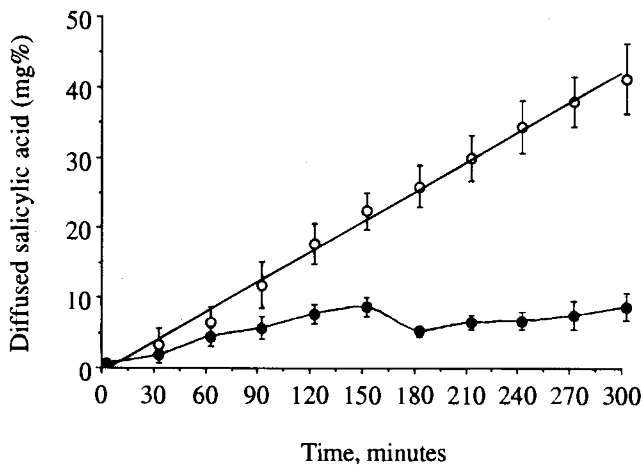


Fig. 3. Diffusion profiles of SA through EC films with (closed circles) or without (open circles) bacteria (fecal *E. coli*) in the acceptor compartment of side-by-side diffusion cells. Shown are the mean values ± S.D. of three measurements taken from different cells.

The results of the *in vivo* insulin release studies from the I-solid carriers after implantation into the rat cecum, as expressed by the rat blood glucose levels, are summarized in Figure 4 (see Tables I and II for detailed study protocols). It can be seen that while in Study II, in which the solid carriers were not coated with pectin films, the mean blood glucose levels dropped to 40.6 ± 21.6% of glucose basal levels within 2.4 ± 1.4 h., the glucose levels did not change in Study I. The positive blood glucose levels (12.0 ± 9.3% of glucose basal levels within 3.25 ± 1.9 h) in study I are typical of the stress situation of the rats after surgery and, in fact, should be considered as the real basal blood glucose levels. This is verified in Study III, in which Formulation 3 which did not contain insulin was implanted into the rat cecum and, similarly, yielded positive blood glucose levels (7.1 ± 16.3% of glucose basal levels within 6.6 ± 1.8 h). The last two studies were conducted after antibiotic treatment of the rats which reduced their GI bacterial population. In study IV, in which Formulation 1 was used, blood glucose levels dropped to 22.0 ± 4.7% of glucose basal levels within 7.4 ± 2.6 h. In study V, where Formulation 2 (no pectin coat) was used, blood glucose levels dropped to 50.9 ± 20.5% of glucose basal levels within 1.8 ± 0.9 h.

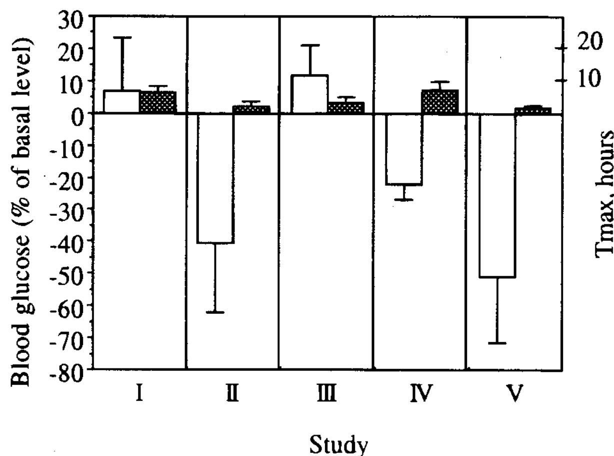


Fig. 4. Peak glucose levels [expressed in % of basal levels (empty columns)] and the time (in hours) to reach the peak glucose levels (cross-hatched columns) after implantation of the three insulin formulations in separate studies (I–V) (see Tables I and II for detailed protocols). Shown are the mean of six studies ± S.D.

The effect of agitation rate on bacterial adherence on pectin films is summarized in Figure 5. This figure clearly demonstrates that maximum bacterial adherence occurred at stationary conditions (RPM = 0), while at maximum speed (an extreme value of 200 RPM), almost no adherence occurred.

DISCUSSION

Colon drug delivery systems are designed to release their drug load in the large intestine, an organ which is characterized by low motility and high bacterial concentrations (10^{11}–10^{13} CFU/ml). Thus, colonic delivery systems, whether coated with a pH-dependent polymeric coat or designed to degrade enzymatically, are expected to function in an environment rich in bacteria. It should, therefore, be realized that the typical colonic microflora could interfere with the performance of solid dosage forms in terms of either drug release or drug delivery degradation kinetics.

In a previous *in vitro* study we found significant retardation in the dissolution rate of pectin matrices in the presence of the pectinolytic bacteria *Klebsiella oxytoca.* Under conditions of moderate agitation the bacteria rapidly adhered to the surface of pectin films (within 20 minutes). Using special *E. coli* strains containing plasmids with pectinolytic enzymes, it was found that the ability of the bacteria to adhere to the films did not correlate with their ability to degrade pectin (13). Similar observations were made by Imad and Gould (20) who studied the adherence of the amylolytic bacterium KB-1 to a variety of synthetic polymers that did (and therefore were considered as biodegradable films) or did not contain starch. They found that in some cases [*e.g.*, starch-poly(methylmethacrylate)], KB-1 bacteria was able to adhere to the films, but the film's starch was not degraded. They concluded that adhesion of KB-1 cells to the starch-containing films was not a prerequisite for enzymatic degradation of the films, and that they may be degraded by enzymes that are secreted by the bacteria or are released by cell lysis. Hoyle and co-workers (21) have already pointed out

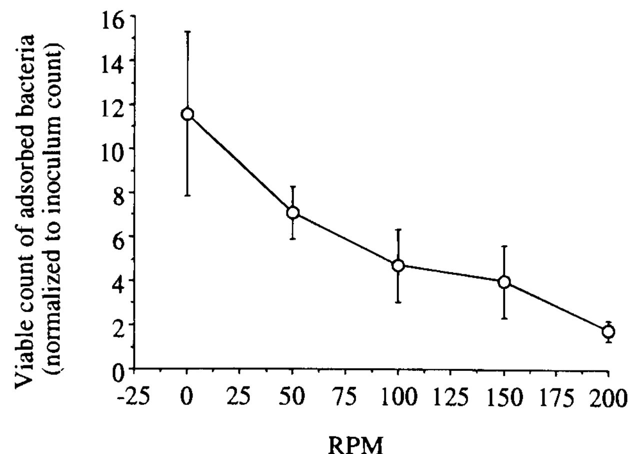


Fig. 5. The effect of agitation rate on *E. coli* adherence on pectin films. Shown are the mean of five studies ± S.D.

that the diffusion of the β-lactam antibiotic piperacilline across membranes colonized with *P. Aeruginosa* was retarded significantly as a result of the formation of a physical barrier by the bacteria in the form of biofilm.

In this study we investigated the role of bacterial adherence in drug diffusion through films representing two approaches in the design of colonic drug delivery: EC, which is used in products such as Pentasa®, (aimed at the delivery of 5-ASA to both small and large intestine), and pectin which was recently suggested as a possible biodegradable colonic drug carrier either as such (16) or as pectin's low water soluble modification calcium pectinate (8). The studies with the EC films (Figures 2–3) verified the observation of Hoyle and co-workers (21). SA, which was used as a drug marker, diffused much slower through films coated with a layer of *E. coli* in diffusion cell experiments. The films were incubated overnight with the bacteria, a typical period for colonic residence time of solid dosage forms. The implication of this observation is that solid carriers such as tablets or pellets may be exposed to bacterial adherence phenomena in the colon which, under certain circumstances (*e.g.*, very low agitation), may cause a severe delay in drug release. The second set of studies included *in vivo* examination of pectin films in the rat [*in vitro* studies were reported earlier (13)]. The diffusion of the drug marker insulin (I), as analyzed by monitoring the pharmacodynamic effect (reduction in blood glucose levels of the rat), from solid drug platforms coated with pectin films also containing AP and SDC, was significantly ($p < 0.05$) retarded in the rat cecum (Figure 4-I), compared with insulin diffusion from drug platforms that were not coated with pectin (Figure 4-II). It can be assumed that the reason for the differences was bacterial adherence to the surface of the solid drug carriers which interfered with I diffusion. This assumption is supported by the observation that after antibiotic treatment a significant ($p < 0.05$) reduction in blood glucose levels was observed (Figure 4-IV). A possible reason for the moderate reduction of blood glucose compared with the reduction observed after implantation of uncoated drug platforms (Figure 4-II and V) either with or without antibiotic treatment is the formation of pectin gel which acts as a diffusional barrier for the I. The results shown in Figure 4-V also indicate that the antibiotic treatment did not alter the absorption of I in the rat cecum. It was expected that bacterial adherence would interfere also with I diffusion from those solid formulations which were not coated with pectin. However, the results obtained show (Figure 4-II and V) that if cell adherence occurred, it was to a much lesser extent than the adherence to pectin-coated dosage forms. It is speculated that the gelling property of the pectin films was the reason for the improved bacterial adherence to pectin surfaces and biofilm formation as compared to bacterial adherence to cellulose (paper) surfaces.

The results summarized in Figure 4 suggest that pectin colonic delivery systems may not function properly (specific delivery of drugs to the colon) unless polymer layers, together with the adhered bacteria, are stripped off. Indeed, Figure 5 demonstrates *in vitro* that the number of cells adhering to pectin surfaces depends on agitation rate. The higher the agitation rate, the lower the bacterial count measured on the pectin films. The highest number of bacteria was measured under totally stationary conditions (agitation rate = 0). It is concluded that after arrival in the colon, biodegradable polymers such as pectin will function optimally as colonic delivery systems only if enzymatic erosion is accompanied by physical erosion (friction, abrasion). Under stationary conditions bacterial adherence to the surface of the dosage form may interfere with both drug diffusion (as shown in the case of EC films) and film erosion, leading to malfunction of the carrier.

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