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BINDING TO AND RETENTION BY MUCOSAL CELLS OF THE *TAMARINDUS INDICA* SEED POLYSACCHARIDE: VISUAL EVALUATION BY MEANS OF INORGANIC AND ORGANIC MARKERS

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ABSTRACT

The aim of this study was to investigate the possibility of using inorganic and organic markers to visualize the ability of the transparent polysaccharide (TSP) polymer isolated from the endosperm of the seed kernel of *Tamarindus indica,* a tree that mainly grows in India and South-East Asia, to bind to human mucosal cells. A layer of human buccal cells was prepared on slides and overlaid by 0.2 ml of 0.6, 0.3, 0.15 and 0.075 % TSP solutions in phosphate buffer and then colloidal carbon black particles were deposited on the slides. The unbound colloidal carbon black particles were cleared by thoroughly washing the slides. The slides were then examined by means of Nomarski interference contrast microscopy in order to visualize the degree of surface retention of the black particles by the buccal cells. The same procedure was followed using *Escherichia coli* as organic markers. The clearly visible binding of black carbon particles to the cells treated with polymer revealed the presence of a thin layer of TSP covering the cells (untreated cells had no black carbon particles binding). The presence of the TSP has also been confirmed by a significant reduction in bacterial adhesiveness. Both markers made it possible to visualize the binding of the thin transparent layer of TSP and its retention, which was proportional to the degree of dilution. Using *Escherichia coli* it has been observed the possibility of counteracting the lock-and-key mechanism of micro-organism adhesion using the bioadhesive properties of this polymer to prevent possible contact between microorganism adhesins and complementary receptors.

INTRODUCTION: Polysaccharides are polymers consisting of repeating carbohydrate units. They may be linear or branched and, when soluble in water, they swell and form highly viscous solutions. This capacity is used in various technological and industrial fields (particularly pharmaceutical technology and pharmacology) because polymeric high-viscosity solutions frequently cover nasal, buccal, vaginal and gastrointestinal mucosal tissue with a thin transparent film in order to protect it and control drug absorption or prolong local drug delivery which can be advantageous in the treatment of local conditions. Adhesion is defined as the state in which two surfaces are held together by interfacial forces [1, 2], with the term bioadhesion being used if one or both of the adherents are biological.

A bioadhesive can therefore be defined as a biocompatible substance that is capable of interacting with biological materials and being retained by them or holding them together for an extended period of time [1, 3].

The ability of bioadhesives to bind to, and be retained by mucosal surfaces also means that they can prevent the surfaces from coming into direct contact with particulate matter in the environment, and is used for instance to prevent airway mucosa from coming into contact with airborne micro-organisms, such as viruses, bacteria, fungi, pollens, or other chemical or physical pollutants present in the air and flowing through the nose and mouth during inspiration.

The aim of this study was to investigate the possibility of visualizing the ability of the tamarind seed polysaccharide to bind to mucosal surfaces, using human buccal cells as a model for mucosal adhesion [4-6].

Inorganic and organic visual markers were used to facilitate visual detection rather than other methods that more particularly measure tensile adhesive strength (peak detachment force).

The novelty of this visual procedure is the use of a suspension of colloidal carbon black in water (inorganic marker), which does not stain human buccal cells but is able to deposit itself on the polysaccharide and thus reveals its adhesion to the cells. A further novelty is the use of the cells of the bacterium *Escherichia coli* (organic marker) and the interference of the lock-and-key mechanism of adhesion induced by the polymer to mucosal cells that can be clearly seen by means of microscopy.

The Tamarind seed polysaccharide (TSP) was isolated from the endosperm of the seed kernel of *Tamarindus indica,* a tree that mainly grows in India and South-East Asia. TSP belongs to the xyloglucan family [7] and is a high-molecular-weight, non-ionic, neutral, branched polysaccharide, whose structural unit consists of a cellulose-like backbone (β-(1→4)-D-glucose with a α-(1→6)-D-xylose linked to the glucose residue.

Some of the xylose residues (50%) are substituted by β-(1→2) galactose residues in position C-2 [8]. These chemical residues are similar to that of mucin MUC-1 and episialin [9]. Native TSP has a strong tendency to aggregate when dispersed in aqueous solvents [7]. These aggregates consist of lateral assemblies of single polysaccharide strands [7].

MATERIALS AND METHODS:

Collection of human buccal cells : Healthy non-smoking volunteers were required not to eat or drink for at least 60 minutes before the mucosa of each cheek was gently scraped with a sterile plastic spatula, which was subsequently twirled in 2 ml of phosphate-buffered saline (PBS) (0.02 M phosphate and 0.15 M NaCl, pH 7.3) to dislodge the buccal cells. The cell suspension obtained by pooling the cells from three or four subjects was washed three times to free it from debris and non-adherent bacteria by means of low-speed centrifugation (260g, 10 min, 21°C). PBS was added to the washed epithelial suspension in order to reach a concentration of $3x10^5$ cells/ml, as determined by direct microscopic counts in a Bürker chamber (Passoni, Milan, Italy).

Inspection of binding and retention properties using an Inorganic Marker: The high viscosity of the TSP solutions prevented us from using the conventional method of putting the preparation under study in contact with the buccal cells suspension by means of incubation for a period of time and then separating the cell pellet from the supernatant by means of centrifugation.

In a control test, the suspension of human cells was filtered on a cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany) (pores 8 µm, dia 25 mm), pressed onto a microscope slide to create a layer of buccal cells to which 0.2 ml of a suspension of colloidal carbon black in water (commercial Pelikan indian ink) was added as an inorganic marker and incubated at 37°C for five minutes. After this time, the unbound colloidal carbon black particles were cleared by thoroughly washing the slide by dropping 10 ml of PBS released by gravity from a graduated pipette at a distance of 20 cm. This washing procedure was repeated three times. The slide was then examined by means of Nomarski interference contrast microscopy in order to visualize the normal degree of surface retention of the black particles by the buccal cells [10].

To visualize the thin transparent film of TSP, testifying its ability to bind to and be retained by the transparent cells, a layer of buccal cells was prepared on a slide as previously described, and overlaid by 0.2 ml of 0.6, 0.3, 0.15 and 0.075 % TSP solutions in phosphate buffer.

After incubation at 37 °C for five minutes, the slide was thoroughly washed by dropping 10 ml of PBS released by gravity from a graduated pipette at a distance of 20 cm. This washing procedure was repeated three times, after which colloidal carbon black particles were deposited on the slide as previously described and incubated at 37°C for five minutes. The unbound colloidal carbon black particles were cleared by thoroughly washing the slide three times as previously described. The slide was then examined by means of Nomarski interference contrast microscopy in order to visualize the degree of surface retention of the black particles by the buccal cells.

Inspection of binding and retention properties using bacteria as Organic Markers: *Escherichia coli* ATCC 25922 and two strains of *E. coli* isolated from human urinary infections were used to test the binding of the TSP and its retention by buccal cells. In this case, the bacteria were used as organic markers to investigate the interference of TSP with the bacterial lock-and-key (adhesin-receptor) mechanism. Suspensions of each organism were prepared from overnight cultures in tryptic soy broth (Sigma, Milan, Italy) under static conditions at 37°C.

The organisms were harvested, washed three times in PBS, and adjusted to $3x10^8$ organisms/ml, as determined by direct microscopic counts in a Petroff-Hausser chamber (Thomas Scientific, Swedesboro, NJ, USA). In a control test, the suspension of human cells was filtered on a cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany) (pores 8 µm, dia 25 mm), pressed onto a microscope slide to create a layer of buccal cells to which 0.2 ml of a suspension of bacterial suspension ($3x10^8$ organisms/ml) was added and incubated at 37°C for 60 minutes.

After this time, the unbound bacteria were cleared by dropping 10 ml of PBS released by gravity from a graduated pipette at a distance of 20 cm. This washing procedure was repeated three times, and the slide was then stained with Gram stain, and examined by means of Nomarski interference contrast microscopy. As differences in bacterial strains and cell surface characteristics (different donors) lead to variations in the number of bacteria attaching to individual buccal cells, bacterial adhesion was determined by counting the total number of bacteria adhering to 50 randomly chosen cells in each sample [11]. Buccal cell suspensions not incubated with bacteria were always included in order to establish the number of bacteria already attached at the time of cell collection (natural adhesion). In order to visualize whether the TSP covered the buccal cells, a layer of buccal cells was prepared on a slide as previously described, and overlaid by 0.2 ml of 0.6, 0.3, 0.15 and 0.075 % TSP solutions in phosphate buffer.

After incubation at 37 °C for five minutes, the slide was thoroughly washed by dropping 10 ml of PBS released by gravity from a graduated pipette at a distance of 20 cm. This washing procedure was repeated three times, and the bacteria were then deposited on the slide as previously described and incubated at 37°C for 60 minutes. After this time, the unbound bacteria were cleared by thoroughly washing the slide as previously described. The slide was then stained with Gram stain and examined by means of Nomarski interference contrast microscopy.

The total number of bacteria was determined as previously described in order to measure the degree of surface retention by the buccal cells.

Scanning Electron Microscopy: The interference of the TSP with bacterial adhesion was also observed using scanning electron microscopy (SEM). The samples were prepared under the different test conditions described above, put on round coverslips and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1, for six hours. After dehydration, the coverslips were coated with 200 Å of gold and observed through a scanning electron microscope.

Data Analysis: The differences in the mean values ($\pm$ SEM) of bacterial adhesion of three separate repetitions for each strain and each test dilution were compared using the Student's t test and one-way ANOVA followed by multiple paired comparisons using Dunnett's test. The differences were considered statistically significant when the p value was ≤ 0.05.

RESULTS AND DISCUSSION: As the aim of this study was to highlight the mucosal cell binding and retention capacity of TSP, the description of the results will be mainly limited to the images, and only summary data will be given concerning the bacterial adhesion test.

Figure 1A is a micrograph showing the density of the black particles, and **Figure 1B** shows control buccal cells after incubation with the carbon black particles. The fact that very few or no particles were deposited on the surface indicates that they were not spontaneously retained by the cells. When the challenge was repeated after the cells had been incubated with 0.6 % TSP, the background was free but patches of particles were clearly visible on the cell surfaces, thus revealing the retention capacity of the thin TSP layer (**Figure 1C**). The same was observed after incubation with the other TSP concentrations, although the retention was proportionally less (**Figures 1D-F**). The possibility of the simple superimposition of the particles on the cells was easily ruled out because under the microscope if the marker simply overlaps the cells, tapping the slide will separate cell and the marker; this does not occur when the two are "glued" together as in our findings.

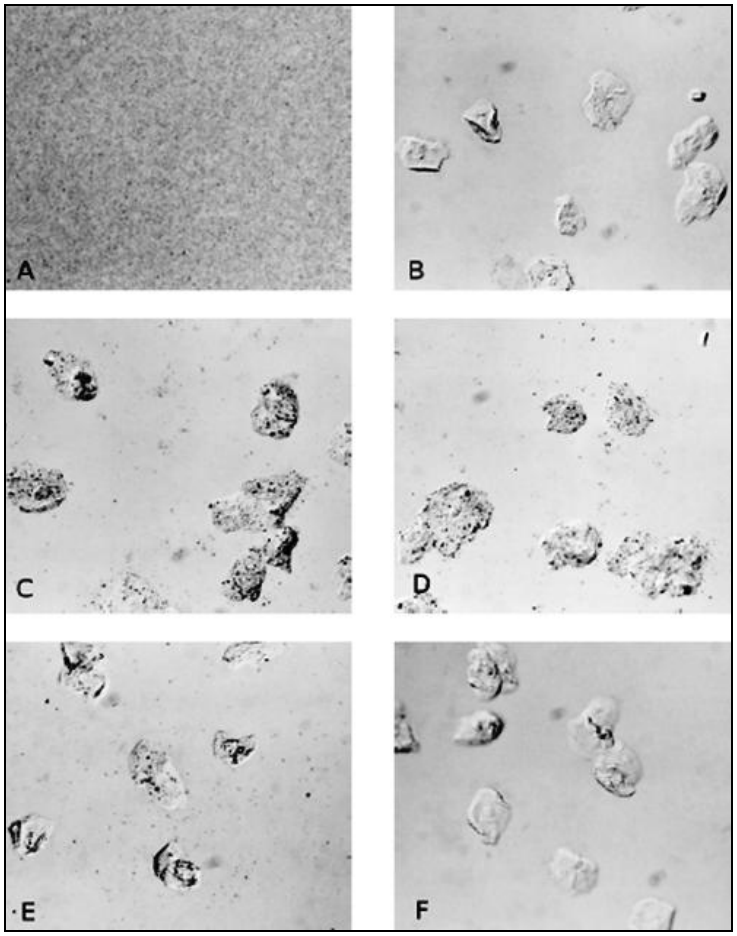

FIGURE 1: LIGHT MICROSCOPY SHOWING THE DEPOSITION OF CARBON BLACK PARTICLES ON BUCCAL CELLS BEFORE AND AFTER INCUBATION WITH TSP.

A) Homogeneous distribution of carbon black particles in the absence of buccal cells. B) Control cells after challenge with carbon black particles (without TSP), showing no deposition. C) Diffuse particle retention after challenge with TSP at 0.6%. D-F) Particle retention after challenge with TSP at 0.3, 0.15 and 0.075 % (x250).

The second part of the study, in which bacteria were used as organic markers, was also started with 0.6 % TSP (**Figure 2**). There was a statistically significant reduction in bacterial adhesion after the cells had been incubated with this and other TSP concentrations down to 1.5 % (**Table 1**).

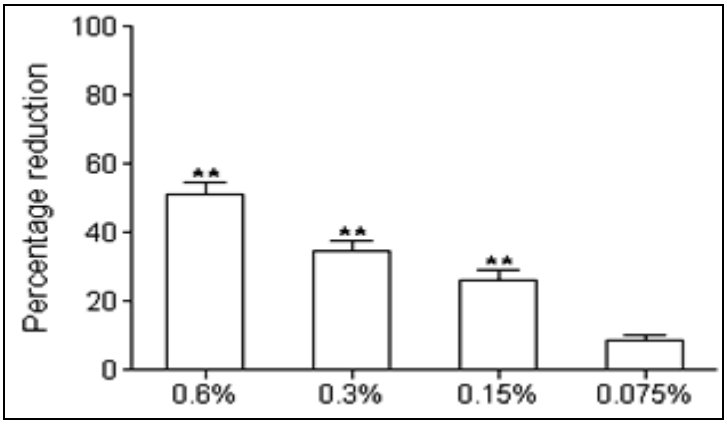

FIGURE 2: EFFECTS OF TSP ON THE ADHESION OF *E. COLI* TO HUMAN BUCCAL CELLS

(X axis: TSP concentrations; Y axis: percentage of reduction of bacterial adhesion) (** = p≤0.01).

TABLE 1: EFFECTS OF TPS ON THE ADHESIVENESS OF *E. COLI* TO HUMAN BUCCAL CELLS

Strain (*E. Coli*)	Control	TSP			
		0.6%	0.3%	0.15%	0.075%
ATCC 25922	2680	1074	2014	2248	2519
	2730	1144	1478	1750	2556
	2840	1166	1856	2026	2498
Clinical isolate	1990	1312	1592	1690	2069
	2100	1280	1512	1700	1932
	1810	1048	1248	1376	1592
Clinical isolate	2180	959	1264	1482	1962
	2020	1010	1252	1454	1838
	2350	940	1292	1504	1996
Mean ± SEM	2300.00 ±123.18	1103.67** ±44.17	1500.89** ±93.09	1692.22** ±95.75	2106.30 ±113.48

**= p≤0.01 vs control

This can be attributed to the retained layer of the polymer covering the receptor molecules on the cell surface, which prevents the bacterial adhesins from expressing their lock-and-key mechanisms, reduces bacterial adhesion and reveals the presence of the polymer layer. The SEM observations confirmed these findings (**Figure 3**).

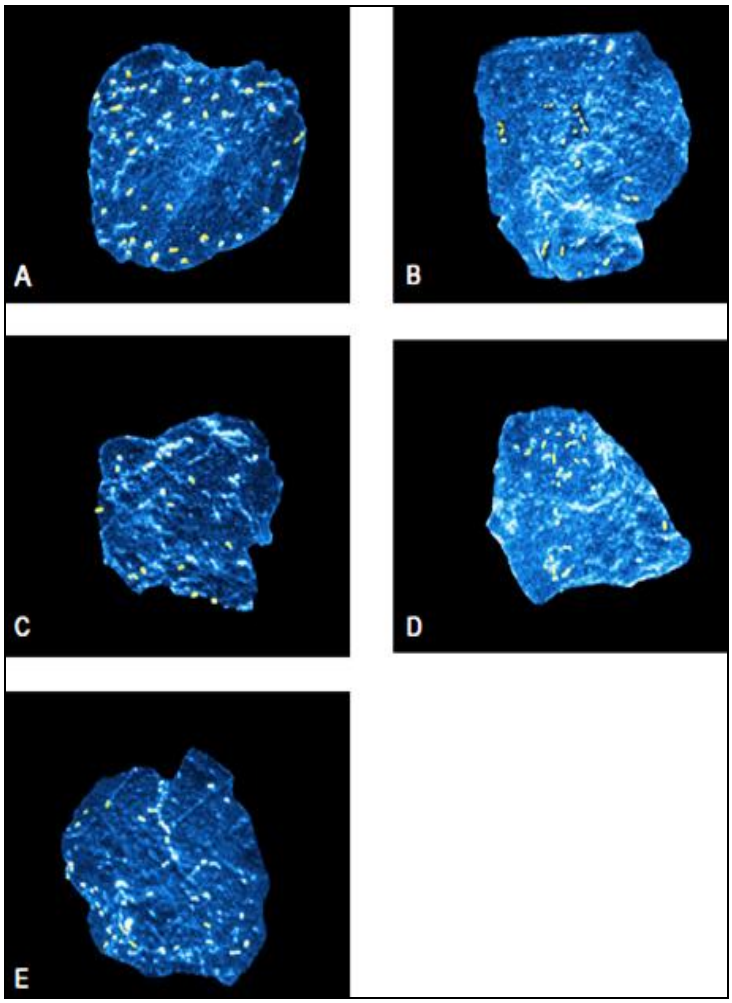

FIGURE 3: SCANNING ELECTRON MICROGRAPHS SHOWING THE EFFECTS OF TSP ON *E.COLI* ADHESION TO HUMAN BUCCAL CELLS.
A) *E.coli* adhesion without TSP incubation. B-E) After incubation with TSP at 0.6, 0.3, 0.15 and 0.075 % (x 1600).

A number of methods have been used to screen the bioadhesion of polymers. Classic tensile and shear testing methods mainly investigate physisorption and chemisorption properties, and can sometimes lead to contradictory results because of the different types of forces involved.

Direct visual approaches, which are quicker and more suitable for revealing surface cell coverage, have been previously used to evaluate the adhesion and retention of polymers following aqueous dispersion [12], the activity of bioadhesive preparations in the oral cavity [6], and the ability of a commercial preparation to cover human buccal cells [10].

Similar methods using colloidal gold staining [13] or radiolabelled markers have also been explored, such as gamma scintigraphy [14, 15] and magnetic resonance imaging [16, 17].

Bacterial markers have been previously used to investigate the effect of hydroxypropylmethylcellulose [10, 18] and poloxamer [19], and atomic force microscopy (AFM) has also recently been used by Patel *et al.*,[20] to investigate the covering ability of hydroxypropylmethyl cellulose. They observed that untreated cells have surfaces covered by small “crater-like” pits and indentations, whereas treated cells appear to have lost the craters and indentations, thus indicating the presence of a covering effect.

A polysaccharide polymer such as TSP that can be dispersed on and retained by cell surfaces is useful because it covers and protects small surface lesions of the mucosa. It also offers the potential benefit of preventing the adhesion of bacteria, fungi and viruses approaching the mucosal environment by preventing their adhesins from reaching the complementary receptors located on the surface of buccal cells.

Our examinations using inorganic or organic markers revealed that the binding of buccal cells may vary. This was probably because the observed cells were generally separated from each other, creating a discontinuous binding surface; the distribution of the polymer should be more homogeneous in a continuous mucosal layer such as that existing *in vivo*.

Our visual approach showed that this TSP does not simply cover the cell surface, but is also retained in a manner that is proportional to its dilution.

The interactions between polymeric materials and mucosal tissue surfaces are complex and have not yet been fully elucidated. However the most widely accepted theories include the molecular adsorption theory, which says that adhesion is due to the combined result of secondary forces such as Van der Waals dispersion forces, hydrogen binding and related forces [21, 22]; the wetting theory, which states that the intimate contact between the adherents depends on their wetting equilibrium and interface tensions [23]; the electronic theory, based on the fact that electron transport across the interface induces the formation of a double layer of electric charge at the bioadhesive interface [24]; the diffusion/interpenetration theory, which says that, because of the concentration gradient, bioadhesive polymer chains penetrate at rates that depend on the diffusion coefficient of a

macromolecule through a cross-linked network and the chemical potential gradient [25, 26]; and the fracture theory, which relates the difficulty of separating two surfaces (after adhesion) to the strength of adhesive bond, which is equivalent to tensile fracture strength [25].

Given the variety of bioadhesion phenomena and bioadhesives, the final result is probably attributable to combinations of the different mechanisms, which variously contribute to the formation of adequately strong interactions between bioadhesives and biological surfaces.

The properties of polymeric solutions are closely connected to the final architecture of the solute. Aqueous solutions of TSP, which has a branched chain (mucin-like) structure may be described by the model of bundle-shaped (multistranded) lateral aggregates of single polymeric chains, whereas the aggregates seem to be more spherical at higher degrees of aggregation [27]. As it has been observed that in solutions mucins adopt a random-coil conformation occupying a time-averaged spheroidal domain [28], the similarity between the shape of TSP aggregates and that of mucins suggested a mucomimetic property of TSP.

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