Saliva in health and disease, chemical biology of

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Advanced Article

Article Contents

Saliva is a bodily fluid secreted by three pairs of major salivary glands (parotid submandibular and sublingual) and by many of minor salivary glands. Saliva is supplemented with several constituents that originate from blood serum, from intact or destroyed mucosal and immune cells, and from intact or destroyed oral microorganisms that result in a complex mixture of a variety of molecules. Saliva plays an important role in acquired pellicle formation on tooth surfaces, crystal growth homeostasis, bacterial adhesion, plaque formation, and—because of its lubricating effect—in maintaining mucosal integrity of the oral and upper gastrointestinal mucosal surfaces. It also plays an important role in physico-chemical defense, antimicrobial defense, and wound healing. Many saliva constituents including proteins, carbohydrates, lipids, and ions interact under fine regulation to fulfill these important tasks. Local and systemic disorders may disturb and interrupt these complex balanced functions, which can lead to mucosal and tooth damages. In other cases, systemic disorders induce salivary changes without any significant local effects. Many such changes are of high diagnostic interest because they can be rather specific to the causing conditions and can be used for screening and early diagnosis of several local and systemic disorders.

saliva, *tooth surface interaction*, *defense function*, *diagnostics*, *analysis*

Saliva is a major determinant of the oral environment and serves as an easily available diagnostic tool of systemic conditions. Consequently, more intense saliva research can be observed in recent decades, which leads to a high amount of scientific data presented by numerous engaged researchers of this far-reaching field. With the rapid growth of knowledge, a need exists to summarize the obtained data of this interesting field. This article provides a brief introduction to the most important aspects of the chemical biology of saliva.

Origin of Salivary Chemical Components

Salivary components can originate from several sources, which leads to a rather complex collection of molecules. To understand the importance and meaning of a certain component, it is crucial to know the origin and the excretion mechanism of the component.

Constituents of salivary gland origin

Constituents of salivary gland origin (i.e., water, ions, proteins, carbohydrates, lipids) can be released from major salivary glands such as parotid, submandibular, and sublingual glands, and from minor salivary glands of the labial, buccal, lingual, palatoglossal, and palatal mucosa (1).

Based on the features of secreted primary saliva, secretory endpieces (acini) of salivary glands can be characterized as serous, seromucous, or mucous. The acini of parotid gland are mainly serous and seromucous, those of submandibular gland are mainly seromucous, and those of sublingual gland are mainly mucous. The acini of the minor salivary glands have various features that depend on their location (1).

Primary saliva is modified in the intercalated, striated, and excretory (collecting) ducts that lead from the acini to the mouth. Water and electrolyte transport into the saliva is believed to occur in the intercalated ducts. Striated ducts are responsible for electrolyte transport such as secretion of potassium and

reabsorption of sodium ions. A transport of proteins like IgA, lysozyme, and kallikrein [and may be Hsp70 (2)] probably exists in the striated duct as well. An electrolyte transport in the excretory (collecting) ducts is also suspected (1).

Constituents of other origin

Each blood constituent may enter the oral cavity via intraoral bleeding. Serum exudates also reach saliva either from the gingival crevicular fluid or through the oral mucosa (mucosal transudate) and from the salivary glands via transcellular diffusion and ultrafiltration (via tight junctions) (3). Oral microbes and their fermentation products, enzymes, RNA, DNA, and structural elements are also usual constituents of the saliva (4). Fragments from the keratinized mucosal surfaces, mucosal cells with intact cell organelles from nonkeratinized surfaces, and some immune cells are also present. Cellular fragments, cytoplasmic products, enzymes, structural elements, membranes, RNA, and DNA of these cells are also usual salivary constituents. Certain amount of expectorated bronchial and nasal secretum, constituents of foods, administered drugs, smoke (from smoking), toothpastes, mouth rinses, and molecules released from dentures can also be found (2, 4).

Molecular Participants of Saliva Chemical Biology

According to the above, many constituents exist in human saliva. Although all may deserve scientific interest, only some have the focus of our attention. The most important constitutes are summarized below.

Inorganic components

Water is the most abundant constituent of saliva (∼94 %). The pH value of resting whole saliva is slightly acidic, which varies between pH 5.75 and 7.05, and it increases with increasing flow rate up to pH 8. Besides flow rate, the pH also depends on the concentration of salivary proteins, bicarbonate (HCO_3^-) and phosphate (PO_4^{3-}) ions that have considerable buffering capacity. Bicarbonate concentration is ∼5–10 mM/L in resting conditions, and it may increase up to 40–60 mM/L with stimulation, whereas phosphate concentration is ∼4-5 mM/L in saliva rather independently from the flow rate (5). Besides bicarbonate and phosphate, a significant amount of other ions are present to maintain the slightly hypotonic osmolarity of saliva. The most important ions are sodium (1–5 mM/L resting; 100 mM/L stimulated), chloride (5 mM/L resting; 70 mM/L stimulated), potassium (15 mM/L resting; 30–40 mM/L stimulated), and calcium (1.0 mM/L resting; 3–4 mM/L stimulated). Many other ions such as ammonium (NH_4^+), bromide, copper, fluoride, iodide, lithium, magnesium, nitrate (NO_3^-), perchlorate (ClO_4^-), thiocyanate (SCN^-), and so on can be found in the whole saliva in lower concentrations (4, 5). (Data are summarized in Table 1.)

Table 1 The most important inorganic components of saliva

Component	Resting saliva	Stimulated saliva
Water	∼94%	∼94%
pH	5.75–7.05	up to 8.0
Bicarbonate	5–10 mM/L	up to 40–60 mM/L
Phosphate	4–5 mM/L	4–5 mM/L
Sodium	1–5 mM/L	up to 100 mM/L
Chloride	5 mM/L	up to 70 mM/L
Potassium	15 mM/L	30–40 mM/L
Calcium	1 mM/L	3–4 mM/L

Proteins

Human whole saliva has a protein content of about 0.5 to 3 mg/mL, and parotid saliva has a protein content of about 0.4 to 4 mg/mL, whereas submandiblar and sublingual saliva of about 0.6 to 1.5 mg/mL. The protein concentration is rather stable and independent from the flow rate (5). Besides maintaining osmolarity and buffer capacity, salivary proteins are also involved in several specific functions. The number of distinct salivary proteins is roughly between 100 and 140 (6, 7), from which roughly 30–40 % are produced by the salivary glands, whereas other proteins are originate from serum, from mucosal and/or immune cells, or from microorganisms (6). The most important proteins of glandular origin are alpha-amylase, glycoproteins with blood-group substances, cystatins, epidermal growth factor (EGF), gustin, histatins (HRPs), lactoferrine, lysozyme, mucins (MUC5B, MUC7; older terms: MG1, MG2), salivary peroxidase, proline-rich proteins (PRPs) and statherin. The most important serum derived proteins are albumin, $alpha_1$-antitrypsin, blood-clotting factors (VIII; IXa; XI) and members of the fibrinolytic system (proactivators, traces of plasminogen activator). Most important proteins that originate from immune cells are myeloperoxidase, calprotectin (Ca^{2+} binding L1 leukocyte protein), cathepsin G, defensins, elastase, immunoglobulins (90% to 98% sIgA, 1% to 10% IgG, a few IgM, IgD, IgE). Finally, the most important protein constituents of microbial- unknown- or mixed origin are $alpha_2$-macroglobulin, cystein peptidases, DNases, RNases, kallikrein, secretory leukocyte protease inhibitor (SLPI), fibronectin, molecular chaperone (Hsp70), and streptococcal inhibitor. (Data are summarized in Table 2.)

Carbohydrates

A significant amount of protein bound carbohydrates exists in the saliva. Some proteins may contain carbohydrates up to 80% of the molecule (i.e., MUC5B mucins) but 10–40% of carbohydrate moiety is rather usual in the case of any glycoproteins (4). The most important constituents are aminosugars, galactose, mannose, and sialic acid (N-Acetylneuraminic acid). Carbohydrate chains of mucins contain predominantly sialic acid and sulphate residues, although chains with blood group antigen properties contain about equal amounts of 6-deoxygalactose, glucosamin, galactosamin, and galactose (5). Other usual constituents of the carbohydrate chains of salivary glycoproteins

Table 2 Origin of the most important salivary proteins

Glandular origin	Serum derived	Immune cell	Bacterial, unknown, mixed
Alpha-amylase	Albumin	Myeloperoxidase	$Alpha_2$-macroglobulin
Blood-group proteins	$Alpha_1$-antitrypsin	Calprotectin	Cystein peptidases
Cystatins	Blood-clotting factors	Cathepsin G	DNases
EGF	Fybrinolytic system	Defensins	RNases
Gustin		Elastase	Kallikrein
Histatins		Immunoglobulins	Protease inhibitor SLPI
Lactoferrine			Fibronectin
Lysozyme			Salivary chaperon Hsp70
Mucins			Streptococcal inhibitor
Salivary peroxidase			
Proline-rich proteins			
Statherin			

are also N-acetylgalactosamine, N-acetylglucosamin, and glucuronic acid (5). The total amount of protein-bound carbohydrates in the saliva is 300–400 μg/mL, of which the amount of sialic acid is usually about 50 μg/mL [up to 100 μg/mL (8)]. The most important function of protein-bound carbohydrates is the increase of viscoelasticity of the saliva, prevention of proteolysis through holding proteases at a distance, prevention of acid precipitation in case of several glycoporteins (i.e., acid soluble blood group antigens, mucins), and labeling/antigen function.

Lipids

Whole saliva contains about 10–100-μg/mL lipids (9). The most frequent lipids in the saliva are glycolipids (i.e., neutral and sulphated glyceroglucolipids), neutral lipids (i.e., free fatty acids, cholesteryl ester, triglycerides, and cholesterol), and a somewhat lower portion of phospholipids (i.e., phosphatidylethanolamine, phosphatidylcholine, sphingomielin, and phosphatidylserine) (10). Salivary lipids are mostly of glandular origin, but some (such as cholesterol and may be some fatty acids) are believed to diffuse directly from serum (11). Lipids originate from several membranes such as secretory vesicles, microsomes, lipid rafts, and other plasma and intracellular membrane fragments of lysed cells and bacteria, although the lower percentage of phospholipids indicate that the salivary lipids are not primarily of membrane origin (9). A large portion of salivary lipids is associated with proteins, especially to high molecular weight glycoproteins (i.e., mucins) and to PRPs (12). Salivary lipids may play a role in the acquired pellicle, dental plaque, calculus, sialolith, and caries formation.

Other molecules

As mentioned, many other molecules exist in the saliva, including nucleic acids (RNA, DNA), several hormones, growth factors and neurotransmitters, amino acids and their derivatives, urea, lactate, citrate, vitamins, creatinine, prostaglandins, several drugs, and chemical constituents of foods, cosmetics, tooth pastes, dental materials, and several other molecules originated from body and environment.

Chemical Biology of Saliva in Health and Disease

Saliva constituents play a role in several oral processes, and they perform important defense functions in the oral cavity. Moreover, saliva may be used for diagnostic purposes. The most important knowledge related to these fields will be summarized briefly in the following section.

Saliva and bacterial adhesion

The basis of bacterial adhesion is given by the acquired pellicle formation on tooth surfaces. This pellicle is a thin (∼0.5–1 μm) layer of several salivary proteins with calciumhydroxide-binding properties. The most important such proteins are salivary amylase, cystatins (S, SA, and SN type), histatin (HRP1), mucine (MG1), acidic PRPs, statherin, and immunoglobulins (sIgA) (4, 7).

The surface binding of these proteins occurs mostly through ionic interaction of positively charged groups of the proteins' polypeptide chain and the negatively charged tooth surface (globular proteins wring on the tooth surface during binding). Although tooth surface is negatively charged, in some cases negatively charged protein regions are responsible for binding (i.e., N-terminal region of PRPs). Calcium bridging (Ca^{2+} complex formation) between the negative charges may be a mechanism of such binding (13).

First, bacterial adhesion (usually gram-positive cocci and filamentous bacteria) occurs primarily through a Ca^{2+} complex formation between carboxyl (COO^-) and phosphate (HPO_3^-) groups of bacterial surface and acquired pellicle, although van der Waals' forces and repulsive electrostatic forces are also present. Some specific bacterial surface proteins also serve as adhesins for specific receptors on acquired pellicle. Pellicle-integrated immunoglobulins also bind bacteria specifically.

Saliva and bacterial biofilm (plaque) formation

After the adhesion of the first layer of bacteria (i.e., *Streptococcus mutans*) the bacterial accumulation process is initiated by the activity of secreted extracellular glucosyltransferases (GTFs) of *S. mutans*. In the presence of sucrose, GTFs synthesize several forms of high-molecular-weight branched extracellular glucans (i.e., dextrane), which leads to sticky polysaccharide products resulted in stronger binding to the surface and facilitation of adhesion of more bacteria via glucan-binding proteins of the bacterial surface. Food rests in the saliva may serve as store of sucrose during this process (4).

Saliva and crystal growth homeostasis

In general, saliva (as well as plaque fluid) is supersaturated with respect to calcium-phosphate salts, and they prevent tendency to dissolve mineral crystals of teeth. Moreover, precipitation of calcium-phosphate salts that include hydroxyapatite may also occur (demineralization) in early lesions of tooth surfaces injured by acidic bacterial products (i.e., lactic acid). Salivary fluoride facilitates calcium-phosphate precipitation, and such crystals (i.e., fluorapatite) show lower acid solubility properties that lead to an increased caries preventive effect. The increase of pH (i.e., buffer capacity and pH of saliva, as well as ureolysis in dental plaque) also facilitates crystal precipitation and demineralization (4, 13).

Similarly, supersaturation of saliva with respect to calcium phosphate salts is the driving force of calculus (i.e., mineralized dental plaque) and sialolith (i.e., salivary duct ”stones”) formation. In these cases, negatively charged phospholipids play a crucial role: Ca^{2+} ions bind to the negative charges of such lipids, and inorganic phosphate associates with the bound calcium tha forms a Ca-phosphate–phospholipid complex, which is an excellent nucleus of calcium-phosphate deposition. Salivary proteins may also play a role in this process because such complex formation occurs predominantly on lipids that are protein associated. The increase of pH facilitates these processes (13).

Because calcium-phosphate precipitation would lead to a ”confluent growth” of tooth surfaces and intensive formation of dental calculus and sialolith, the precipitation must be controlled. For such purposes, calcium and/or hydroxyapatite binding proteins such as calprotectin, histatins (HRP1), statherin, acidic PRPs, and cystatins (S, SA, and SN type) are present in the saliva. All inhibit crystal growth, whereas statherin also inhibits spontaneous unseeded precipitation (nucleation inhibition). Interestingly, dental plaque-bound immunoglobulins also inhibit crystal growth during calculus formation. Mg^{2+} ions also have some nucleation inhibitory effect (4, 13).

Saliva and surface protection

Besides taking part in acquired pellicle formation on tooth (denture, implant) surfaces, MUC5B type mucins cover all oral surfaces with a ∼10–20-μm thick layer. In addition, MUC5B type mucins form a hydrophilic viscoelastic gel (already in low concentration) that causes a high viscosity matrix of saliva. These properties of mucins (MUC5B), together with similar effects of glycosilated PRPs, accomplish the lubricating effect of saliva that defends against physical injuries during chewing (4, 14). Salivary proteins, especially basic PRPs, bicarbonate ions, and phosphate ions may also act as buffers against acids of nourishment and/or bacterial fermentation. PRPs and especially HRPs are potent precipitators of tannins, which are a widespread phenolic plant compound (flavonoid) of nourishment with unpleasant taste and protein-precipitating properties (15). Protease inhibitory effect of saliva (i.e., HRP5 against trypsin-like proteases, cystatins against cysteine proteinases, and SLPI against serine proteases) may also serve as surface defense by decreasing the proteolytic degradation of surface proteins and salivary defense proteins. Salivary chaperone Hsp70 is also a potent defense protein against cell surface damage; moreover, Hsp70 can repair aggregated and/or denatured salivary proteins (2). Peroxidases also protect host cells that transform H_2O_2 (produced by microorganisms and during immune/inflammatory reactions) to reactive anion hypothiocyanate ($OSCN^-$) that has a stronger antibacterial effect but a smaller cell-damaging effect than H_2O_2 (16). The diluting effect of saliva and the oral clearance (i.e., swallowing toward the stomach and/or expectoration) of many proteins, bound or free molecules, and microbes also serve as an effective surface defense mechanism.

Antimicrobial effects of saliva

A network of antimicrobial salivary defense includes numerous salivary proteins. Although some defense molecules are present in a rather low concentration in whole saliva, it should be considered that local concentrations of these proteins nearby the mucosal surfaces (mucosal transudate), periodontal sulcus (gingival crevicular fluid), and oral wounds and ulcers (transudate) may be much greater (2). Furthermore, the effects are addable, synergistic, and in many cases reinforced by immune and/or inflammatory reactions (2, 16, 17).

Some defense proteins are involved primarily in immune activation. Salivary immunoglobulins take part in elimination of bacteria fungi and viruses through specific immune binding and agglutination. Immunoglobulins act via the antibody-induced ozone formation (18). Molecular chaperone Hsp70 acts as a danger signal that leads to a specific immune answer and complement activation and takes part in the antigen presentation (bacterial, micotic, and viral) (2). Cystatin C has chemotactic properties, and it plays a role in antigen presentation of dendritic cells present in oral mucosa. Moreover, cystatin S, C, and D show antiviral activity; cystatin C, SA, and SN show antiparasitic activity; and cystatin S shows antibacterial activity.

Other proteins are responsible for nonimmune elimination of microbes (4, 16, 17). Salivary amylase is proposed to perform inhibitory effect on growth of microorganism. Calprotectin has bactericide and fungicide properties. HRPs show aspecific antibacterial and antifungal activities. Lactoferrine has bacteriostatic effect. Lysozyme is bacteriolytic for gram-positive bacteria. Secretory leukocyte proteinase inhibitor shows antibacterial, antifungal and antiviral activity. Defensins possess antimicrobial and cytotoxic properties. Mucins, (especially MUC7) are highly affine to microorganisms, entrap and agglutinate bacteria, fungi and viral particles. Peroxidases have antiviral antifungal and bacteriostatic properties through producing reactive anion

Table 3 The most important salivary proteins with antimicrobial properties

Name	Properties
Immunoglobuline	Specific immune binding, agglutination, antibody-induced ozone formation
Salivary chaperon Hsp70	Danger signal, complement activation, antigen presentation
Cystatin C	Chemotactic, antigen presentation
Alpha-amylase	Inhibition of microbial growth
Calprotectine	Bactericide, fungicide
Histatins	Antibacterial, antifungal
Lactoferrine	Bacteriostatic
Lysozyme	Bacteriolytic
Protease inhibitor SLPI	Antibacterial, antifungal, antiviral
Defensins	Antimicrobial, cytotoxic
Mucins	Agglutination
Peroxidases	Bacteriostatic, antifungal, antiviral
Proline-rich proteins	Aspecific binding of bacteria, fungi, and viruses

hypothiocyanate. Acidic PRPs bind bacteria, basic PRPs bind fungi (e.g., Candida albicans), and viruses, whereas glycosilated PRPs bind bacteria and viruses that indicate a role of PRPs in clearance of these microorganisms toward the stomach (4, 16, 17). (Data are summarized in Table 3.)

Saliva and wound healing

Besides prevention of wound infections through the above antimicrobial effects, saliva plays other roles in the healing of oral wounds as well. Salivary EGF speeds up the healing process by its angiogenetic and cell proliferating effects (19, 20). Other growth factors present in saliva (3) such as transforming growth factor beta, fibroblast growth factor, insulin like growth factors, and nerve growth factor also contribute to the healing process. Furthermore, saliva contains several blood clotting factors (IXa, VIII, XI) at a level comparable to plasma, and saliva can replace platelets in the thrombin generation (5). This property of saliva is highly important in the oral wound healing because, although saliva dilutes blood-clotting factors of blood origin, blood-clotting can be initiated. A relatively high amount of salivary kallikrein (5) is suggested to play a role in vasodilatation around mucosal injuries to facilitate healing and defense of the injured area.

Saliva in dental caries

Besides the presumable alterations of bacterial adhesion, plaque formation, and salivary defense mechanisms detailed previously, some more or less-specifically detectable changes of saliva are in connection with caries formation, and **it** may be used for recognizing risk patients and to maintain prevention. Decreased saliva flow rate, decreased buffer capacity, increased number of *S. mutans* and *Lactobacilli* in saliva are usually associated with increased caries prevalence. Similarly, decreased level of certain salivary proteins such as proline-rich proteins (PRP1, PRP3), histatin 1, and statherin is associated with significantly higher caries-susceptibility (3, 21).

Saliva and periodontal disorders

Several important effects of saliva on bacterial adhesion, plaque and calculus formation, and elimination of microorganisms are described previously. Besides these effects and measuring several marker proteins, saliva may be used for periodontal disorder screening. The levels of proteolytic granulocyte enzyme elastase, protease inhibitor alpha$_1$-antitrypsin,, and elastase inhibitor alpha$_2$-macroglobulin may increase considerably under gingivitis and/or periodontitis. Moreover, the level of alpha$_2$-macroglobulin is also a good indicator of an individual's periodontal status (22). Salivary level of 3-hydroxy-fatty acids (lipid constituent of lipopolysacharide endotoxin of several anaerobic bacteria) are also good indicators of chronic periodontitis (23). Albumin may also correlate with gingival inflammation (24). Although the periodontal diseases be diagnosed only by dental examination, the latest data on the role of periodontal diseases in cardiovascular and cerebrovascular conditions and also in premature birth may increase the significance of "quick screening" from the saliva (3).

Saliva and xerostomia

Five main reasons for subjective dry mouth sensation (xerostomia) exist, such as salivary gland disorders, systemic disorders, medication, radiation therapy, and aging. In healthy humans, the resting flow rate is around (or somewhat below) 1 mL/min, although in some conditions, like dehydration, sleeping, relaxation, or altered mental states (i.e., hypnosis, photo-acoustic stimulation) the flow rate may not exceed 0.25 mL/min (25). Under stimulation, the flow rate increases in healthy subjects to the usual value of 1.5–2.3 mL/min, but it may increase to 3.7 mL/min (26). Xerostomia usually appears when resting unstimulated whole saliva flow rate is less than 0.1–0.2 mL/min and stimulated flow rate is less than 0.4–0.7 mL/min. In other cases (∼25% of patients), the resting flow rate decreases, but the stimulated flow remains normal. In other patients (∼22%), both resting and stimulated flow rate is normal (27). In serious cases, saliva demonstrates low pH and buffer capacity, increased total protein albumin and sodium concentration, decreased amylase/protein ratio, and high lactobacillus and yeast

Table 4 The most important salivary changes in several systemic conditions

Condition	Usual but not specific changes in the saliva
Anxiety	Decrease of flow rate
Depression	Decrease of flow rate
Acute stress	Decrease of sIgA, Increase of amylase, salivary chaperon Hsp70, stress hormones Prompt changes of mucins' adhesive properties
Sjögren's syndrome	Decrease of flow rate, phosphate Increase of sodium, chloride and several salivary proteins
Cystic fibrosis	Increase of electrolytes, lipids, and prostaglandin E_2 Unusual form of EGF
Graft-versus-host disease	Decrease of flow rate, sIgA, and IgM, Increase of sodium, magnesium, salivary proteins, and IgG

concentration (27). The concentrations of MUC5B and MUC7 type mucins are also decreased (14).

Salivary changes under medication

Numerous kinds of medications such as anticholinergics, antidepressants, antipsychotics, diuretics, benzodiazepines, antihypertensive agents, muscle relaxants, analgesics, and antihistamines have been reported to induce xerostomia, although some (antipsychotics, benzodiazepines, and antihypertensive drugs) may also induce siallorhea (28). Antiepileptic drugs also induce significant changes in saliva; however, in this case the level of flow rate and pH usually remains normal. Phentoin, valproate, and carbamazepine medications increase the amylase and total protein concentration and decrease the cystatin S concentration (but not the cystatin C). A degradation of the glycan moiety of salivary mucins and other glycoproteins, and a decreased effectivity of mucine-induced aggregation of bacteria was also shown under multiple antiepileptic medications (24). However, it should also be considered that degradation and decreased bacterial aggregation was probably caused by poor oral hygiene in this group.

Salivary changes in systemic conditions

Anxiety and depression may lead to decrease in salivary flow rate and consecutive xerostomia. Acute stress conditions also induce significant salivary changes such as a decrease in secretory IgA (29), increase in salivary amylase (25, 30) and molecular chaperone Hsp70 (25) concentrations, and prompt changes of bacterial adherence to salivary mucins. In Sjögren's syndrome, low level of resting and stimulated flow rate as well as increased salivary level of sodium, chloride, IgA, IgG, lactoferrin, albumin, β2 microglobulin, lipids, cystatine C, cystatine S, prostaglandin E_2, interleukin-6, soluble interleukin-2 receptor, and kallikrein was reported. A decreased level of phosphate in the saliva was also found. Cystic fibrosis induces elevation of electrolytes (sodium, chloride, calcium, and phosphorus) and lipid levels of submandibular saliva that lead to increased calculus formation. An unusual form of EGF with poor biologic activity and abnormally elevated salivary prostaglandin E_2 was also found in cystic fibrosis patients (3). Graft-versus-host disease causes a mean reduction of 55–90% of salivary flow rate with elevated concentration of sodium, magnesium, total protein, albumin, EGF, and IgG in the saliva, whereas the amount of salivary IgA and IgM decreases, and no change exists in potassium, calcium, and phosphate concentrations (31). (Data are summarized in Table 4.)

Salivary diagnostics of systemic diseases

In some cases, saliva can be used as a highly effective diagnostic tool of systemic conditions. Serum-free hormone levels in the case of several nonpeptide hormones like cortisol, testosterone, estriol, estradiol, progesterone, aldosterone, androstendion, dihidroandostendion, and insulin can be calculated from salivary hormone levels (3, 32). Saliva levels of small peptide type neurotransmitters such as met-encephalin, substance-p and β-endorphin (33), and melatonin (a single amino acid derivative) may also refer to plasma levels. Salivary levels of epinephrine, norepinephrine, and dopamine although do not correlate too much with serum levels, but increase specifically under stress conditions. Monitoring of the systemic level of several medications is also possible from saliva. Similarly, medication and drug abuse and level of active or passive smoking and ethanol consumption can also be monitored. Screening of virus infections with detection of specific antibodies against viruses (i.e., hepatitis, HIV) in mucosal transudate enriched saliva is also a simple, well-tolerated, and accurate method (3). Tumor markers (i.e., c-erbB-e, p53 antigen, CA125) present in the saliva may be also used for screening and early diagnosis of malignancies that appear in several regions of the body (i.e., not exclusively oral tumors) (3). The fact that, in addition to the normal salivary transcriptome core (∼180 mRNA), a high amount (∼3.000) of other mRNA is detectable under several systemic conditions, and it will be of high diagnostic value in the future (32, 34). Similarly, oral fluid also provides an available source of microbial or human DNA, although the DNA content is rather low. This finding is useful for biomarker profiling of oral bacteria, oral, or systemic diseases and for forensic identification (32). (Data are summarized in Table 5.)

Table 5 The most important diagnostic possibilities of systemic diseases

Disease	Specific indicator
Hormonal alterations	Nonpeptide hormones
Stress conditions	Cortisol, epinephrine, norepinephrine, dopamine
Abuse	Drug or derivatives
Infections	Virus-specific antibodies
Tumor (oral and other)	General and local tumor markers
Other disorders	Disorder-specific mRNA

Chemical Techniques Used in Saliva Analysis

The methodology of salivary analysis is wide ranging and includes nearly all techniques used commonly in other fields of chemical biology. The methods used most frequently for saliva analysis are summarized briefly below.

Collection of saliva

Sample collection should be made at standardized time, according to the diurnal cycle (and/or the response and recovery time) of the analyte. Subjects should not eat within 60 minutes prior to sample collection. For recovery of salivary glands, alcohol, caffeine, and dairy products should also be avoided. Resting saliva can be collected avoiding any chemical (i.e., acids), physical (i.e., pressure, warm, cold), biologic (i.e., taste, chewing), and psychologic (i.e., imagination of a meal) stimulation. Stimulated saliva is collected most widely with chewing stimulation (i.e., paraffin wax), and/or with taste stimulation (i.e., candy, lemon). Whole saliva can be collected simply by drooling into a vial with forward tilted heads or by allowing the saliva to accumulate in the mouth and then expectorate it into a vial. Isolated parotid saliva may be collected with direct cannulation of the parotid duct or with the use of parotid cap (a plastic container with a pocket that enables some negative pressure for stabilizing the device on the mucosal surface). Mixed saliva of submandibular and sublingual gland may be collected with direct cannulation of submandibular duct. Saliva of minor glands may be collected with pipettes or with small piece of adsorbent. A fluid enriched in mucosal transudate and gingival crevicular fluid can be collected placing an adsorbent pad between the cheek and the lower gum.

Handling and storage of saliva

Saliva is usually homogenized on a vortex mixer for one minute and precleared by centrifugation (i.e., 10.000 × g; 4°C; 10 min.) to remove food rests, bacteria, mucosal cells, and other particles. Saliva may also be cleared by filtration using small (i.e., 0.2 μm) pore size filters, but only small amounts, because the filter pores are blocked by high molecular weight components in saliva. Precleared saliva can be stored on ice (+4°C) without significant changes of enzyme activity (i.e., amylase) or protein degradation only for few hours. Addition of protease inhibitors is advantageous for time consuming analysis procedures. Freezing may lead to significant protein precipitation, even if quick freezing (i.e., liquid nitrogen) is used. Frozen sample can be stored at −20°C without any more damage for a few days only. Somewhat longer storage is possible in liquid nitrogen or at −80°C.

Detection of ions

The pH value can be measured with hydrogen-selective electrodes; however, it should be noted that, the pH value of the saliva is dependent on the level of dissolved CO_2 thus–for a true pH value to be obtained—saliva must be collected without a loss of CO_2 to avoid measuring a pH value higher than real (5). The free (unbound) form of other ions like sodium and calcium can also be measured with ion selective electrodes. With the use of flame photometry total amount of sodium, potassium and calcium can be measured in the saliva. With the use of atomic absorption spectrophotometry the total amount of calcium, magnesium, copper and some other ions (i.e., constituents of dentures like zinc, iron, cobalt, and chrome) is detectable. Photometric (colorimetric) methods can be used for measuring the total amount of chloride, calcium, bicarbonate, and phosphate. In case of calcium and phosphate a high percentage (∼50% and up to 20%, respectively) may be bound to proteins and/or lipids (5, 35). Determination of the ratio between free and bound forms of calcium can be performed by comparing the data of flame photometry and electrode measurement. In case of phosphate, an exact discrimination is only possible after a careful isolation of proteins and lipids from saliva fluid.

Analysis of salivary proteins

The protein content of the saliva is determined usually with modified biuret reaction [Lowry method (36)]. Qualitative protein analysis is carried out most frequently with gel electrophoresis, especially sodium-dodecyl-sulphate poliacrylamide gel electrophoresis, and isoelectric focusing in both cases either in one-dimensional (1-D) or in two dimensional (2-D) forms. Similarly, 1-D and 2-D high performance liquid chromatography (HPLC) is also used widely. These methods are combined frequently with immunologic methods such as Western-blot analysis (in the case of gels) and enzyme linked immunoassay (ELISA, EIA) and radio immunoassay in the case of whole saliva samples and saliva fractions (i.e., HPLC fractions). Measurements of enzymatic activity with the addition of substrates are common methods for determination of salivary amylase and lysozyme. Newer approaches use in-gel-tryptic-digestion of the separated proteins (7). Digestion is followed by extraction of the resulted peptides and fragmentation with tandem

mass spectrometry (MS/MS) in a predictable fashion that allows computational determination of the peptide sequence (37). The identification of the protein from which the peptide was derived can be reached by protein database searching. In the latest direct analysis of large protein complexes and multidimensional protein identification technologies, tripsin digestion is the first step, followed by chromatography of the peptide fragments (i.e., biphasic or triphasic microcapillary columns and HPLC) and MS/MS analysis (38).

Analysis of carbohydrate structures

The carbohydrate moiety can be isolated after degradation of the protein and isolation of carbohydrate carrying particles via gel filtration and/or other chromatographic methods. Qualitative and quantitative analysis of the carbohydrate chains can be carried out after enzymatic digestion with glycosidases and after determination of the several carbohydrate components with the standard chemical methods based on colorimetry or gas-liquid chromatography (39). The structure mapping of carbohydrate chains can be done with the use of sequential enzymatic digestion by exoglycosidases (i.e., β-D-galactosidase, β-N-acetylhexosaminase, α-D-mannosidase, etc.), and reconstruction of the supposed chain sequence with the analysis of the released carbohydrate components. In addition to chemical methods immunologic methods like ELISA, immunoblotting and lectin blotting are used.

Analysis of salivary lipids

Salivary lipids should be extracted prior analysis by means of chlorophorm-methanol (2:1) according to Folch's method (40). Thereafter, lipids may be separated and quantified with thin-layer chromatography (9) or with gas-liquid chromatography (23). Salivary lipids may also be fractionated with silicic acid column chromatography.

Analysis of other salivary constituents

Many other constituents are present in the saliva and may be studied. For such purposes, related literature is possessing, taking into consideration the basic knowledge of saliva biologic chemistry summarized briefly in this article.

AQ3

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Cross-References

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