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Notes

Mucoadhesive properties of the mussel adhesive protein[1]

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Abstract

The mucoadhesive properties of the mussel adhesive protein (MAP), a 130-kDa protein produced by the blue mussel (*Mytilus edulis*) to provide firm adhesion to underwater surfaces, were evaluated by measuring the maximum adhesive force of detachment for protein-coated cover glasses from porcine intestinal mucosa in a neutral physiological buffer using a modified tensiometer. In this simple test, air-dried films of MAP performed as good as analogously prepared films of polycarbophil, which is one of the best mucoadhesive polymers known so far and therefore was chosen as reference. If MAP films were dried and stored in an N_2-atmosphere, mucoadhesion was significantly improved by nearly doubling the force of detachment under otherwise the same experimental conditions.

Apart from the presence of positively charged lysine residues and a high content of hydroxylated amino acids, the remarkably strong adhesion of this protein to mucosal surfaces is probably due to the presence of *o*-dihydroxy-benzyl (carbachoyl) residues, as the molecule contains about 10–20% di-hydroxy-phenylalanine (Dopa). However, oxidation of carbachoyl residues to *o*-chinons under influence of air or appropriate enzymes, which should allow for nucleophilic addition reactions with primary and secondary amines or mercaptans present in the substrate, did not increase mucoadhesion. Together with its favorable safety profile, MAP appears as an interesting new compound for the development of mucoadhesive drug delivery systems, preferentially if these can be manufactured and stored under non-oxidative conditions.

Keywords: Bioadhesion; Mucoadhesion; Mussel adhesive protein; Polycarbophil; Tensiometer method

Since the introduction of the bioadhesion concept to the pharmaceutical sciences in the 1980s (Gurny and Junginger, 1990; Lenaerts and Gurny, 1990), the search for compounds with suitable bio- or mucoadhesive properties for the design of

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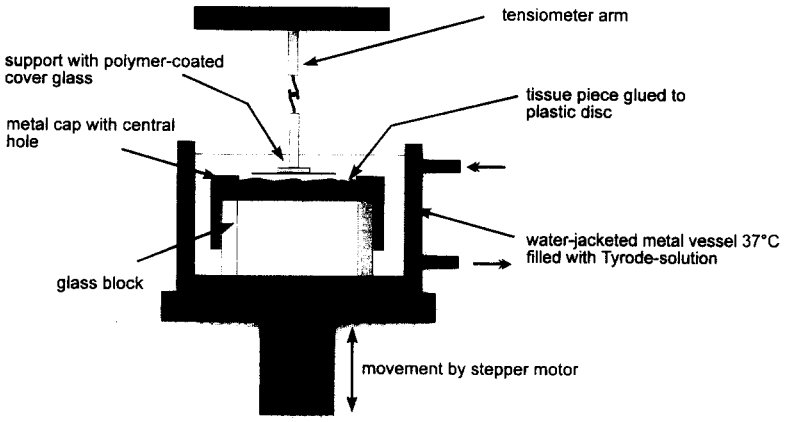

Fig. 1. Experimental setup to measure mucoadhesion of polymer-coated cover slips in terms of the the maximum vertical force of detachment from porcine intestinal mucosa in a physiological buffer at 37°C. Contact time was 60 s.

such dosage forms has become an important issue. So far, the most potent mucoadhesives have been identified among natural and synthetic polymers. One important class of mucoadhesives are the poly(acrylates), in particular some high-M_w derivatives of poly(acrylic acid), such as carbomer and polycarbophil (Ch'ng et al., 1985). Another class are poly(saccharides), such as some cellulose derivatives (Smart et al., 1984), hyaluronic acid (Saettone et al., 1989) or chitosan (Illum et al., 1994; Lehr et al., 1992). However, apart from some preliminary results reported for the hydrophobic corn protein zein (Mathiowitz et al., 1994), it seems that proteins have rarely been investigated as potential mucoadhesives.

Mussel adhesive protein (MAP) is produced by the blue mussel (*Mytilus edulis*) in order to attach itself to any underwater surface. Bioadhesion of this animal is remarkably strong, resisting high shear forces, e.g. on ship hulls or rocks on the coastal surf. Up to now several promising results have been reported for the use of MAP as an attachment factor in in vitro cell cultures (Olivieri et al., 1990) or as a bonding adhesive in transplantation surgery (Pitman et al., 1989). However, mucoadhesive properties of MAP, i.e. its adhesion to mucosal epithelial tissues as would be desirable for various drug delivery applications, seem not to have been investigated yet.

MAP of 65% nominal purity was a kind gift of Magnus Quist, Swedish BioScience Laboratory. The protein was received as a 0.1% (w/v) solution in 5% acetic acid and was stored in the dark at 4°C. A 0.1% (w/v) aqueous solution of polycarbophil (PCP, Noveon AA1, kind gift of B.F. Goodrich, Cleveland, USA) was used for comparison because of the well-known strong mucoadhesion of this synthetic polymer (Ch'ng et al., 1985; Lehr et al., 1990).

Cover glasses ($\varnothing = 10$ mm) were coated by pipetting an aliquot of either polymer solution to the center of one cover slip. The applied solution was allowed to dry, leaving a thin mucoadhesive film. For the first sets of measurements the polymer films were dried overnight at room temperature in air, and later in a N_2-atmosphere to avoid uncontrolled oxidation. As a further control, non-coated cover slips were used.

Mucoadhesion was quantified by measuring the force of detachment for the polymer-coated cover slips from pig duodenal mucosa using a modified surface tensiometer, as previously described (Lehr et al., 1990) (Fig. 1). The testing medium was physiological Tyrode buffer of pH 7.4 and 37°C. Duodenum from freshly slaughtered pigs was received from a nearby slaughterhouse, stored at 4°C in oxygenated Krebs-Ringer buffer and used for the experiments within 48 h. The cover slip was hung on the tensiometer arm and brought in contact with the tissue by raising the platform with the tissue until the force measured at the tensiometer was zero. After 60 s of polymer–tis-

sue contact, the platform was lowered at a constant rate, and the force exerted on the tensiometer arm was plotted. The maximum vertical force recorded until the cover slip polymer became detached from the tissue was used as a parameter for mucoadhesion.

Tyrosinase (Sigma, catalog # T-7755, dissolved at 7500 units/ml in 50 mM phosphate buffered saline (PBS), pH 7.2) was used to investigate the effect of enzymatic oxidation of MAP on its mucoadhesion. Unless stated otherwise, 200 μl of the enzyme solution were applied to the dried MAP-coated cover slips and left there for a certain period of time. Then the solution was pipetted off, and the mucoadhesion experiment was performed immediately.

The first set of experiments was designed in order to find out whether MAP has any mucoadhesive properties at all. The air-dried films of MAP showed about the same force of detachment as comparably thick films of PCP (Fig. 2).

Though already promising, these results let us to the question whether the mucoadhesive properties of MAP could possibly be further improved. According to the supplier's product manual, tyrosinase treatment may be used to improve MAP's bonding strength in ophthalmic and other biomedical applications. However, in our experiments, it turned out that tyrosinase treatment of air-dried MAP films at the recommended enzyme concentration and for various periods of time had no effect on their mucoadhesion (Fig. 3).

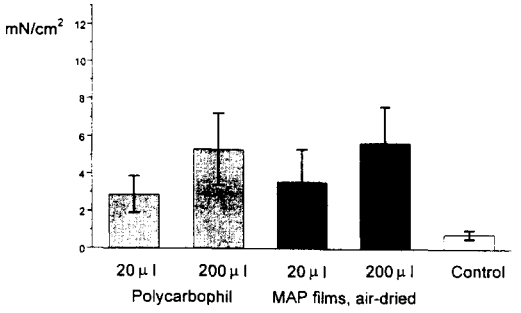

Fig. 2. Mucoadhesion of polymer films of varying thickness, obtained by overnight drying of aqueous polymer solutions at room temperature without any further treatment (mean ± 95% confidence intervals (C.I.), $n = 7$, control = glass cover slip without any coating).

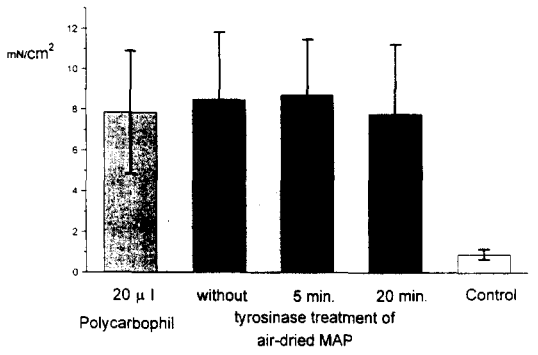

Fig. 3. Effect of tyrosinase treatment on mucoadhesion of air-dried MAP films (mean ± 95% C.I., $n = 7$).

In order to better control the possibly crucial oxidation of MAP, the drying conditions were altered: instead of air-drying, the cover slips were dried in an exsiccator under a slight stream of nitrogen gas. The same batch of nitrogen-dried films was split, and half of them were treated with tyrosinase solution for 5 min while the other half were measured without enzyme treatment. Surprisingly, the N_2-dried MAP films without tyrosinase treatment performed significantly better than both the tyrosinase-treated MAP and the reference compound PCP (Fig. 4). In another set of experiments, N_2-dried MAP films were stored for 1 week either in N_2 or air. For the MAP films stored in air, mucoadhesion was clearly decreased

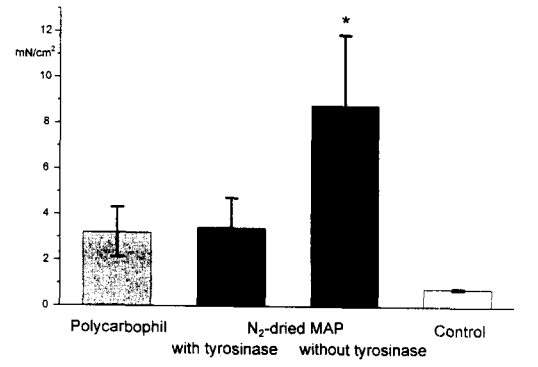

Fig. 4. Mucoadhesion of MAP films dried in an N_2-atmosphere (mean ± 95% C.I., $n = 6$). Asterisk indicates significance compared to all other treatments (analysis of variance (ANOVA) and subsequent multiple range test based on 95% LSD).

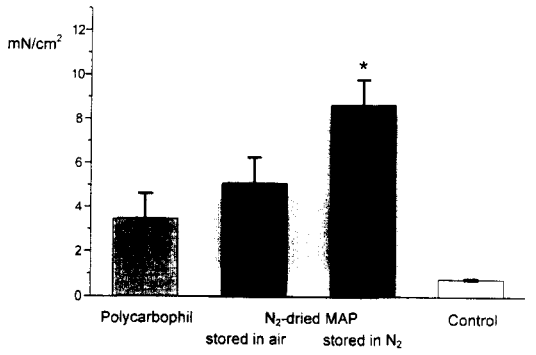

Fig. 5. Mucoadhesion of MAP films after 1 week of storage under different conditions (legend as in Fig. 4).

compared to the MAP films kept under N_2, and was no longer statistically different from the reference polymer PCP (Fig. 5).

MAP is a 130-kDa protein, composed of 75–85 repeated hexa- and decapeptides in tandem linear array. The primary sequence of the prominent decapeptide is shown in Fig. 6 (Waite, 1987). Hence, MAP contains a relatively high portion of hydroxalkyl- (about 40%), aminoalkyl- (about 20%), and phenolic (about 20%) functional groups. A large portion (about 75%) of the phenolic groups are *o*-dihydroxybenzyl or catechoyl functionalities, such as 3,4-dihydroxy-phenylalanin (DOPA), which are rarely found in natural proteins.

The results of this first evaluation show that MAP has mucoadhesive properties, i.e. it was found to adhere to mucosal tissue in the presence of excess amounts of a physiological buffer at neutral pH. Under the conditions of such an in vitro test, the mucoadhesion of MAP was even superior to polycarbophil, one of the best mucoadhesive polymers known at present, provided the protein was protected from premature oxidation. Oxidation may either occur during drying of aqueous solutions in order to obtain MAP films, during storage of the dried films, or under the influence of enzyme catalysts, such as tyrosinase.

Several theories have been discussed to explain the adhesive properties of MAP to various, including biological, substrates (Olivieri et al., 1990; Waite, 1987). First, its 20% lysine content gives a positive electrical charge to the molecule at neutral pH and may therefore assist bioadhesion by electrostatic attraction to usually negatively charged cell surfaces and to mucin. Next, the aliphatic OH-groups of serin, threonin and hydroxyproline, apart from the phenolic mono- and dihydroxybenzoyl groups, make the molecule relatively hydrophilic and capable of undergoing intermolecular OH-bonds. Both hydrophilicity and OH-bonds, which are common to many other mucoadhesive polymers (e.g. poly(acrylic acid), hydroxypropyl cellulose, chitosan) appear as necessary, but not sufficient (e.g. poly(vinyl alcohol) is not mucoadhesive), conditions to give mucoadhesive properties.

Finally, the most peculiar feature of MAP compared to other mucoadhesive polymers (and to other proteins as well) is the presence of catechoyl groups from DOPA. Waite (1987) has reviewed and discussed the possible energetic interactions of the DOPA catechoyl groups in MAP, which are (a) hydrogen bonds, (b) metal–ligand complexes, (c) Michael-type addition of primary or secondary amines and mercaptans after oxidation of DOPA to an *o*-chinone, and (d) quinhydrone-charge-transfer complexes stabilized by π-interactions with other aromatic amino acids. From these four interactions, the last one (d) might be less relevant to mucoadhesion, because there are no substantial amounts of aromatic amino acids present in mucus. In contrast, the role of hydrogen bonding (a) in mucoadhesion is widely accepted (see above). Interaction with metal ions (b), in particular Ca^{2+}, are known to affect mucoadhesive and other properties of some mucoadhesive polymers, in particular of poly(acrylic acid) derivatives (Leung and Robinson, 1988, 1990; Lueßen et al., 1994b). We have, however not further investigated this aspect of MAP mucoadhesion in this study.

Besides the formation of metal complexes by the (native) DOPA catechoyl groups, their *o*-chinon oxidation products have been suggested to be important for the curing (i.e. intramolecular crosslinking to improve cohesive strength) of the adhesive by intra-molecular reactions with nucleophilic groups, e.g. with lysine residues (interaction (c)). The same type of molecular interaction, however, may also occur with primary or sec-

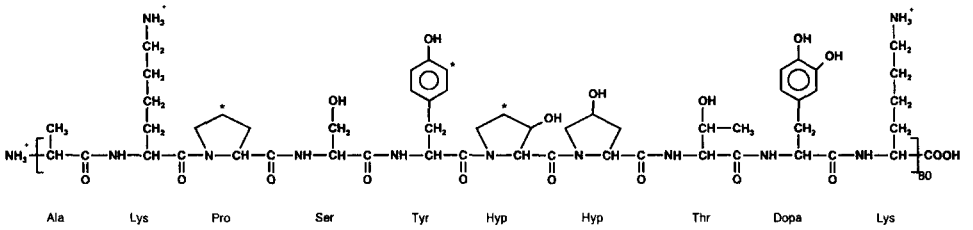

Fig. 6. The decameric repeating unit of MAP. The dots represent positions of additional hydroxylation (from Waite (1987), redrawn).

ondary amines or mercaptans at the interface to biological substrates. Catechol oxidase has been reported to be produced in significant quantities by the mussel to act as such a curing agent (Waite, 1985). Therefore, it has been suggested that similar tyrosinase derived from mushroom (synonyms: monophenol monooxygenase, phenol oxidase, catechol oxidase, EC 1.14.18.1) which is commercially available, be mixed with MAP in order to improve bonding strength in biotechnical applications (M. Quist, personal communication). As oxidative processes in our experiments, either during air drying or under influence of this enzyme, worsened rather than improved the mucoadhesion of MAP, the contribution of *o*-chinon reactions to mucoadhesion appeared to be less important compared with the other hypothetical mechanisms.

Notably, even mucoadhesion of oxidized MAP films was still as good as of polycarbophil films, but a significant improvement of MAP mucoadhesion could be observed when the protein was prevented from premature oxidation during drying and storage of the films. This suggests an important role of the non-oxidized carbachoyl groups for the mucoadhesion of MAP. According to a model proposed by (Olivieri et al., 1992), adhesion of MAP is initiated by (9)-DOPA which acts as a primary binding unit and displaces adherent water from the substrate due to its strong hydrophilicity. Subsequently, the molecule may change its conformation in such a way that also the (5)-tyrosine or (5)-Dopa residue approximates the substrate surface for a secondary stabilization and reinforcement of the adhesive bond. This hypothetical model for MAP adhesion to various surfaces is in good agreement with the results observed here in case of mucoadhesion.

In this first evaluation, MAP impresses with an excellent mucoadhesive bonding strength compared with the hitherto 'golden standard' of poly(acrylic acid) derivatives, such as polycarbophil. It may even be speculated that the mucoadhesion of MAP or MAP-like polypeptides may still be improved by better purification or preparation methods. But it also appears that non-oxidative conditions for manufacturing and storage of MAP-based drug delivery systems will be necessary to fully exploit its mucoadhesiveness, which may be a certain disadvantage compared with other mucoadhesive polymers.

The potential use of MAP as a mucoadhesive excipient for dosage forms will also depend on several other factors. First, from in vitro cell culture or in vivo transplantation experiments reported in the literature (Olivieri et al., 1990; Pitman et al., 1989), the compound appears to be non-toxic and well tolerated by biological systems, but still its safety will have to be further investigated. Second, MAP can nowadays be produced by biotechnological processes, allowing for a relatively cheap price, dependent on purity and quantity (Filipula et al., 1990). Upon better understanding of the molecular mechanisms of MAP bio-/mucoadhesion, its relatively complex structure may possibly be even further reduced to a simpler peptide sequence, which can be manufactured as a bulk product by chemical synthesis (Olivieri et al., 1992).

While we have here only evaluated the potential of MAP with respect to a possible fixation of dosage forms on mucosal tissues, there is increas-

ing evidence that mucoadhesive polymers are often multifunctional, e.g. by being capable of altering the barrier properties of tight epithelial tissues and/or to act as enzyme inhibitors (Lueßen et al., 1994a). Such biological effects have not been addressed in the present pharmaceutical evaluation of MAP, though they illustrate the additional potential of bioadhesion technologies for the controlled delivery of macromolecular drugs, in particular peptides and proteins or DNA/RNA molecules (Lehr, 1995). This certainly will keep the search for novel and better bioadhesives alive.

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