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Controlled covalent surface immobilisation of proteins and peptides using plasma methods

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ABSTRACT

Coated layers of biologically active molecules on synthetic biomaterials and biomedical devices can promote a variety of desirable biological reactions by the host body or the biological medium, such as cell and tissue attachment or deterring bacterial biofilm formation. Such coated layers should be immobilised covalently in order to avoid competitive displacement phenomena, and the use of surface-activating plasmas or plasma polymer interlayers with suitable chemical surface groups has proved to be very convenient means of grafting bioactive molecules onto solid materials surfaces. We review selected work on the covalent immobilisation of proteins and peptides onto solid biomaterial surfaces and describe efforts towards plasma methods that allow biomolecules to be covalently captured in a single step. After reviewing a number of approaches, we discuss in more detail the use of plasma polymer interlayers that possess aldehyde or epoxide surface groups; these groups react readily with amine groups on proteins and peptides without undesirable side reactions, and avoid other issues such as crosslinking. We also emphasise the importance of detailed surface analysis to verify that covalent grafting has indeed taken place, and to assess the surface density of grafted molecules. With suitably chosen peptides or proteins, such covalently grafted layers can support the surface attachment of delicate cells, or combat bacterial biofilm formation.

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1. Introduction

It has become clear in biomaterials research that synthetic polymers elicit nonspecific responses by the host body; at best a biomedical device is tolerated without becoming integrated into natural tissues, but often there is an immune response, thrombosis, and other adverse events [1]. Consequently, much research has focussed on coating biomedical device surfaces with molecules that can elicit a desirable biological response, such as the attachment and spreading of human cells and tissue [e.g.,2]. Much research has also focussed on producing surfaces that do not activate blood clotting or complement activation [e.g.,3]. Immobilised proteins, particularly antibodies, have also been of great interest for the development of biosensors and other bio-diagnostic devices. Moreover, more recently the problem of bacterial infections on biomedical devices

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has become a topic of much research interest; again, bacteria readily colonise a wide variety of synthetic materials and it is necessary to utilise specific biological molecules and mechanisms to combat this [4].

Proteins perform highly specific functions, and thus it is of much interest to utilise proteins as surface-coated layers on biomaterials in order to mask the underlying synthetic material surface and its nonspecific interfacial interactions with the apposing "biology". The aim is to reproduce a known specific biological action of that protein in a two-dimensional surface-located manner. However, this raises the question whether a protein coated onto a biomaterial surface is still capable of performing that function; steric hindrance or conformational changes upon coating may render it incapable of specific interactions with, for example, cell surface receptors. Hence a key aim of biomaterials science is to understand the roles that coated proteins play at the interface of materials. It is self-evident that coating methods for protein layers must be gentle and preferably use aqueous buffered solutions, close to natural biological fluids, so as to avoid stressing proteins. In some cases the biological function is produced by a relatively short amino acid sequence within a protein, and the use of peptides instead of a full protein can be advantageous, in that they are less sensitive to conformational disturbance resulting in loss of function.

While established coating processes such as solution coating can be used to coat proteins onto biomaterials surfaces, this may often not be an ideal approach, as it is well known that competitive adsorption–desorption processes occur (the so-called Vroman effect, first studied for blood proteins) [5]. Thus, a protein pre-coated onto a biomedical device surface may become displaced off the surface with time upon contact with biological media such as blood, which results in a loss of the intended control over the surface properties as various other proteins form a nonspecific mixed layer on the surface. It has indeed been shown that adsorbed molecules offer only limited benefit whereas covalently grafted molecules showed durable activity [6]. Accordingly, we consider it essential to design strategies that produce covalently immobilised layers of bioactive molecules, and to ensure that the surface-bound molecules are indeed covalently attached rather than just physisorbed.

Clearly, for many uses of biomaterials and biosensors, it is necessary to establish methods for creating coatings with gentle and straightforward coupling of biomolecules to surfaces, so as to preserve the structure and functions of immobilised molecules. Yet, covalent grafting is often not straightforward since biomaterials are designed to be non-reactive, and hence rarely possess reactive surface groups capable of participating in a reaction leading to covalent bond formation with a partner group on a protein. To overcome this limitation, a number of research groups have utilised either surface activation approaches to create suitable reactive surface groups, or thin interlayers that on one hand adhere tightly to the bulk biomaterial and on the other hand provide a suitable chemical group on their surface. Plasma based approaches possess distinct advantages, and surface activation by plasma treatments or plasma ion implantation has been used for biomolecule immobilisation. Alternatively, a coating method of choice is plasma polymerisation as plasma polymer layers usually adhere well on a wide range of substrate materials. Moreover, it is often possible to transfer a plasma coating approach to different substrates with little re-optimisation, whereas plasma surface treatment effects can vary considerably between substrate materials; this generic transferability is very attractive for industrial utilisation. The purpose of this article is to review approaches and show examples where these design considerations have enabled the creation of innovative functional biomaterials coatings.

2. General considerations for the covalent grafting of proteins and peptides

As mentioned, it is highly desirable to apply straightforward and gentle conjugation reactions for proteins, which are often very expensive and conformationally fragile. There is extensive literature on bioconjugation chemistry detailing reactions with proteins in solution, and efforts on surface grafting have usually been based on such precedents.

By far the most frequently used bioconjugation reaction involves the carbodiimide-mediated formation of amide bonds from an amine group on one partner and a carboxyl group on the other reaction partner. Often a combination of a carbodiimide and a succinimide is used, to create an intermediate succinimidyl ester. However, this intermediate has a limited lifetime, of the order of 20 min at room temperature, and without the succinimide there is an unstable acylisourea intermediate that can undergo a side reaction to a stable acylurea [7]. Nevertheless, catalysed amide formation has been used extensively, though regrettably the issue of side products is not addressed when using this approach for producing coatings, presumably due to the analytical challenges. It remains an open question how much published data might have been affected by unrecognised adsorbed side products.

One way to use carbodiimide mediated covalent linking is with a carboxyl group on the surface and an amine group on the protein (most proteins have a number of lysine amino acids). In this case, proteins must diffuse to the surface and react with the activated intermediate (from the carboxyl group) before it decays. Another way that has been used is to activate carboxyl groups on the protein and aim to react them with amine groups on biomaterials surfaces. A major problem with this approach is that activated carboxyl groups can react with amine groups on the same or another protein, thus achieving crosslinking rather than surface immobilisation. Crosslinked protein multimers can precipitate onto surfaces, and there may be instances in the literature where the conclusion of successful molecular grafting via this approach has been erroneous. Appropriate surface analysis methods should be applied to investigate whether the coverage corresponds to a monolayer or larger amounts.

In summary, while carbodiimide mediated amide formation for interfacial bonding has been used extensively, this approach is subject to a number of concerns such as side reactions whose relative contributions are known from solution studies to vary strongly with reaction conditions but could vary again in a confined near-surface environment, and are difficult to analyse in the case of surface coatings. It seems much preferable to use other reactions for the formation of interfacial bonds for grafting proteins. Carbodiimide coupling has often been considered favourably because it can be performed in buffered aqueous solution and thus avoids the need for reaction conditions that could denature proteins.

However, there are other reactions that can also be performed using buffered aqueous protein solutions. Of these, reaction between an aldehyde group and an amine group to form an imine interfacial bond has been of significant interest. To avoid crosslinking of proteins, the aldehyde group should be on the biomaterial surface and thus again amine groups of lysines serve as reaction partners. Amine groups also react readily with epoxide groups, and this approach likewise is well suited for the gentle aqueous covalent linking of proteins to surfaces, with lysine residues reacting with surface epoxide groups to form a stably immobilised protein or peptide layer. Both types of immobilisations will be discussed below.

However, proteins often contain multiple lysine residues, and hence all these routes lead to proteins being linked onto biomaterials surfaces via multiple covalent bonds, which might reduce their ability to undergo conformational motions for a "lock-and-key" fit for specific interaction with another protein from a biological solution, such as in biosensing or integrin docking. Whether this is a problem can be hard to predict and generally the approach has been to try these relatively simple linking methods before considering more stereospecific linking approaches. One such example involves linking sulfhydryl groups, on cysteine residues, with thiol or epoxide groups on biomaterials surfaces. This can provide more control over the attachment location on the protein, but on the other hand most proteins have far fewer cysteine residues than lysine