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Use of polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline for the preparation of a hapten–protein conjugate for antibody development

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ABSTRACT

Polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ), a polymer-supported covalent coupling reagent, was successfully employed for the first time in the bioconjugation of an example hapten (phytanic acid derivative) to a carrier protein (bovine serum albumin (BSA)) within the context of immunogen preparation for antibody development. The ability of the prepared example phytanic acid derivative–BSA conjugate to bind an anti-phytanic acid antibody was confirmed using an enzyme-linked immunosorbent assay (ELISA).

Small molecules (haptens < 1000 Da) generally do not trigger an immune response since their low molecular weight is not sufficient to elicit a response from the recipient's immune system. However, it is possible to trigger antibody production against such haptens by conjugating the haptens to large carrier proteins, resulting in the production of an immunogen that is of sufficient size to be recognised by an immune system.[1] Chemistries used for covalent attachment of haptens to carrier proteins are well established for many functional group types available on both carrier proteins and haptens. Covalent attachment may be accomplished either by direct conjugation between an existing functional group on the hapten and the protein carrier[2] or by more complex methods involving modification of the hapten to introduce suitable coupling groups and/or insertion of linker arms to present additional molecular features.[3] A fundamental characteristic of an immunogen is the number of hapten molecules covalently attached to the carrier protein. The optimal number of hapten attached to the carrier protein has been debated concerning relevance to immunogenicity.[4]

Often, bioconjugates intended for use as immunogens are prepared by directly coupling carboxylic acid-containing haptens or hapten derivatives to primary amine groups of lysine residues on proteins using ethyl dimethylaminopropylcarbodiimide (EDC) or dicyclohexylcarbodiimide (DCC).[5] However, this process can lead to undesirable side reactions such as crosslinking of the protein and *N*-acylurea formation on the protein.[6]

Over the past decade, interest in the development of new polymer-supported reagents has increased,[7] predominantly because these reagents combine the traditional advantages of solution-phase chemistry with the convenience of solid-phase handling. Thus using polymer-supported materials, unreacted reagents and by-products remain on the resin and can be easily removed by filtration at the end of the reaction. Polymer supported coupling reagents that are currently available include several immobilised carbodiimides such as PS-EDC, PS-DCC, PS-TBTU, and PS-BOP.[7,8] However, the two latter reagents imply the release of uronium or phosphonium salts into solution during coupling, clearly an undesirable occurrence for an immobilised reagent, as these by-products have to be removed.

Recently, Bradley et al. reported[9] polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ) as an efficient coupling reagent for amide bond formation. Encouraged by its features and advantages, including that it does not require any pre-activation, addition of any other additives or formation of volatile by-products and high stability under general laboratory conditions, the object of the present study is to demonstrate the application of PS-IIDQ for the first time in the bioconjugation of a hapten to protein to form an immunogen. This study

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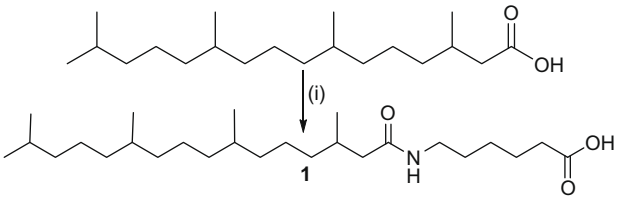


Scheme 1. (i) $NH_2(CH_2)_5COOH$, EDC, NHS and DMF at room temperature with overnight stirring.

Table 1
Different initial molar ratios of protein and hapten used to prepare conjugates PA-1 to PA-4

Code	Protein quantity	Hapten quantity	Protein–hapten molar ratio
PA-1	0.14 μmol (9.4 mg, 940 μL)	3 μmol (60 μL)	1:21
PA-2	0.13 μmol (8.8 mg, 880 μL)	6 μmol (120 μL)	1:46
PA-3	0.12 μmol (8.2 mg, 820 L)	9 μmol (180 μL)	1:75
PA-4	0.11 μmol (7.6 mg, 760 μL)	12 μmol (240 μL)	1:109

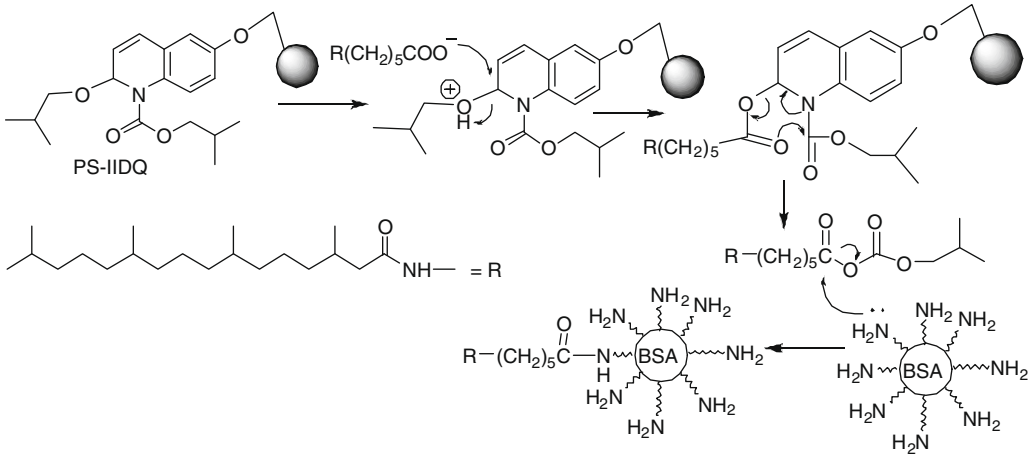
will use a phytanic acid derivative[10] as a representative example of a hapten and BSA as the protein carrier to assess its performance compared to existing supported carbodiimide approaches—specifically PS-DCC and PS-EDC.

PS-IIDQ was synthesised according to a previously reported method.[9a] Briefly, hydroxyquinoline was coupled onto Merrifield's resin using excess potassium carbonate in dimethyl acetamide (DMA) under reflux for 6 h to form PS-quinoline. The product was treated with isobutyl chloroformate in the presence of Hunig's base at 0 °C for 3 h to yield a highly reactive intermediate that was quenched by the addition of isobutanol. The reagent was characterised by NMR and FT-IR. The results were in agreement with reported data.[9a] For a single batch of PS-IIDQ, the loading was determined to be 1.6 mmol/g. This was based on the loading of Merrifield's resin used to prepare PS-IIDQ and on the assumption that 100% conversion of Merrifield's resin to PS-IIDQ was achieved. The phytanic acid derivative (**1**) was synthesised as shown in Scheme 1 and was characterised by IR, NMR, and MS.[11] The synthesis of the hapten–protein conjugates using PS-IIDQ is shown schematically in Scheme 2. The quantity of PS-IIDQ required for the bioconjugation was 2 equiv. To determine the optimal conditions for effective hapten–protein conjugation, conjugates were prepared[12] at four different molar ratios (1:21, 1:46, 1:75, and 1:109 and coded PA-1 to PA-4) as shown in Table 1. In order to assess the coupling performance of PS-IIDQ, PS-DCC and PS-EDC were also used separately as coupling reagents, that is, as controls. The free amino groups in the protein before and after the conjugation were determined by reaction with TNBSA.[13] Reaction of primary amines with TNBSA forms a highly chromogenic trinitrophenyl derivative that can easily be quantified by colorimetric read-out at 335 nm. The result of the TNBSA analyses of the conjugates is shown in Table 2. Degree of hapten conjugation to carrier protein was calculated from the absorbance values at 335 nm (which is the characteristic absorption peak of the TNP group) using the following equation: substitution (%) = $[A_{control}-A_{conjugate})/A_{control}] \times 100$. Control experiments were performed in parallel and consisted of protein alone and protein mixed with hapten without PS-IIDQ. These controls were also reacted with TNBSA, and confirmed that no conjugation occurred.

The resulting conjugates were additionally characterised by MALDI-MS.[14] As shown in Figure 1, spectra were successfully obtained for all four conjugates (PA-1 to PA-4). The molecular weight of native BSA was determined to be 66,451, while that determined on the basis of the published protein sequence was 66,432.9.[15] The relative increase in the molecular weight of the conjugates was manifested as a gradual mass peak shift as a function of hapten to protein ratio. This is interpreted as an increase in the hapten density of the conjugates and therefore provides a method for determining the number of haptens per protein molecule. The observed value for BSA was used in the following equation to determine the hapten density: number of haptens = (conjugate MW-BSA MW)/(hapten MW-18); the value of 18 refers to the loss of a water molecule from the phytanic acid derivative in the formation of the amide bond. The molecular weight of each conjugate was calculated from the peak centroid using the software provided with the MS instrument.

The findings of MALDI-MS analysis in terms of hapten density were in close agreement with the data obtained from the TNBSA spectrophotometric method of analysis. In the following consideration only the MALDI-MS data will be considered.

Comparing the results obtained with PS-IIDQ to those obtained from two other polymer-supported carbodiimides, that is, PS-EDC and PS-DCC, under identical conditions, and using a hapten–protein concentration ratio of 46:1, the PS-IIDQ exhibited the highest hapten density of 12:1 compared to 9:1 for PS-EDC and 7:1 for PS-DCC. The highest hapten density of 22:1 occurred using a hapten–protein concentration ratio of 109:1 and PS-IIDQ. Considering the three-dimensional structure of BSA, only 26 ε-NH_2 groups of the total 59 lysines residues in BSA are present at the protein



Scheme 2. PS-IIDQ-supported preparation of a hapten (phytanic acid derivative)-protein (BSA) conjugate.