Advances in Artificial Antigen Synthesis: Post-print

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Abstract

In recent years, immunoassay techniques employing small molecular compounds as haptens have been widely applied in fields such as food, pharmaceuticals, and environmental protection, achieving satisfactory detection results. Only after conjugation with carriers to form artificial antigens can small molecular compounds indirectly induce B cell proliferation and differentiation via T cell epitopes, thereby generating specific antibodies. Efficient synthesis of artificial antigens constitutes the prerequisite and key to immunoassay. This review comprehensively summarizes recent domestic and international advances concerning the design and synthesis methodologies of small molecular haptens, carrier selection, hapten-carrier conjugation strategies, as well as purification and characterization techniques for artificial antigens.

Full Text

Preamble

Progress in the Synthesis of Artificial Antigens

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Abstract

In recent years, immunoassay technologies employing small molecular compounds as haptens have found extensive applications in food and drug safety, environmental protection, and other fields, achieving favorable detection outcomes. Small molecular compounds possess only reactivity but lack

immunogenicity—they cannot directly induce B cell proliferation and differentiation to produce specific antibodies without T cell epitopes. Consequently, these compounds must be conjugated with carrier macromolecules to form artificial antigens that acquire immunogenicity and can indirectly stimulate B cells via T cell epitopes. Efficient synthesis of artificial antigens is a prerequisite and critical factor for ensuring successful immunoassay performance. This review summarizes recent advances in artificial antigen synthesis, covering hapten design and synthesis methods, carrier selection, hapten-carrier coupling strategies, and purification and characterization techniques.

Keywords: hapten; artificial antigen; carrier selection; antigen purification; antigen identification

1. Introduction

Immunoassay technology (IA) is an analytical method based on the specific recognition and reversible binding between antigens and antibodies. It has attracted considerable attention due to its high specificity, sensitivity, relatively simple operation, rapid detection speed, and low cost. This technology is widely applied not only in the detection of macromolecular compounds such as bacteria, nucleic acids, and proteins, but also plays a crucial role in analyzing small molecular compounds with relative molecular masses below 1000. In recent years, numerous immunoassay techniques targeting small molecules have emerged in fields including food safety [1-3], antibiotic residues [4,5], environmental pollution [6-7], drug detection [8], and pharmaceutical research and development [9-11].

Unlike macromolecular compounds such as proteins and nucleic acids, small molecules exhibit reactivity but lack immunogenicity because they do not possess T cell epitopes and cannot directly induce specific antibody production. Therefore, these compounds are termed haptens (incomplete antigens) and must be conjugated with carrier macromolecules to form artificial antigens that acquire immunogenicity. These artificial antigens can then indirectly induce B cell proliferation and differentiation through T cell epitopes, ultimately generating specific antibodies.

Efficient synthesis of artificial antigens is the prerequisite and key to ensuring successful immunoanalysis. Multiple factors influence the yield and quality of artificial antigens, including hapten design for target analytes, carrier selection, hapten-carrier coupling methods, and purification strategies—all of which subsequently affect the specificity and immunoreactivity of the resulting antibodies. Based on these considerations, this review summarizes recent domestic and international advances in artificial antigen synthesis, covering hapten design and synthesis, carrier selection, hapten-carrier coupling, and artificial antigen purification, aiming to provide theoretical and experimental references for related research.

2. Hapten Design and Synthesis

2.1 Principles of Hapten Design

Many target small molecules cannot be directly used as haptens due to stability issues, necessitating the use of structural analogs. Hapten design should therefore maximize similarity to the original target molecule in terms of molecular structure, characteristic functional groups, stereochemistry, electron distribution, electron cloud density, and hydrophobicity to ensure that the resulting antibodies can specifically recognize the target analyte. For example, natural phosphohistidine exists in two forms— -phosphohistidine (-pHis) and -phosphohistidine (-pHis)—with phosphate groups attached to nitrogen atoms in the histidine imidazole ring, forming N-P bonds that are extremely unstable under acidic conditions [Figure 1: see original paper]. Consequently, these molecules cannot be directly used as haptens for preparing specific antibodies against phosphohistidine proteins. Muir and colleagues [12,13] addressed this by replacing the imidazole ring in -pHis with triazole and pyrazole rings and substituting the labile N-P bond with a stable C-P bond, generating two phosphohistidine analogs, pTze and pPye. Antibodies raised against these haptens demonstrated good recognition of phosphohistidine proteins, with the pPye antibody showing superior performance due to its ring structure being more similar to -pHis.

The immunogenicity of an antigen positively correlates with the structural complexity of the hapten molecule. Introducing unsaturated stereostructures such as benzene rings, heterocycles, and branched chains can increase hapten complexity, highlight antigenic determinants, and significantly enhance immunogenicity to produce high-titer antibodies, though this may compromise specificity. Kim et al. [14] investigated the effect of hapten heterology on immunoassay sensitivity for the organophosphorus insecticide fenthion and found that haptens with five methylene groups in the side chain produced antibodies with 3.5-fold higher sensitivity than those with three methylene groups . Goodrow et al. [15] reported that haptens containing benzene rings achieved a 30% success rate in antibody preparation, compared to only 9% for those lacking benzene rings.

2.2 Selection of Hapten Linkers

A linker (spacer arm) is required as a mediator between the hapten' s characteristic structure and the carrier protein, typically a carbon chain that ensures adequate exposure of the small molecule on the artificial antigen surface for optimal immune recognition. If the hapten contains active groups such as amino, thiol, carboxyl, or hydroxyl groups, it can be directly conjugated to the carrier protein. Otherwise, appropriate active groups must be introduced at the linker terminus based on immunological principles and the structural characteristics of the target molecule, while ensuring the linker does not induce "linker-specific antibodies."

The selection of hapten linkers should generally follow these principles:

1. Linker attachment position should be distant from characteristic functional groups. This maximizes exposure of critical structural features, minimizes interference with specific antigenic determinants, and improves antibody affinity and specificity. Research demonstrates that different attachment sites result in varied spatial structures and electron distributions, leading to differences in antibody specificity, affinity, and titer. Two primary mechanisms explain this phenomenon: first, attachment too close to characteristic structures (similar to using overly short linkers) prevents adequate exposure of the hapten' s features on the antigen surface, impairing immune recognition; second, different attachment sites alter the hapten' s electronic properties through distinct electronic interactions with the linker, affecting immune recognition and antibody characteristics. Therefore, attachment site selection must balance both structural exposure and electronic effects.

Jia Min [16] carefully considered linker attachment positions in designing stilbestrol haptens. Three stilbestrol analogs exist: diethylstilbestrol (DES), hexestrol (HEX), and dienestrol (DIS) [Figure 2: see original paper]. Linkers could be introduced at either the alkyl or phenolic hydroxyl positions. However, attachment at the alkyl position would destroy the characteristic double bond structure in DES and DIS, resulting in weak antibody specificity, while the alkyl position in HEX is difficult to activate. In contrast, attachment at the phenolic hydroxyl position is readily achievable for all three compounds and preserves their specific structures, yielding antibodies with strong specificity.

2. Linker length should be appropriate. Current literature suggests an optimal length of 3–6 atoms [17]. Overly short linkers cause steric hindrance from the carrier that masks the hapten' s characteristic structure, impairing immune recognition. Conversely, excessively long linkers may undergo "folding" due to internal or external influences, preventing full extension of the hapten and partially masking its structure from immune recognition. Li et al. [5] demonstrated that a six-atom carbon chain linker for a furazolidone metabolite hapten produced superior results compared to a one-atom linker, yielding higher immune recognition and specificity. Shi et al. [18] obtained similar conclusions when studying artificial antigens for 2-methyl-4-chlorophenoxyacetic acid (M) and quinclorac (Q), using haptens with 0, 4, and 6 carbon linkers conjugated to BSA. They also found that increasing linker length significantly improved coupling ratios when hapten 主体结构 was constant, while the hapten 主体结构 itself affected coupling efficiency at equivalent linker lengths [Figure 3: see original paper].

Kim [19,20] investigated linker length effects on antibody performance and found no significant differences in affinity, specificity, or sensitivity for linkers ranging from 2–6 atoms under otherwise identical conditions. Lai et al. [21] successfully generated specific antibodies using a zero-length linker for phthalate ester haptens conjugated to BSA.

3. Linkers should preferably contain terminal active groups (amino, thiol, carboxyl, hydroxyl, nitro, carbonyl, etc.) while avoiding conjugated dou-

ble bonds or heterocycles to minimize linker recognition and enhance hapten-specific antibody response. Wang et al. [22] used a linker containing alkyl branches, tertiary amino groups, and carbonyl groups in nortriptyline hapten preparation [Figure 4a: see original paper]. Li et al. [23] introduced benzene rings and phenolic hydroxyl groups into a 3-amino-2-oxazolidinone hapten linker, resulting in significantly improved antibody specificity [Figure 4b: see original paper].

4. Linker stability with the parent molecule must be ensured to prevent cleavage during preparation or immunization. Reaction conditions should be mild, products easily purified, yields high, and costs low to enable large-scale production.

These principles and examples illustrate that optimal linker length remains unresolved and often requires orthogonal experimentation to determine the most suitable length and terminal active groups for specific parent structures.

2.3 Hapten Preparation, Purification, and Characterization

Hapten preparation involves chemical modification of small molecules lacking linkers or requiring group protection. Current methods primarily focus on group transformation at the attachment site to introduce linkers. Common chemical modification strategies include: (1) oxidation (e.g., hydroxyl to aldehyde); (2) reduction (e.g., nitro to amino); (3) hydrolysis (e.g., tertiary amino to amino, ester to carboxyl); (4) addition (e.g., double bond addition); and (5) substitution (e.g., halogen replacement) .

Zhang et al. [26] employed substitution, addition, and hydrolysis simultaneously in synthesizing tetrabromobisphenol A haptens, with the synthetic route shown in [Figure 5: see original paper].

Reported hapten purification methods include thin-layer chromatography (TLC), column chromatography, and extraction. While TLC yields pure products, its limited preparative scale restricts application. Column chromatography and extraction are more widely used due to simple operation, low material consumption, and recyclable solvents.

Common characterization methods include infrared spectroscopy, UV spectroscopy, ESI-MS, NMR, elemental analysis, and HPLC-MS. Combined mass spectrometry and NMR enables rapid determination of hapten structure and molecular weight.

3. Hapten-Carrier Coupling

3.1 Role of Carriers

Carriers play a crucial role in artificial antigen synthesis. Since haptens have small molecular weights and cannot elicit immune responses independently, they rely on the carrier' s specificity and immunogenicity to induce immune responses

and hapten recognition. Carrier selection should consider: (1) sufficient active groups for hapten coupling; (2) weak immunogenicity; (3) good solubility; (4) non-toxicity to the organism; and (5) low cost and ready availability.

3.2 Types of Carriers

Currently used carriers fall into three categories: proteins, polypeptides, and macromolecular compounds. The most commonly used protein carriers are bovine serum albumin (BSA), ovalbumin (OVA), and keyhole limpet hemocyanin (KLH). BSA is most frequently employed due to its stable physicochemical properties, good immunogenicity, low cost, abundant free amino groups, and excellent solubility across various pH, ionic strength, and organic solvent conditions. Although KLH exhibits the best immunogenicity, its high cost limits application. Recent studies have explored alternatives including bovine -globulin (BGG) [8], rabbit serum albumin (RSA) [27], thyroglobulin [28], porcine serum albumin (PSA) [29], and limulus polyphemus hemocyanin (LPH) [30].

Beyond proteins, researchers have investigated synthetic polypeptides such as poly-L-lysine (PLL) [3] as carrier alternatives. Compared to protein carriers, polypeptides offer higher free amino density, milder coupling conditions, higher coupling efficiency, and lower intrinsic immunogenicity, resulting in antibodies with higher hapten recognition and fewer anti-carrier components. Additionally, macromolecular polymers like carboxymethyl cellulose and polyvinylpyrrolidone [31] have been reported as carrier substitutes, though proteins remain predominant in most research.

3.3 Coupling Principles and Methods

The fundamental principles for artificial antigen synthesis include uniform and stable coupling products, easy purification, mild reaction conditions, and minimal impact on antigenic characteristic groups. Reported coupling methods include chemical coupling, chemobiological methods, and immunological labeling, with chemical coupling being most common .

Most artificial antigens involve one carrier protein conjugated to one hapten, producing highly specific antibodies but limiting detection of derivatives from the same parent compound, such as antibiotics. Sai et al. [52] addressed this limitation by sequentially coupling amoxicillin and chlortetracycline to BSA to create a dual-hapten immunogen. The resulting antibody cross-reacted with six penicillins (amoxicillin, penicillin G, ampicillin, penicillin V, carbenicillin, and sulbenicillin) and four tetracyclines (tetracycline, oxytetracycline, doxycycline, and chlortetracycline), with cross-reactivity ranging from 24–100%. Enzyme-linked immunosorbent assay demonstrated simultaneous detection of all ten drugs in milk with detection limits of 0.4–0.37 ng/mL, opening new avenues for small molecule immunoassay development.

4. Purification and Characterization of Artificial Antigens

Following successful synthesis, artificial antigens require purification to enhance immune recognition and specific antibody production while reducing non-specific responses. Reported purification methods include dialysis, centrifugation, gel chromatography, ion exchange chromatography, column chromatography, and salting-out. Currently, dialysis and gel chromatography are most commonly used. Dialysis offers excellent purification with simple operation but requires longer time. Gel chromatography is faster but more complex, requiring component tracking to identify target products.

To confirm successful hapten-carrier conjugation, various characterization methods are employed, including NMR, IR, UV, fluorescence spectroscopy, circular dichroism, gel-HPLC, atomic force microscopy, elemental tracing, elemental analysis, polyacrylamide gel electrophoresis, ESI-MS, MALDI-TOF-MS, and Coomassie brilliant blue staining. Among these, NMR, UV, and IR spectroscopy are most frequently used.

5. Summary and Outlook

Artificial antigens with good immunogenicity are critical to immunoassay success. Numerous examples of design, synthesis, purification, and characterization methods have been reported domestically and internationally. However, due to the structural diversity of small molecules and the complexity of immune responses, multiple parameters—including linker attachment sites, linker length, carrier type, coupling method, and coupling ratio—must be systematically designed and optimized through immunological activity testing for each target compound. This process is often labor-intensive, costly, and subject to experimental variability.

Therefore, employing computational assistance, molecular simulation, and network database analysis to design hapten structures, model their properties, and predict relationships between molecular structure and immunological characteristics represents an important future direction for achieving predictive hapten design and artificial antigen synthesis.

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Note: Figure translations are in progress. See original paper for figures.